COMPENSATORY ANTIVIRAL MECHANISMS IN PRIMARY MOUSE CELLS

IRF9 AND NITRIC OXIDE: IMPORTANT ANTIVIRAL MEDIATORS IN THE ABSENCE OF KEY SIGNALLING MOLECULES

By

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ABSTRACT

The innate host response to virus infection is largely dominated by the production of type I interferons (IFNs). Fibroblasts, considered nonprofessional immune cells, respond to virus infection after recognition of viral components such as double-stranded (ds)RNA. The constitutively expressed transcription factor IFN regulatory factor 3 (IRF3) is rapidly activated and type I IFNs are produced. In the absence of IRF3, it was found that IFNs are still produced. This thesis identifies IRF9 as the transcription factor responsible for IFN production in the absence of IRF3 based on its ability to bind the murine (m)IFNβ promoter determined via oligonucleotide pull-down assays.

In the absence of both IRF3 and IRF9, primary fibroblasts are deficient for IFN signalling. Surprisingly, significant inhibition of virus replication following dsRNA treatment of cells deficient for IRF3 and IFN signalling was recently observed with the large DNA virus herpes simplex virus type 1 (HSV-1) being more susceptible to inhibition than the small RNA virus vesicular stomatitis virus (VSV). As nitric oxide is known for its nonspecific antiviral effects against DNA viruses, involvement of this molecule in the antiviral response to HSV-1 in the absence of IRF3 and type I IFN induction and signalling was investigated. Here it is shown that in the absence of IRF3 and IFN, nitric oxide constitutes a major component of the innate response against HSV-1 in response to dsRNA in primary fibroblasts. In these cells, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IRF1 regulate inducible nitric oxide synthase (iNOS) expression, subsequently producing nitric oxide. As most viruses encode strategies to render their environment IRF3 and/or IFN deficient, it appears that IRF9 and

nitric oxide serve as secondary responses to protect the host against viral infection. These data emphasize the importance and requirement of the host to employ multiple strategies to overcome infection.

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Cue the Oscar "Get off the Stage" music.

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LIST OF ABBREVIATIONS

AMG	Aminoguanidine hydrochloride
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATF-2	Activating transcription factor 2
CBP	Cyclic-AMP-responsive-element-binding protein-binding protein
CREB	Cyclic-AMP-responsive-element-binding protein
Ct	Cycle threshold
DEAE-dextran	diethylaminoethyl-dextran
DETA	Diethylenetriamine
DETA-NO	DiethylenetriamineNONOate
DF	DharmaFECT
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
dsRNA	Double-stranded RNA
EC_{50}	Half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse-radish peroxidase
HSV-1	Herpes simplex virus type I
IFN	Interferon

IFNR	Type I interferon α/β receptor
IKKi	I_kB kinase i
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Interferon-inducible protein
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
L-NIL	N6-(1-iminoethyl)-L-lysine, dihydrochloride
LPS	Lipopolysaccharide
M	Mean
MDA-5	Melanoma-differentiation-associated gene 5
MEF	Mouse embryo fibroblast
MHC	Major histocompatibility complex
mIFNβ	Murine β-interferon
MOI	Multiplicity of infection
mRNA	Messenger RNA
N	Number
ND	Not determined
NDV	Newcastle Disease virus
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase

OAS	2', 5'-oligoadenylate synthetase
OM/DEAE	OptiMEM® Reduced Serum Medium with diethylaminoethyl-dextran
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFU	Particle forming unit
PKR	Double-stranded RNA-dependent protein kinase
Poly I:C	Polyriboinosinic:polyribocytidylic acid
PRD	Positive regulatory domain
PRR	Pathogen recognition receptor
PVDF	Polyvinylidene diflouride
qRT-PCR	Quantitative real-time polymerase chain reaction
RIG-I	Retinoic acid indicible gene I
RIP-1	Receptor interacting protein 1
RLR	Retinoic acid indicible gene I-like receptor
RNAseL	2-5A-dependent ribonuclease
Scr	Scrambled IRF1 oligomer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Short interfering RNA
STAT	Signal transducers and activators of transcription
TBK	Tank-binding kinase
TIR	Toll/interleukin-1 receptor
TLR-3	Toll-like receptor-3
TNF	Tumor necrosis factor

TRAF	Tumour necrosis factor receptor-associated factor
TRIF	Toll/interleukin-1 receptor domain-containing adaptor inducing interferon β
VRE	Virus response element
VSV	Vesicular stomatitis virus
WNv	West Nile virus
WT	Wild type
α-MEM	Alpha minimal essential medium

CHAPTER 1. INTRODUCTION

1.1 Viruses and the Host Immune System

Microorganisms that infect a vertebrate host are initially detected by the innate immune system. It is well recognized that immune detection of pathogens focuses on highly conserved molecular patterns that are distinct from the host (Janeway, 1989). Germline encoded pathogen recognition receptors (PRRs) have evolved to recognize components specific to pathogens, such as bacteria and viruses, as the initial stage of host defense. PRRs recognize conserved microbial components known as pathogen-associated molecular patterns (PAMPs) that are essential to the survival of the microorganism and hence, difficult for the pathogen to alter over time. In terms of viral infection, host PRRs have evolved to recognize viral nucleic acids or viral replication components such as double-stranded (ds)RNA (Stetson & Medzhitov, 2006). They may also recognize viral envelope glycoproteins (Trinchieri & Sher, 2007). Different PRRs detect specific PAMPs, show distinct patterns of expression, and activate specific signalling pathways that lead to distinct responses against the invading pathogen.

1.2 Recognition of dsRNA by TLRs and RLRs

dsRNA is a common PAMP and is produced from many viruses as a replication intermediate (Alexopoulou et al., 2001). This nucleic acid is recognized by PRRs such as toll-like receptor (TLR)-3 and the retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) which include RIG-I and melanoma-differentiation-associated gene 5 (MDA-5).

Mammalian TLRs are a family of transmembrane proteins. Thus far, 10 and 12 functional TLRs have been identified in humans and mice, respectively; TLR-1 through TLR-9 are conserved in both species. TLRs were originally discovered in vertebrates based on their homology with Toll, a molecule known to play a critical antimicrobial role in *Drosophila melanogaster* (Medzhitov et al., 1997). Members of the TLR family of receptors recognize PAMPs through the leucine rich repeats in their luminal domains and signal through the cytoplasmic toll/interleukin (IL) 1 receptor (TIR) domain (Kawai and Akira, 2006).

TLR-3 was originally identified based on its ability to recognize polyriboinosinic: polyribocytidylic acid (poly I:C; Alexopoulou et al., 2001), a synthetic analog of dsRNA that mimics viral infection and induces antiviral immune responses by promoting the production of both type I interferons (IFNs) and inflammatory cytokines (Field et al., 1967, Matsumoto & Seya, 2008). TLR-3 localizes in the endoplasmic reticulum (Johnsen et al., 2006) and recognizes natural, synthetic, and *in vitro* transcribed dsRNA (Alexopoulou et al., 2001; Kawai & Akira, 2006). Class A scavenger receptors, which are localized on the cell surface, are projected to deliver extracellular dsRNA to the endosome (DeWitte-Orr et al., 2010). TLR-3 subsequently moves to the dsRNA-

containing endosome where ligand recognition occurs (Johnsen et al., 2006). The TIR domain of TLR-3 enables recruitment of the adaptor TIR-domain-containing adaptor-inducing IFNβ (TRIF), which signals to one of tumour necrosis factor (TNF) receptor-associated factor (TRAF)-6, receptor interacting protein (RIP)-1, or tank-binding kinase (TBK)1/I_kB Kinase i (IKKi). Signalling leads to activation of the transcription factors activating transcription factor (ATF)-2/cJun, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IFN regulatory factors (IRFs) downstream (Fitzgerald et al., 2003; Yamamoto et al., 2003, Kawai and Akira, 2006), as shown in figure 1.

Alternatively, RIG-I and MDA-5 are receptors for cytoplasmic dsRNA (Yoneyama et al., 2004). The cytosolic RLRs distinguish dsRNA based on its length and origin. RIG-I recognizes RNA from a variety of viruses, while MDA-5 preferentially binds RNA from picornaviruses and the synthetic dsRNA, poly I:C (Kato et al., 2006). Furthermore, RIG-I preferentially binds short dsRNA, whereas MDA-5 binds longer lengths of dsRNA (Kato et al., 2008).

Regardless of the PRR that recognizes dsRNA, stimulation of either pathway leads to activation of IRFs, which contribute to early defense against viral infection by induction of type I IFNs (Yoneyama et al., 2004). The induction of type I IFNs, specifically IFNβ, in fibroblasts has been the subject of much research in virology. Overall, the IRF family of transcription factors contains very important players in host protection as they regulate IFN expression.

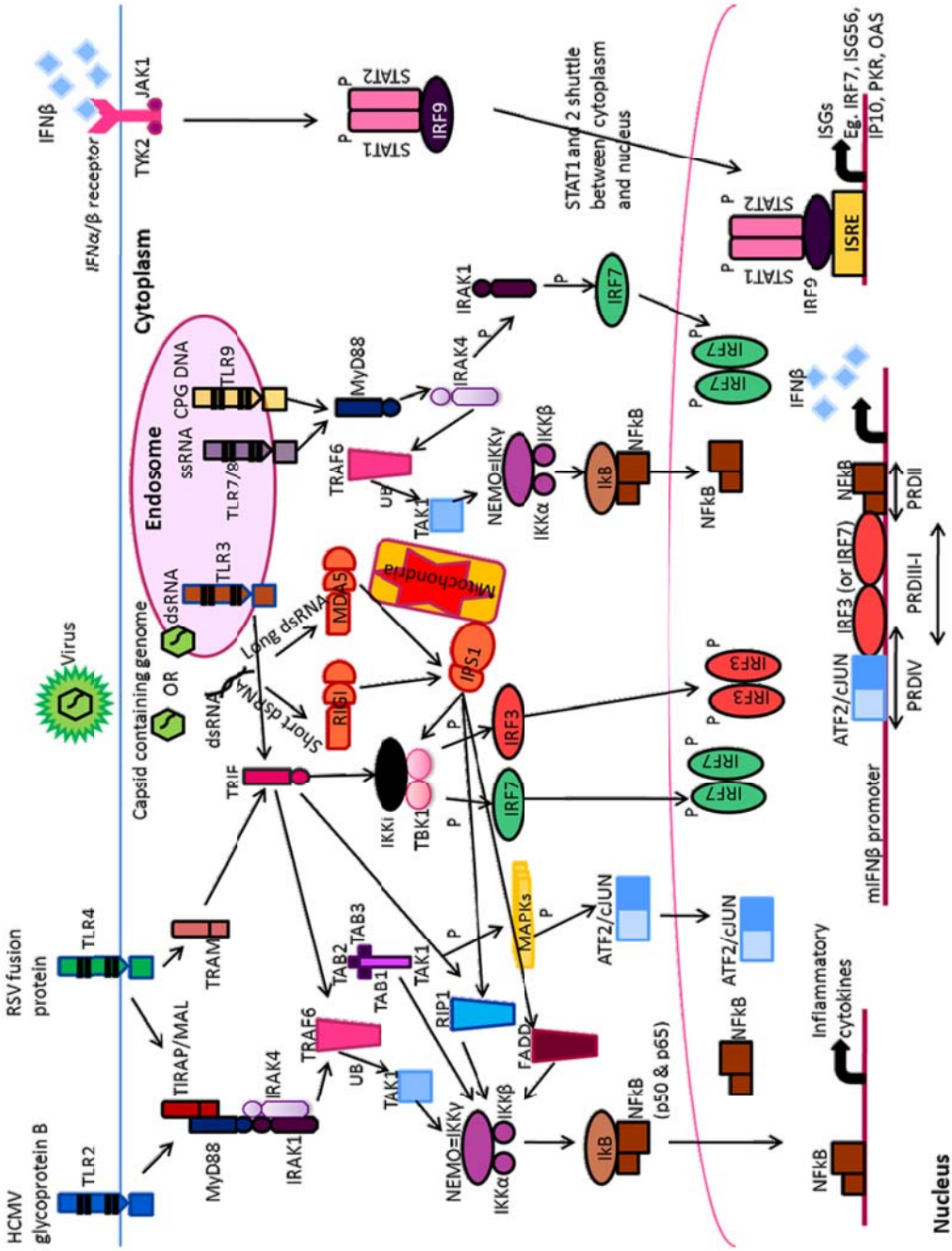


Figure 1. Signalling pathways initiated in response to various viral PAMPs. Signalling mediated by dsRNA recognition initiates the production of antiviral factors such as IFNβ, which is made upon binding of ATF-2, NF-κB and IRFs to the promoter. This initiates a secondary arm of IFNβ-mediated signalling that activates the IFN stimulated gene factor 3 (ISGF3) complex, which binds to the IFN stimulated response element (ISRE) to induce transcription of IFN stimulated genes (ISGs).

1.3 IRF Family

Understanding how an infection elicits an immune response via PRRs has progressed significantly in the last decade. It is widely recognized that IRFs are important in regulating the immune response to viral pathogens by mediating type I IFN expression (Honda & Taniguchi, 2006, Kawai & Akira, 2006). IRFs regulate expression of IFNβ by binding to the IFNβ promoter, depicted in figure 2, to either prevent or promote transcription of IFNβ (Miyamoto et al., 1988, Sato et al., 2000, Yanai et al., 2005). Some IRFs are constitutively expressed, while the expression of others is inducible (Miyamoto et al., 1988, Sato et al., 2000, Yanai et al., 2005, Honda & Taniguchi, 2006). There is variation within the IRF family and the genes they target. Ultimately, however, all characterized IRFs work to protect the host in the response to pathogens. Each member of the IRF family shares extensive homology in the N-terminal DNA binding domain, characterized by five tryptophan repeat elements located within the first 150 amino acids of the protein. The DNA binding domain of IRFs mediates binding to GAAANN and AANNNGAA sequences, termed the ISRE in ISGs (Hiscott, 2007). In addition to their role in immune regulation, IRFs are also involved regulation of the cell cycle, apoptosis, and tumor suppression (Honda and Taniguchi, 2006). These functions are described in table I.

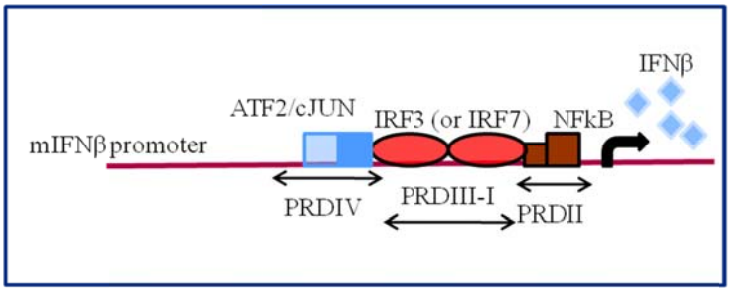


Figure 2. The murine (m)IFNβ promoter. The promoter contains binding sites called positive regulatory domains (PRD) I-IV. PRDI and PRDIII bind IRFs, PRD II binds NF-κB and PRDIV binds ATF-2/cJUN. Depending on the IRF, binding generally results in production of IFNβ as opposed to its negative regulation. Modified from Bonnefoy *et al.* (1999).

Of the nine known members of the IRF family of transcription factors, most members of this family are known to positively regulate type I IFN production (Honda & Taniguchi, 2006). This thesis will discuss only a subset including IRF1 (Miyamoto et al., 1988), IRF3 (Sato et al., 2000), IRF7 (Sato et al., 2000), and IRF9 (Kawakami et al., 1995, Harada et al., 1996). Although IRF5 and IRF8 positively regulate IFNβ, IRF5 is not activated in response to poly I:C (Honda & Taniguchi, 2006, Paun & Pitha, 2007) and expression of IRF8 is restricted to hematopoietic cells (Chen-Feng et al., 2009). As this thesis focuses on innate responses in primary fibroblasts to dsRNA, neither IRF5 nor IRF8 were investigated.

Table I. Summary of known IRFs, their expression, target genes and function. Circled IRFs are known to positively regulate the production of type I IFNs. ND, not yet determined. Modified from Honda & Taniguchi, 2006.

IRF	Expression	Target genes	Function of target gene
IRF1	Constitutive and inducible by IFNγ	iNOS, GBP1	Promotes antibacterial & antiviral innate immunity
		IL12	Promotes Th1 cell responses
		CDKN1A	Controls cell cycle
IRF2	Constitutive	IFNβ	Attenuates type I IFN responses
IRF3	Constitutive	IFNα, IFNβ, IFIT1, CXCL10	Promotes antibacterial & antiviral innate immunity
IRF4	Constitutive and inducible by TLR ligation	IL4, GATA3	Controls T cell function
IRF5	Constitutive and inducible by type I IFNs and by TLR ligation	IL6, IL12, TNFα, CXCL2	Promotes inflammation
IRF6	ND	ND	ND
IRF7	Constitutive and inducible by type I IFNs	IFNα, IFNβ	Promotes antiviral innate immunity
IRF8	Constitutive and inducible by IFNγ	IL8, IL12	Promotes Th1 cell responses
IRF9	Constitutive	ISGs	Mediates type I IFN responses

1.3.1 IRF1

IRF1 was discovered in 1988 (Miyamoto et al., 1988). It is located in the cytoplasm and can translocate into the nucleus by TLR signalling (Miyamoto et al., 1988, Honda & Taniguchi, 2006). IRF1 expression is constitutive and IFN-inducible in most cell types (Honda & Taniguchi, 2006). It was originally found to induce an antiviral state by upregulating IFNβ (Miyamoto et al., 1988, Kimura et al., 1996); it also upregulates, albeit at lower levels, IFNα in hematopoetic cells (Miyamoto et al., 1988). However, studies in IRF1$^{-/-}$ MEFs demonstrated induction of IFNβ in a normal manner, implying that it is not essential to type I IFN induction (Matsuyama et al., 1993).

IRF1 has become better known for its role in cell growth and oncogenesis. It suppresses oncogene induced transformation and is required for DNA damage-induced growth arrest (Tanaka et al., 1994, Tamura et al., 2008).

1.3.2 IRF3 and IRF7

IRF3 and IRF7, which are highly homologous, have gained much attention as crucial mediators of type I IFN gene expression elicited by viruses. Discovered in late 1995, IRF3 is constitutively expressed (Au et al., 1995). In contrast, IRF7, which was discovered in 1997 based on its association with Epstein-Barr Virus latency, is expressed in small amounts and strongly induced by type I IFN-mediated signalling (Honda & Taniguchi, 2006). Following viral infection, serine residues in the C-terminal region of IRF3 are phosphorylated, thereby activating IRF3. IRF3 resides in the cytosol in latent form and undergoes nuclear translocation following viral infection and phosphorylation

(Lin et al., 1998, Tamura et al., 2008). Phosphorylated IRF3 forms either a homodimer or a heterodimer with IRF7, enabling the IRF to interact with the co-activators CBP (cyclic-AMP-responsive-element-binding protein (CREB)-binding protein) or p300 to form a holocomplex in the nucleus which then binds type I IFN gene promoters. Consequently, efficient transcription of target genes is initiated (Sato et al., 1998, Hoshino et al., 2006, Honda & Taniguchi, 2006).

Secreted IFNs induce a secondary arm of IFN-mediated signalling, discussed in subsequent sections, including increased IRF7 transcription (Decker et al., 1991, Goodbourn et al., 2000). Similar to IRF3, IRF7 resides in the cytosol and undergoes phosphorylation of its C-terminal region following viral infection. After forming either a homodimer or a heterodimer with IRF3, it translocates to the nucleus (Honda & Taniguchi, 2006).

IRF3 is a potent activator of the IFNβ gene, and the IFNβ-inducible IRF7 efficiently enhances transcription of IFNβ in fibroblasts following initial IRF3-mediated signalling (Au et al., 1998, Sato et al., 2000). These findings led to a model in which IRF3 initiates a two-step induction of type I IFN genes by a positive-feedback loop whereby induction of IRF7 enhances the antiviral response by allowing the efficient production of type I IFNs during viral infection. However, subsequent generation of $IRF7^{-/-}$ mice demonstrated that the induction of type I IFN genes by ssRNA viruses is severely impaired (Honda et al., 2005a), indicating that IRF7 is a key component of the cytosolic pathway of type I IFN gene induction by these viruses. As such, a model was proposed in which IRF7, although expressed in small amounts, seems to be crucial for

initial induction of type I IFNs. Thus, the homodimer of IRF7 or the heterodimer of IRF7 and IRF3, rather than the homodimer of IRF3, might be more important for type I IFN gene induction by viruses. The IRF3 homodimer was subsequently deemed important for the induction of other genes, such as the gene that encodes IFN-inducible protein (IP) 10 (Nakaya et al., 2001). However, pivotal work by Sato *et al.* (2000) found that in the absence of IRF3, mice are more susceptible to virus infection due to reduced type I IFN expression. Furthermore, the authors generated mice deficient for IRF3 and IRF7 and found they were poorly protected against infection with Newcastle Disease virus (NDV; Sato et al., 2000). This study showed that, as hypothesized earlier, IRF3 functions mainly for IFNβ production in the early phase and subsequently cooperates with IRF7 in later phases of IFN production for positive feedback; ultimately, IRF3 and IRF7 perform non-redundant roles. The importance of IRF3 is particularly emphasized based on the fact that IRF7 is undetectable in most non-hematopoietic cells prior to initial induction of IFNβ. Thus, in cells such as fibroblasts, initial IFNβ production is likely the result of IRF3 activity (Escalante et al., 2007).

1.3.3 IRF9

IRF9, also referred to as ISGF3γ or p48, was discovered between 1988 and 1990. It was originally identified as part of the ISGF3 complex, which was considered one protein until 1990 when a subunit of the ISGF3 complex responding to IFNγ was identified (Levy et al., 1988, Levy et al., 1990). Localization of IRF9 is constitutive in the cytoplasm and upon activation, translocates to the nucleus. This protein may also be induced by type I IFNs in various cell types (Tamura et al., 2008). It associates with signal transducers and activators of transcription (STAT)-1 and STAT2 to form the heterotrimer ISGF3 (Fu et al., 1990). This heterotrimer stimulates type I IFN inducible genes such as 2',5'-oligoadenylate synthetase (OAS), IP10, ISG56 (Honda & Taniguchi, 2006) and IRF7 (figure 1; Lu et al., 2000). IRF9, although known as part of the ISGF3 complex, also binds to the IFNβ promoter to allow for IFNβ transcription (Kawakami et al., 1995, Harada et al., 1996).

1.4 Induction of type I IFNs and Establishment of an Antiviral State

First discovered in 1957, IFNs belong to a family of structurally related cytokines and are found only in vertebrates (Isaacs & Lindemann, 1957, Takaoka & Yanai, 2006). The family of IFNs is subdivided into type I, type II, and type III IFNs; they are differentiated by their signalling through distinct receptors. Type I IFNs are best known for inducing an antiviral, or a protective, state in virus-infected and neighbouring cells (Isaacs & Lindemann, 1957). They also regulate the subsequent cellular immune response (Honda et al., 2005b). Type I IFNs consist of 14 IFNα subtypes in mice (13 in

humans) and a single IFNβ subtype in both species (Honda & Taniguchi, 2006). While IFNα can be induced from lymphoid cells upon viral infection, IFNβ is inducible in most cell types (Stark et al., 1998). Type II IFN consists of the cytokine IFNγ, which is induced by T-cells, natural killer (NK) cells, and neutrophils; IFNγ is primarily associated with the adaptive immune response (Farrar & Schreiber, 1993). Type III IFN, also known as IFNλ, or IL28/29, has a similar biological function to type I IFNs, but is primarily involved in host defense at the epithelial surface (Ank & Paludan, 2009). Once induced, all members of the IFN family interact with a type-specific receptor complex consisting of a pair of heterologous subunits (Takaoka & Yanai, 2006).

Type I IFNs, which will be the focus of this thesis, bind the type I IFN α/β receptor (IFNR), which is composed of two subunits, IFNR1 and IFNR2 (Jaks et al., 2007). Via the Jak-STAT signalling pathway, the ligand-receptor interaction of type I IFNs and IFNR activates ISGF3 (Decker et al., 1991, Rani & Ransohoff, 2005), which, as discussed, is a transcriptional complex consisting of STAT1, STAT2, and IRF9 (Pestka, 1997). IFNs act in an autocrine and paracrine fashion for the purpose of stimulating their cognate receptor on the infected cell as well as the IFNR of cells in close proximity (Reis et al., 1989, Yoneyama et al., 1996, Honda et al., 2005b). This leads to the expression of ISGs which are important for antiviral responses, including increased antigen presentation via upregulation of major histocompatibility complex (MHC) class I molecules, cell death to prevent viral replication, messenger (m)RNA degradation, and translational arrest (Goodbourn et al., 2000). The antiviral response serves to either block or impair viral replication and spread.

In mouse embryo fibroblasts (MEFs), IFNβ is made shortly after virus infection in most cases. IFNα subspecies are not made (DeWitte-Orr et al., 2009). IFNβ transcription is important for protection against many different types of viruses. One of the best understood and most studied example of a virus-inducible transcription unit is the IFNβ promoter, or the virus response element (VRE). The mIFNβ promoter, located -92 to -50 relative to the transcription start site, corresponds with the minimal DNA sequence necessary for virus-induced transcription of IFNβ. The VRE, conserved between mice and humans, is composed of four regulatory domains, PRDI-IV. Transcription of this gene would not be possible without binding of three families of transcription factors to the PRDs of IFNβ promoter, which has binding sites for ATF-2/cJUN, IRFs, such as IRF3, and the p50 and p65 subunits of NF-κB (Bonnefoy et al., 1999, Weill et al., 2003, Honda et al., 2005b; figure 2). Binding of an architectural protein, high mobility group I (HMGI), located near PRDII and PRDIV, with the transcription factors forms an enhanceosome (Du & Manistis, 1994, Bonnefoy et al., 1999). The enhanceosome modifies and repositions a nucleosome that blocks the formation of a transcriptional pre-initiation complex on the IFNβ promoter (Bonnefoy et al., 1999, Hiscott et al., 2007).

1.5 Viral Modulation of the Host Immune Response and Non-Prototypic Antiviral Counter Mechanisms

Early activation of IRF3 and IFNs has been greatly emphasized in combating infection. However, infection of MEFs deficient for IRF3 with NDV induced a number of IRF3-independent direct response genes, including several p200 family proteins (Andersen et al., 2008). Furthermore, we recently published that in response to long dsRNA molecules, an IRF3-independent antiviral response including IFN and ISG production is observed (DeWitte-Orr et al., 2009). Alternatively, ISG induction and antiviral protection can be independent of IFNs due to IRF3 binding directly to the promoter of a subset of ISGs (Grandvaux et al., 2002).

Viruses such as the highly successful human HSV-1 inactivate IRF3 (Melroe et al., 2004, Lin et al., 2004, Melroe et al., 2007, Paladino et al., 2010) and subvert the type I IFN response (Paladino & Mossman, 2009). In fact, over 200 anti-IRF3 and anti-IFN mechanisms encoded by diverse viruses have been identified (Schröder & Bowie, 2005, Noyce et al., 2008, Versteeg & García-Sastre, 2010). Given the importance of IRF3 and type I IFNs in protection against virus infection, it is likely that all viruses encode mechanisms to disable these proteins. Accordingly, there are compensatory mechanisms to protect the host in the event that either of these crucial proteins is compromised. While there have been numerous studies examining either the IRF3-independent or the IFN-independent antiviral response, until recently, it was unknown if the host could be protected if both IRF3 and IFN were absent.

We recently observed a protective response against both DNA and RNA viruses in the absence of IRF3 and IRF9 in primary MEFs (DeWitte-Orr et al., 2009). In response

to dsRNA, MEFs deficient for IRF3 and IRF9 fail to induce IRFs, IFNs or ISGs, suggesting that the antiviral response is independent of the type I IFN system (DeWitte-Orr et al., 2009). In the absence of IFNs and ISGs, there is approximately 60-90% inhibition of vesicular stomatitis virus (VSV) and herpes simplex virus type 1 (HSV-1) replication following poly I:C stimulation. Interestingly, the earlier and more potent response occurs against HSV-1 (DeWitte-Orr et al., 2009). HSV-1 is a large DNA virus well known for its ability to manipulate and evade the host response (Taylor et al., 2002) in comparison to VSV, which a small RNA virus that is highly susceptible to the effects of type I IFNs (Maheshwari et al., 1980, Banks & Rouse, 1992, Davis-Poynter & Farrell, 1996).

1.6 Nitric Oxide

Small oxygen species such as reactive oxygen species and nitric oxide have increasingly been gaining awareness for their role in antiviral protection. In particular, endogenous nitric oxide plays a role in many physiological functions.

Nitric oxide acts as a vasodilator in response to chemical and physical stimuli such as stress in vascular endothelial cells, and is important for regulation of blood flow and pressure (Moncada et al., 1989, Vanhoutte, 1989, Furchgott, 1990, Ignarro, 1990, Vane et al., 1990, Luscher, 1991). Nitric oxide produced by endothelial cells or platelets serves to inhibit platelet aggregation and adhesion, modulates proliferation of smooth muscle cells and inhibits leukocyte adhesion (Moncada & Higgs, 1993). Nitric oxide is also produced by the neurons of the central nervous system. Here, this small molecule

helps with memory formation, pain modulation, and coordination between neuronal activity and blood flow (Garthwaite, 1991, Snyder & Bredt, 1992). Nitric oxide also exerts its effects in the peripheral nervous system. It acts as a mediator released by a widespread network of nerves to regulate neurogenic vasodilation. It also functions in modulation of certain gastrointestinal and respiratory functions (Gillespie et al., 1990). The mechanisms of these physiological actions of nitric oxide are mediated by the activation of soluble guanylate cyclase and subsequent increase of cyclic guanosine monophosphate in target cells (Murad et al., 1990, Ignarro, 1991). This molecule also plays an important antimicrobial role against numerous pathogens (Umezawa et al., 1997, Alam et al., 2002, Charville et al., 2008). Nitric oxide is generated in large quantities during host defense and immunological reactions (Nathan & Hibbs, 1991, Nussler & Billiar, 1993). In this role, generation of nitric oxide was originally observed in activated macrophages where it contributes to their cytotoxicity against tumour cells, bacteria, viruses and other microorganisms (Hibbs et al., 1988, Marletta et al., 1988, Stuehr et al., 1989).

1.7 Antiviral Actions of Nitric Oxide

The antimicrobial effects of nitric oxide have mostly revolved around its antibacterial effects. However, nitric oxide is also effective in clearance of viruses, particularly DNA viruses (Croen, 1993, Bi & Reis, 1995, Zaki et al., 2005). This may help explain the earlier and more potent antiviral effect that we observed against HSV-1 in comparison to VSV (DeWitte-Orr et al., 2009). Nitric oxide exerts direct antiviral effects that contribute to host resistance to viruses such as HSV-1 (Croen, 1993) and certain RNA viruses such as coxsackievirus (Zaragoza et al., 1999) and dengue virus type II (Takhampunya et al., 2006). Direct cytostatic and cytotoxic actions result from inhibitory actions of nitric oxide on key enzymes in the respiratory chain and synthesis of DNA in target cells (Hibbs et al., 1990, Nguyen et al., 1992). This molecule may also lead to release of other toxic substances such as peroxynitrite by interacting with oxygen-derived radicals (Hibbs, 1992). Nitric oxide may additionally induce oxidative stress. Cytotoxicity resulting from nitric oxide-induced oxidative stress may not only cause immunosuppression and immunopathology, but also cellular and organ dysfunctions (Akaike & Maeda, 2000).

Antiviral effects of nitric oxide can be independent of IFNs and ISGs (Akaike & Maeda, 2000). However, nitric oxide may be produced indirectly by IFNγ activation of nitric oxide synthases (NOSs).

1.8 NOS Involvement in the Formation of Nitric Oxide

As indicated in figure 3, NOSs are important for the production of nitric oxide, as they oxide the amino acid L-arginine to synthesize nitric oxide. Three NOS subtypes are known, inducible (i), endothelial (e), and neuronal (n).

The nomenclature of the three subtypes is an oversimplification. For example, eNOS is found in platelets (Radomski et al., 1990) and certain neuronal populations in the brain in addition to vascular endothelial cells (Dinerman et al., 1994). The isoenzyme nNOS has been found in the epithelium of bronchi and the trachea (Kobzik et al., 1993), as well as in the skeletal muscle (Kobzik et al., 1994). Some differences have also been uncovered between iNOSs from different tissues within the same species (Mohaupt et al., 1994). Although eNOS is generally described as constitutive, it can also be induced during chronic exercise and pregnancy (Sessa et al., 1994, Weiner et al., 1994). Moreover, iNOS is generally considered inducible, but is constitutively expressed in tissues such as human bronchial epithelium, rat kidney cells, and certain fetal tissues (Kobzik et al., 1993, Mohaupt et al., 1994, Baylis et al., 1994).

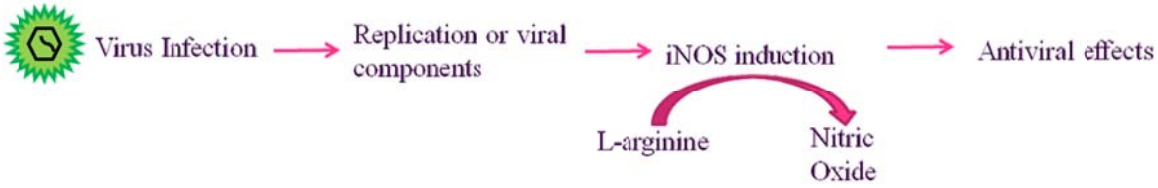


Figure 3. IFN-independent antiviral effects of nitric oxide. Viral replication or viral components such as dsRNA trigger NOS that oxidizes L-arginine to synthesize nitric oxide.

In response to virus infection, it is typically iNOS that is induced (figure 3; Nussler & Billiar, 1993, Akaike & Maeda, 2000, Majano et al., 2001, Lee et al., 2009, Mishra et al., 2009). Alternatively, in many virus infections, iNOS expression can be indirectly regulated by induction of IFNγ, resulting in overproduction of nitric oxide. Regardless of whether iNOS is activated directly or indirectly, its induction depends on different transcription factors. While there are differences in which transcription factors activate iNOS between species, NF-κB and IRF1 seem to be consistent central targets for activators of iNOS expression. Lipopolysaccharide (LPS), IL-1β, TNFα, as well as oxidative stress have been shown to activate NF-κB in different cells types for iNOS induction. IRF1 has also been shown to directly interact with the iNOS promoter in murine systems (Pautz et al., 2010). For example, in response to dsRNA, IRF1 has been shown to be important for iNOS transcription in murine macrophages. However, it has also been shown to be dispensable for iNOS expression and nitric oxide production by mouse islet cells (Blair et al., 2002).

1.9 Study Objectives & Hypotheses

Overall, the goal of this thesis was to better characterize the antiviral response to dsRNA in MEFs. MEFs were employed for numerous reasons. Importantly, MEFs are a primary culture; they are valuable in that they can be readily harvested from mice deficient for key regulators, such as various IRFs and PRRs. Additionally, immortalized cell lines may produce more or less IFN than non-immortalized cells (Nunez et al., 1999, Wang et al., 2009); it was important for the chosen cell line to mimic a normal state. Finally, immortalized cells often have mutations in their type I IFN signalling pathways (Lehman et al., 1993, Fridman & Tainsky, 2008). As such, primary MEF cultures were employed in these studies.

Our previous publication led to two new lines of investigation. The first was to identify the mechanism by which IFNβ is made in the absence of IRF3. The second was to determine the type of antiviral response generated in the absence of IRF3 and IRF9, and hence the type I IFN system. Thus, there are two main hypotheses explored in this thesis. ***First, I hypothesized that in the absence of IRF3, IRF9 compensates in the production of IFNβ and subsequent antiviral protection. Second, in MEFs devoid of IRF3 and IRF9, I hypothesized that nitric oxide is responsible for the early protection observed against HSV-1.*** Thus, this thesis will present two aims of the research conducted:

1. To determine if IRF9 binds to the IRF binding site of the IFNβ promoter in the absence of IRF3;
2. To determine if nitric oxide is involved in the IRF3- and IFN-independent antiviral response.

1.10 Implications of the Study

The immediate and non-specific immune response is important for effective clearance of infection. Type I IFNs have a significant impact on host defense mechanisms; viruses have thus evolved numerous evasion strategies to overcome its effects, as discussed. By studying virus and host interactions, and elucidating archetypical as well as atypical antiviral pathways, it is possible to further current understanding of both viral pathogenesis and host immunology. This project is important in that it may provide researchers with data that can advance the characterization of virus-host interactions. This is essential to further understanding of not only host innate immunity, but also of viruses. This work may lead to further research on viruses that may have therapeutic potential.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cells and Viruses

With the exception of IRF3$^{-/-}$9$^{-/-}$ MEFs, all MEF cultures were derived by Derek Cummings; IRF3$^{-/-}$9$^{-/-}$ MEFs were a generous gift from Dr. Tadatsugu Taniguchi (University of Tokyo). Each culture was propagated in α-MEM (alpha minimal essential medium). The α-MEM was supplemented with 15% fetal bovine serum (FBS; Invitrogen), 100 U•ml^{-1} penicillin, 100μg•ml^{-1} streptomycin and 2mM L-glutamine, and will herein be referred to collectively as complete MEF media. Each culture was incubated at 37°C in a 5% CO_2 humidified incubator.

VSV expressing green fluorescent protein (GFP), provided by Dr. Brian Lichty, (McMaster University) was used in antiviral assays. HSV-1 (KOS strain) expressing GFP (HSV-1gfp) was also used and propagated on Vero cells (Minaker et al., 2005). Vero cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS. For viral infections, cells were split and seeded into dishes 24 hours prior to infection. Infections with both VSVgfp and HSV-1gfp utilized a multiplicity of infection (MOI) of 0.1 particle forming units (PFU) per cell and occurred in serum-free α-MEM for 1 hour. This amount of virus was the maximal dose for which signal saturation in untreated cells did not occur. Following 1 hour of infection, the viral inoculum was removed and replaced with DMEM containing 1% methylcellulose. GFP fluorescence intensity was measured on a Typhoon Trio (GE Healthcare) 24 hours later and quantified using ImageQuant TL software.

2.2 Synthesis of dsRNA

Different lengths of dsRNA were transcribed *in vitro* from West Nile virus (WNv) genome fragments using a Megascript RNAi kit (Ambion). One microgram of PCR fragments amplified from portions of the cloned WNv genome were used as a template for dsRNA synthesis (table II). The length of the dsRNA is denoted in the name; that is, E200, E1000, and NS3000 were transcribed with lengths of 200bp, 1000bp, and 3000bp, respectively. The lengths of 200 and 1000bp were derived from the E protein sequence and the 3000bp length was derived from the NS3-NS4B sequence.

Table II. Primers used for the production of dsRNA. This table is modified from DeWitte-Orr *et al.* (2009).

dsRNA	Source Gene	Length (bp)	Primers
E200	WNv E	200	Sense: TCCTCCAACTGCGAGAAACGTG Antisense: AAAGGAGCGCAGAGACTAGCCG
E1000	WNv E	1000	Sense: TCCTCCAACTGCGAGAAACGTG Antisense: ACACATGCGCCAAATTTGCC
NS3000	NS3-NS4B	3000	Sense: CATGACAACCAACCCCCACGCATGATG Antisense: GCGGGCGTGATGGTTGAAGGTGT

These studies also used poly I:C (GE Healthcare); it is a synthetic dsRNA and its average length was determined to be approximately 4500bp by marker size comparison using agarose gel electrophoresis and a 1-kb Plus DNA ladder (Fermentas).

2.3 Treatment with dsRNA

Cells were treated with dsRNA diluted in OptiMEM® Reduced Serum Medium (OM; Life Technologies) in the presence of 50μg/ml diethylaminoethyl-dextran (DEAE-dextran; Pharmacia) for 1 hour; this combination will herein be referred to as OM/DEAE. Following one hour of treatment, cells were washed twice in complete MEF media. After the second wash, cells were incubated in complete MEF media for additional indicated amounts of time to allow for production of antiviral factors unless otherwise noted. Equal molar amounts of dsRNA were used to ensure an equal number of molecules per dsRNA length. DEAE-dextran was used in dsRNA-untreated controls in all experiments to ensure that the polymer alone was not influencing subsequent cellular responses.

2.4 Preparation of Whole-Cell Extracts and Western Blot Analysis

For preparation of whole-cell extracts, cells grown to 90% confluency were mock- or poly I:C-treated for 3 hours, except where indicated. Cells were subsequently washed twice and harvested in 1x phosphate-buffered saline (PBS). A centrifugation step followed at 200 x g for 3 minutes at 4°C. Pellets were resuspended in whole-cell extract buffer consisting of 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at a pH of 7.4, 100mM NaCl, 10mM β-glycerophosphate, 0.2% Triton-X 100, 5mM NaF, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 2mM dithiothreitol, and 1x protease inhibitor cocktail (Sigma). The cell suspension was lysed on ice for 15 minutes and centrifuged again for 10 minutes at 13000 x g at a temperature of 4°C. Extract concentrations were determined using a Bradford assay kit (Bio-Rad) and 40μg

of denatured extract were run on 10% polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membrane and probed with the specified IRF antibodies (table III).

Table III. Details of IRF antibodies used in Western blot and immunofluorescence analysis. HRP = horse radish peroxidase

IRF Antibody	Source	Dilution for Western Blot	Secondary Antibody for Western Blot	Dilution for Immuno-fluorescence	Secondary Antibody for Immuno-fluorescence
IRF1	Santa Cruz Biotechnology, Inc.	1:200	Anti-rabbit HRP 1:1500	1:50	Anti-rabbit IgG Alexafluor 488 1:1000
IRF3	Lab of Dr. Takashi Fujita (Japan)	1:1000	Anti-rabbit HRP 1:3000	N/A	N/A
IRF7	Santa Cruz Biotechnology, Inc.	1:200	Anti-rabbit HRP 1:1500	1:50	Anti-rabbit IgG Alexafluor 488 1:1000
IRF9	Santa Cruz Biotechnology, Inc.	1:200	Anti-rabbit HRP 1:1500	1:100	Anti-rabbit IgG Alexafluor 488 1:1000

2.5 Antiviral Assays

For antiviral assays, cells were treated with dsRNA diluted in 50µg/ml OM/DEAE or with OM/DEAE alone as a mock treatment. The concentration of dsRNA used in antiviral assays ranged from $8.0x10^{-5}$nM to 3.0nM.

Following either 6 hours or 24 hours of dsRNA pretreatment, cells were challenged with VSVgfp or HSV-1gfp as described above for antiviral assays. HSV-1gfp was used as the challenge virus for all work completed with IRF3$^{-/-}$9$^{-/-}$ MEFs and with some antiviral assays performed with IRF9$^{-/-}$ MEFs. After 24 hours of infection at the specified parameters, plates were scanned on the Typhoon Trio. Using the analysis toolbox mode of the ImageQuant TL program, fluorescence in each of the wells was quantified; a higher amount of fluorescence corresponded with increased virus replication. Baseline values of the mock-treated cells were subtracted from each of the other treatment groups and infection as a percentage of the untreated control was determined. The percent of infection was plotted against dsRNA concentration as the mean ± standard error of the mean (SEM; equation 1), and the half-maximal effective concentration (EC_{50}) was determined for each dsRNA length using GraphPad Prism 5.0.

Equation 1:

$$SEM = \sqrt{[(\Sigma (X - M)^2)/N-1]} / \sqrt{N}$$

where X is the value of the sample, M is the mean, and N is the number of samples.

High, medium, and low protection concentrations were determined from graphs displaying the percent of infection as a function of dsRNA concentration by examination of the plateaus for high and low protection, and the EC_{50} for moderate protection (figure 4).

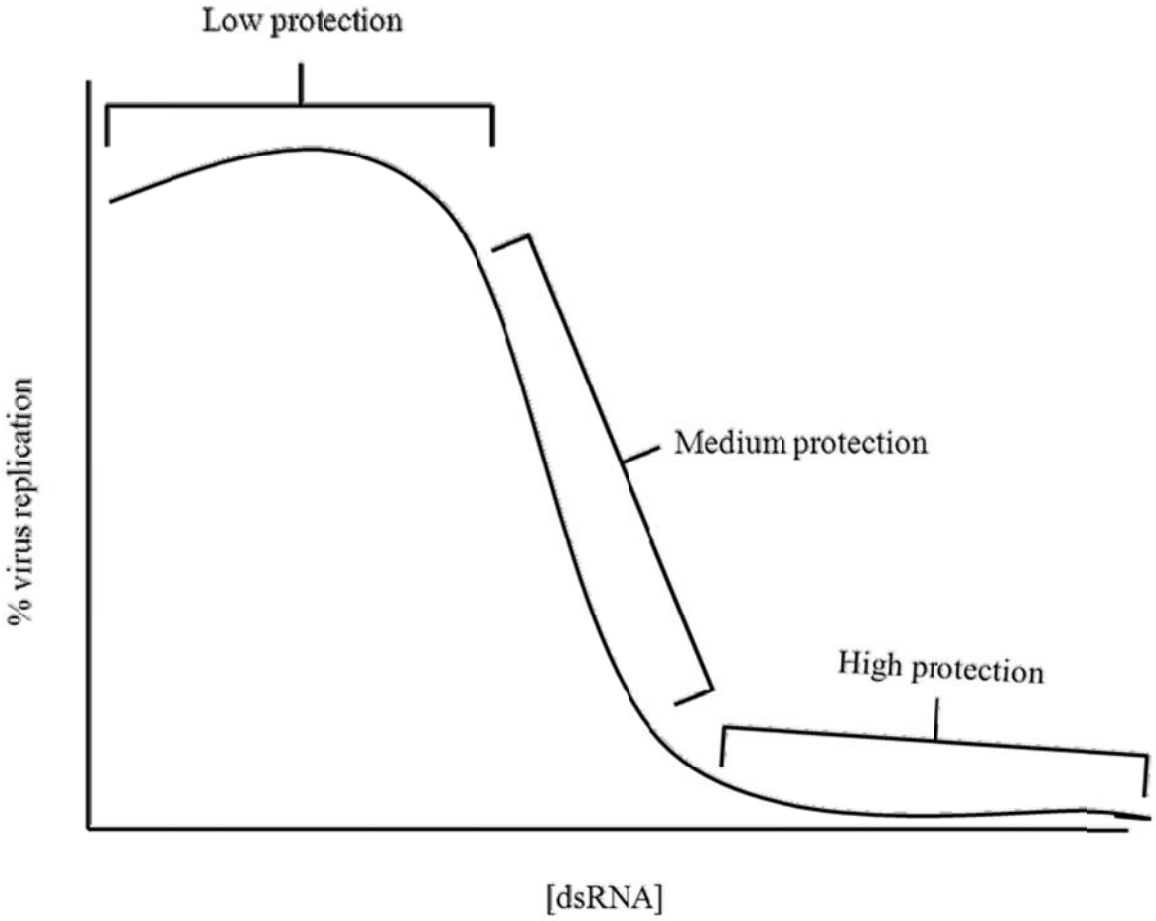


Figure 4. A hypothetical antiviral curve displaying dsRNA concentrations corresponding to areas of high, medium, and low protection in the cell. A high protection range is defined as one that is between 0-20% of the maximal response. A medium protection range is one that is between 21-75% of the maximal response, and a low protection range corresponds to 76-100% of maximal response.

2.6 Quantitative Real Time-PCR (qRT-PCR)

RNA was isolated from cells using TRIzol reagent (Invitrogen). A random 6-mer primer (0.2ng) and 50U of Superscript II (Invitrogen) were used to reverse transcribe 300ng of DNase-treated RNA (DNA-free kit, Ambion) in a total reaction volume of 20µl. Subsequently, qRT-PCR was performed in triplicate using Universal PCR Master Mix and gene-specific TaqMan primers (Applied Biosystems) in a total volume of 25µl. Data were analyzed via the ΔΔ cycle threshold (Ct) method. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the housekeeping gene, and expressed as fold change over the mock-treated group (cells treated with OM/DEAE). TaqMan specific primers used in this study are listed in table IV.

Table IV. Primer information for genes of interest used in qRT-PCR. The assay identification for the gene from Applied Biosystems is provided. Also listed are the transcripts detected and the length of the amplicon for each gene.

Common Name	Assay	Detected Transcript	Amplicon Length
iNOS	**Mm00440502_m1**	**NM_010927.3**	**66bp**
eNOS	**Mm00435217_m1**	**NM_008713.4**	**71bp**
IRF1	**Mm00515191_m1**	**NM_008390.1**	**71bp**
IRF7	**Mm00516788_m1**	**NM_016850.2**	**67bp**
ISG56	**Mm00515153_m1**	**NM_008331.3**	**80bp**
IP10	**Mm99999072_m1**	**NM_021274.1**	**62bp**
IFNβ	**Mm00439552_s1**	**NM_010510.1**	**69bp**
GAPDH	**Mm99999915_g1**	**NM_008084.2**	**107bp**

2.7 NF-κB Inhibitor Preparation

The NF-κB inhibitor Bay 11-7082 (EMD) was prepared in 1% dimethylsulfoxide (DMSO), and used at concentrations ranging from 1-10μM. Cells were incubated with this compound for 0.5 hours prior to mock and poly I:C treatments. DMSO was included in mock- and poly I:C-treatments.

2.8 Immunofluorescence Microscopy

Cells seeded to 60% confluency on cover slips overnight were either mock- or poly I:C-treated for 3 hours as described previously for IRF nuclear translocation experiments. To determine the efficacy of Bay 11-7082 in inhibition of NF-κB in IRF3$^{-/-}$ 9$^{-/-}$ MEFs, cells were either mock- or poly I:C-treated with or without Bay 11-7082 for 5 hours. Following treatment, cells were fixed with 10% formalin and permeabilized with 0.1% Triton X-100 diluted in 1xPBS for 10 minutes each. An overnight blocking step at 4°C followed in 1xPBS with 2% goat serum. IRF antibodies were added to the coverslips for 1 hour at room temperature; antibody details are found in table III. For Bay 11-7082 efficacy experiments, a 1:200 dilution of NF-κB p65 antibody (Santa Cruz Biotechnology, Inc) was added to coverslips for 1 hour at room temperature. A secondary anti-rabbit IgG Alexafluor 488 antibody diluted 1:1000 (Molecular probes) was hybridized for 1 hour at room temperature. Nuclei were stained with Hoescht dye diluted 1:10000 for 10 minutes. All antibody and Hoescht dilutions were performed in 1xPBS with 2% goat serum. Images were taken and analyzed using a Leica DM IRE2 inverted microscope with Openlab software (Improvision). Nuclear translocation of cells that

received poly I:C or poly I:C and Bay 11-7082 was plotted as a percent of mock-treated cells.

2.9 Cytoplasmic and Nuclear Extract Preparation

For preparation of cytoplasmic and nuclear extracts, cells grown to 90% confluency were mock-treated or treated with poly I:C for 3 hours. Cells were subsequently washed twice in 1x PBS and once in 0.2xPBS. After thorough washing, cells were harvested in hypotonic buffer (10mM HEPES, pH 7.4, 10mM KCl, 1.5mM $MgCl_2$, 50mM NaF, 0.5mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail and 1mM Na_3VO_4). A centrifugation step followed at 12000 x g for 3 minutes at 4°C. The supernatant was collected as the cytoplasmic extract and pellets were resuspended in a high-salt buffer consisting of 20mM HEPES at a pH of 7.4, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 50mM dithiothreitol, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail. The cell suspension was lysed on ice for 30 minutes and centrifuged again for 15 minutes at 13000 x g at 4°C. Extract concentrations were determined using a Bradford assay kit (Bio-Rad).

2.10 Oligonucleotide Pull-Down Assay

Double-stranded oligomers corresponding to the mIFNβ virus response element (VRE), also referred to as the mIFNβ promoter, incorporating only the IRF binding site were designed. These oligomers will be referred to as the short VRE due to the fact that they did not incorporate the NF-κB or ATF-2/cJUN binding sites. The short VRE sense oligomer (5'-GAGGAAAACTGAAAGGGAGAACTGAAAGTGG-3') and the antisense oligomer that was biotin-labeled on the 5' end (5'-CCACTTTCAGTTCTCC CTTTCAGTTTTCCTC-3') were annealed. A total of 2µg of the double-stranded oligomers were incubated with 200µl streptavidin magnetic beads (Dynal Inc.) for 1 hour in TEN buffer (20mM tris(hydroxymethyl)aminomethane, pH 8.0, 1mM EDTA, and 0.1M NaCl) and the unbound DNA was removed by extensive washing with the same buffer. Cytoplasmic and nuclear extracts were pooled in a 1:1 ratio for a total of 350µg of protein. IRFs from pooled nuclear and cytoplasmic extracts were bound to DNA on magnetic beads overnight. Beads were then washed with binding buffer (10% glycerol, 12mM HEPES, pH 7.4, 5mM $MgCl_2$, 60mM KCl, 0.1mM dithiothreitol and 0.1mM phenylmethylsulfonyl fluoride) and the bound proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A total of 40µg unbound protein extract was loaded and served as a positive control. IRFs were identified by Western blot with the respective antibodies as described in table III.

2.11 Supernatant Transfer

Supernatants from mock-treated cells and cells treated with 8.5nM poly I:C for 5 hours were transferred to naïve IRF3$^{-/-}$9$^{-/-}$ MEFs for another 5 hours. Cells were challenged with HSV-1gfp to determine if a soluble factor confers resistance to infection in the absence of both IRF3 and IRF9. Viral replication was quantified as assessed by GFP fluorescence. To ensure no residual poly I:C in the transferred supernatants was conferring resistance to HSV-1 replication, absorbance of the supernatants was measured. In complete MEF media, poly I:C was serially diluted 1:5 with concentrations ranging from 2.2x10^{-5}-8.5nM. This concentration range corresponds with that used in antiviral assays described previously (DeWitte-Orr et al., 2009). A spectrophotometer was used to determine the absorbance of each concentration to derive a linear curve. To compare, absorbance of the supernatants at the time of transfer was also measured.

2.12 Measurement of Nitric Oxide Production

MEFs were seeded in 96-well plates to approximately 70% confluency. After 24 hours, the cells were incubated with poly I:C for 5 hours. Following treatment with poly I:C, the concentration of nitric oxide in the supernatants of MEF cultures was assessed by measurement of NO_2^-, an oxidized metabolite of nitric oxide. For this, a Griess reaction was performed as previously described (Nazli et al., 2010). Standards were prepared with known concentrations of $NaNO_2$ (BDH) ranging from 0-20μM prepared in α-MEM. Griess reagent (Sigma, USA) was prepared according to the manufacturer's instructions and subsequently added to standards and samples. The absorbance of the supernatants in

the plates was read at 550nm after 10 minutes of incubation at room temperature with Griess reagent.

2.13 Nitrite Release

WT and IRF3$^{-/-}$9$^{-/-}$ MEFs were seeded at a confluency of ~80% overnight prior to treatments in 12-well plates for antiviral assays, and in 96-well plates to assess nitrite release via Griess assay. The cells were treated with 0-200μM diethylenetriamineNONOate (DETA-NO; Sigma-Alrdich) or with 0-200μM of the control NONOate, DETA (diethylenetriamine; Sigma-Aldrich) diluted in complete MEF medium for 5 hours. Following treatment, cells were challenged with HSV-1gfp in an antiviral assay and viral replication was quantified as assessed by GFP fluorescence. Fresh DETA or DETA-NO was added after each medium change.

2.14 Inhibition of iNOS

The iNOS inhibitors aminoguanidine hydrochloride (AMG) and N6-(1-iminoethyl)-L-lysine, dihydrochloride (L-NIL) (Sigma, USA), were diluted in complete medium to a final in-well concentration of 10μM. To investigate the efficacy of the iNOS inhibitors, cells were pretreated for 2 hours with iNOS inhibitors prior to a 5 hour treatment with poly I:C. RNA was then collected using TRIzol and prepared for qRT-PCR. Expression of iNOS transcript levels both with and without inhibitor were compared using TaqMan specific primers to determine efficacy of each of the iNOS inhibitors used.

To investigate the role of iNOS in the antiviral response, cells seeded the previous day in 12-well plates to 80% confluency were treated as described above. Cells were subsequently challenged with HSV-1gfp as indicated previously.

2.15 IRF1 Short Interfering (si)RNA

An oligonucleotide specific for IRF1, (5'-CAGACATCGAGGAAGTGAAGGA TCA-3') and a scrambled sequence (scr; 5'-CAGTAGCGAAGGAGTAAGGACATCA-3') were designed (Thermo Scientific). The scrambled sequence was used to account for nonspecific knockdown of IRF1. The selected target sequences were tested so as not to match any known murine gene (other than murine IRF1) sequences by using NCBI nucleotide BLAST at http://www.ncbi.nlm.nih.gov/BLAST/. Transfection was performed according to the manufacturer's protocol. IRF1 siRNA and scrambled siRNA were used at a concentration of 50μM. IRF1 gene expression following knockdown was quantified by qRT-PCR.

2.16 Statistical Analyses

A 1-way analysis of variance (ANOVA) with a Tukey post-test was used to compare the means of 4 concentrations of Bay 11-7082 in inhibition of NF-κB nuclear translocation. A Student's t-test was used to compare the means of two groups where indicated. All analyses were performed using GraphPad Prism 5.0 software.

CHAPTER 3. IRF9 COMPENSATES IN THE ABSENCE OF IRF3 FOR IFNβ PRODUCTION

3.1 Characterization of Endogenous and Induced IRFs in Response to DsRNA

To identify the transcription factor involved in the antiviral response in the absence of IRF3, several IRFs that positively regulate type I IFNs were characterized. First, endogenous and induced levels of IRF1, IRF3, IRF7 and IRF9 were determined by Western blot analysis (figure 5). Simultaneously, IRF-deficient MEFs were characterized to confirm the genotype of each knockout. Levels of IRF1 protein were not altered in response to poly I:C treatment in WT, $IRF3^{-/-}$ and $IRF3^{-/-}7^{-/-}$ MEFs. IRF1 protein was not detected in $IRF1^{-/-}$ MEFs (figure 5a). Levels of IRF3 in WT MEFs also did not change upon treatment with poly I:C for 3 hours. IRF3, as expected, was not present in $IRF3^{-/-}$ and $IRF3^{-/-}7^{-/-}$ MEFs (figure 5b). IRF7 was induced upon poly I:C treatment in WT and $IRF3^{-/-}$ MEFs, and was not detected in $IRF3^{-/-}7^{-/-}$ MEFs (figure 5c). Unlike the other IRFs investigated, IRF9 was found to increase in response to 3 hours of poly I:C treatment in WT, $IRF3^{-/-}$ and $IRF3^{-/-}7^{-/-}$ MEFs. A band corresponding with IRF9 was not found to be present in $IRF9^{-/-}$ MEFs (figure 5d). Deficiency of IRF7 in $IRF7^{-/-}$ MEFs and IRF3 and IRF9 in $IRF3^{-/-}9^{-/-}$ MEFs was also evaluated by genotyping performed by Derek Cummings (data not shown).

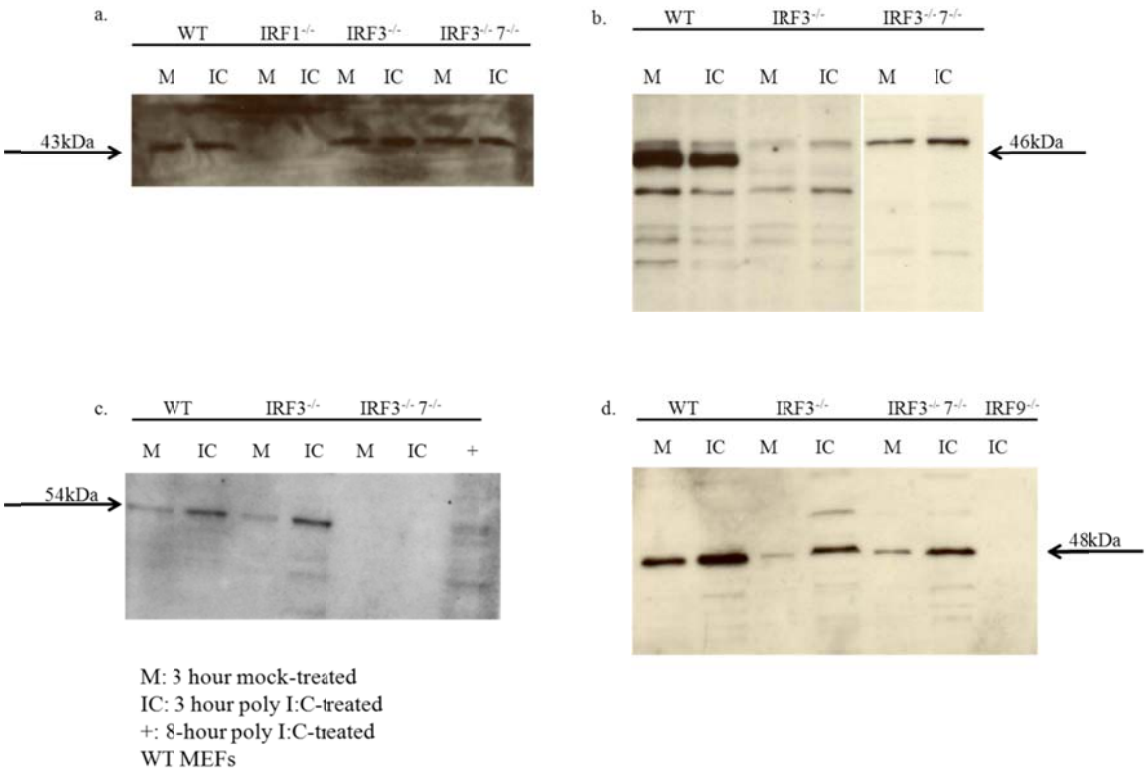


Figure 5. Western blot characterizing endogenous and induced levels of IRFs that positively regulate IFNβ in MEFs after 3 hours of mock- or poly I:C-treatment. Levels of IRF1 (a) IRF3 (b) IRF7 (c) and IRF9 (d) were assayed in the indicated MEF cultures. M: 3 hour mock-treated cultures; IC: 3 hour poly I:C-treated cultures; +: 8-hour poly I:C-treated WT MEFs.

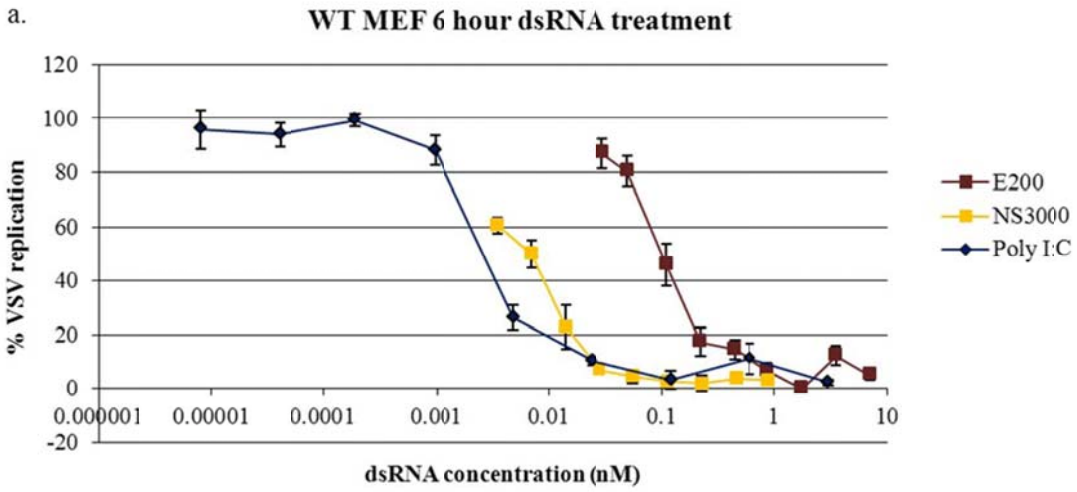
3.2 Evaluation of the ability of IRF1 and IRF7 to compensate for IFNβ and ISG production in the absence of IRF3

Prior to the discovery of IRF3, IRF1 was the presumed antiviral factor as it was discovered based on its capacity to induce an antiviral state by upregulating IFNβ (Miyamoto et al., 1988; Kimura et al., 1996). Studies in IRF1$^{-/-}$ MEFs showed normal production of type I IFNs however (Matsumaya et al., 1993), and IRF3 was later attributed with this ability to regulate IFN production (Sato et al., 2000). Thus, IRF1 was investigated for its ability to compensate for IFNβ production in the absence of IRF3 by siRNA during work performed with Dr. Stephanie DeWitte-Orr during my tenure in the

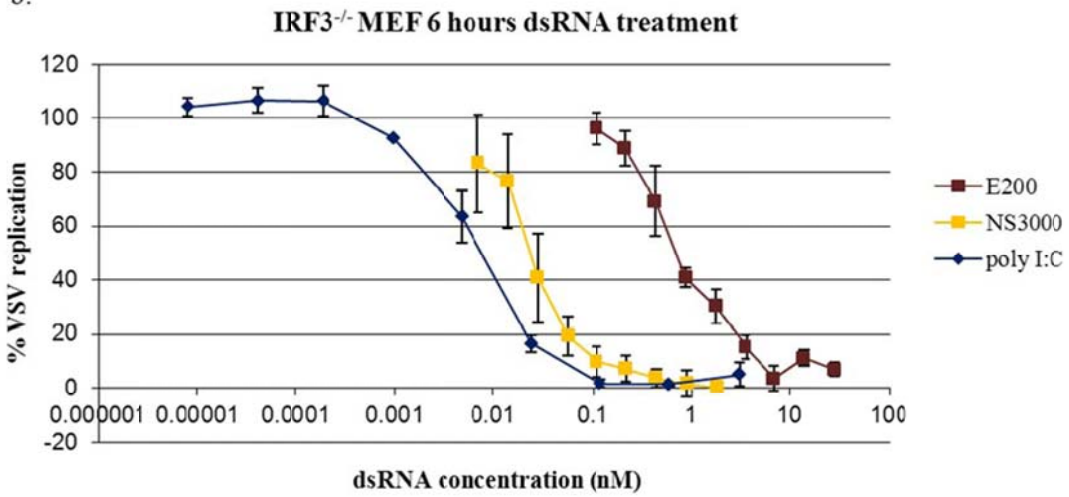
lab as an undergraduate student. We found that rather than diminish ISG production, IRF1 knockdown in IRF3-deficient MEFs dramatically increased ISG production (data not shown). This observation suggests that IRF1 negatively regulates ISG production in the absence of IRF3. To confirm this unexpected result, we are in the process of creating mice deficient for both IRF1 and IRF3.

Based on what is known of IRF7, it was another potential transcription factor worth investigating for involvement in IFNβ production in the absence of IRF3. The ability of IRF7 to compensate for IRF3 was investigated in an antiviral assay in which $IRF3^{-/-}7^{-/-}$ MEFs were pretreated with dsRNA and subsequently challenged with VSV. Antiviral protection data for dsRNA-treated WT (figure 6a) and $IRF3^{-/-}$ MEFs (figure 6b) were included for comparison purposes. Cells deficient for both IRF3 and IRF7 displayed no antiviral protection against VSV after treatment with a 200bp or 3000bp length of dsRNA. With higher concentrations of poly I:C, which averaged 4500bp in length, approximately 50% of pretreated cells were protected against VSV within 6 hours (figure 6c). When $IRF3^{-/-}7^{-/-}$ MEFs were pretreated with dsRNA for 24 hours, a length-dependent protective trend was observed; however, full protection was not observed regardless of the length of dsRNA used or the amount of time cells were pretreated with it (figure 6d).

a.


WT MEF 6 hour dsRNA treatment
% VSV replication
120
100
80
60
40
20
0
-20
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)
E200
NS3000
Poly I:C

b.


IRF3-/- MEF 6 hours dsRNA treatment
% VSV replication
120
100
80
60
40
20
0
-20
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
dsRNA concentration (nM)
E200
NS3000
poly I:C

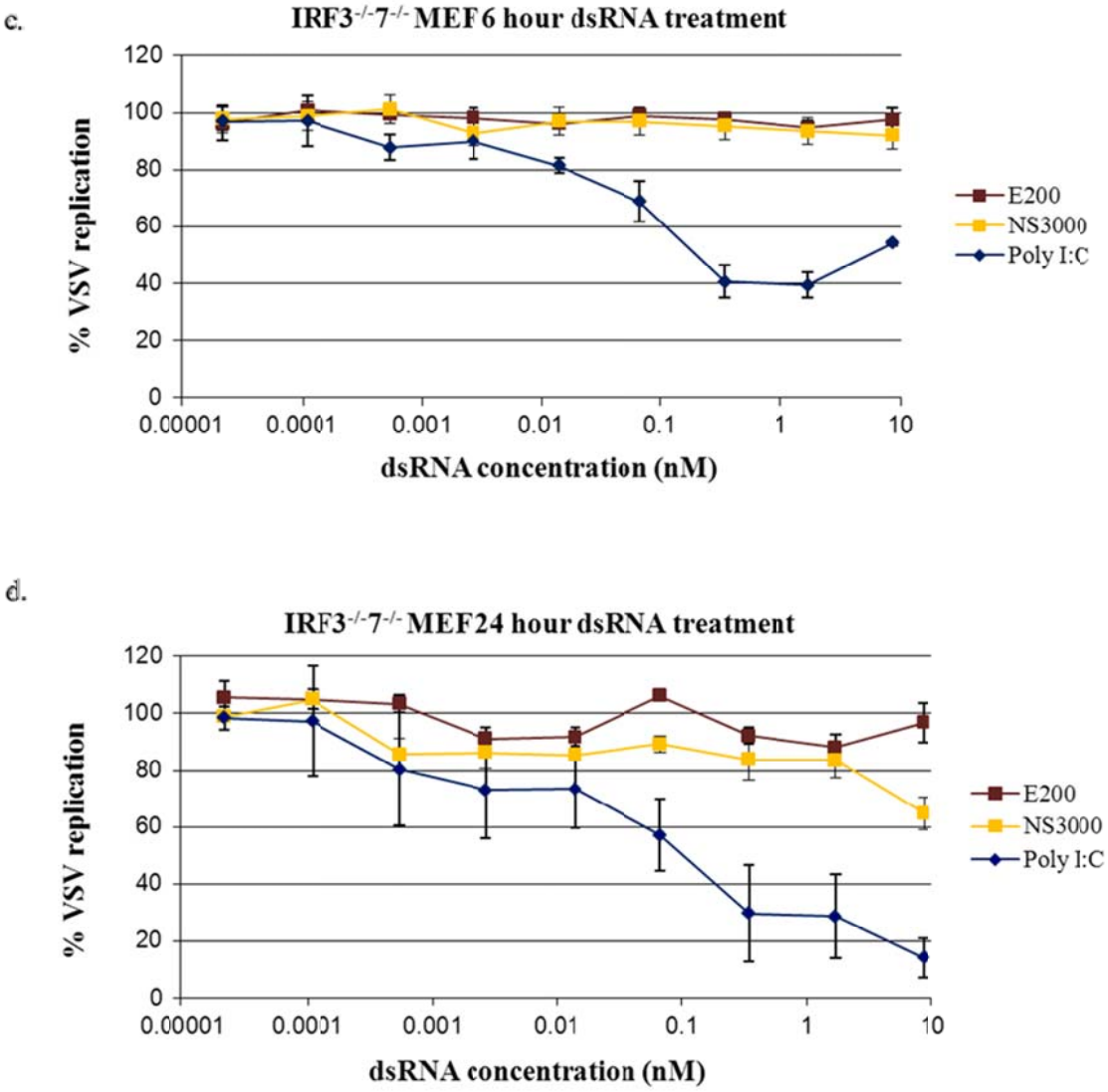


Figure 6. Importance of IRF7 to antiviral protection as determined by antiviral assays. Antiviral protection in WT (a) and IRF3$^{-/-}$ MEFs (b) after 6 hours of poly I:C treatment was compared to protection observed in IRF3$^{-/-}$7$^{-/-}$ MEFs 6 hours (c) or 24 hours (d) hours following dsRNA treatment. Fluorescence from mock-treated, VSV-infected cells was set at 100%. Data are expressed as the mean ± SEM; n=3. Protection data from WT and IRF3$^{-/-}$ MEFs have been published previously (DeWitte-Orr et al., 2009).

As antiviral protection was observed in MEFs deficient of IRF3 and IRF7, it was unknown whether this response was due to IFNβ-mediated production of ISGs. A qRT-PCR assay was performed to determine if IRF3$^{-/-}$7$^{-/-}$ MEFs make IFNβ and ISGs in response to poly I:C treatment. As shown in figure 7, in the absence of IRF3 and IRF7, poly I:C treatment increased ISG56, IP10 and IFNβ mRNA accumulation within 6 hours. Collectively, these data suggest that neither IRF1 nor IRF7 play a dominant role in compensating for the absence IRF3 in the production of IFNβ or ISGs.

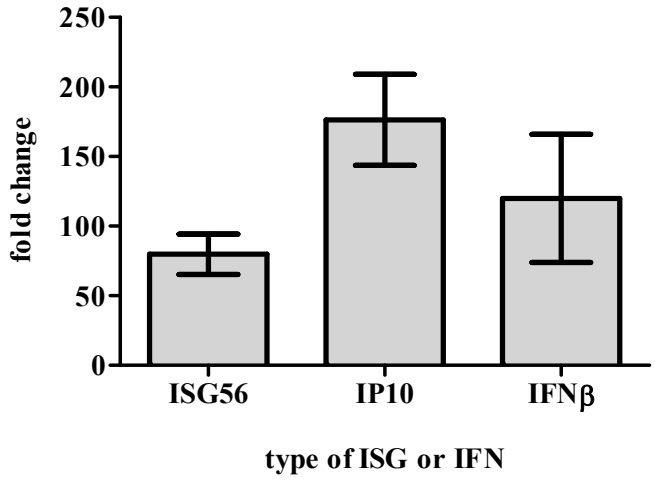


Figure 7. ISG and IFNβ production in IRF3$^{-/-}$7$^{-/-}$ MEF lines. ISG mRNAs were measured by qRT-PCR following 6 hours of poly I:C treatment. Fold changes in transcript levels were compared relative to the housekeeping gene, GAPDH. Data represent the mean ± SEM; n=3.

3.3 Evaluation of the ability of IRF9 to compensate for IFNβ and ISG production in the absence of IRF3

Interestingly, we previously observed partial antiviral protection in MEFs deficient for IRF3 and IRF9. However, these MEFs do not produce detectable levels of IFNs or ISGs in response to poly I:C (DeWitte-Orr et al., 2009). The simplest interpretation of these data is that IRF9 plays an essential role in compensating for IRF3 in IFN and ISG induction. Although IRF9 is known for its role as a component of the ISGF3 complex, it was previously shown to bind to the promoter of the IFNβ gene (Kawakami et al., 1995, Harada et al., 1996).

Consistent with the antiviral assays, it was determined by immunofluorescence microscopy that IRF1 and IRF7 do not significantly translocate to the nuclei of WT, $IRF3^{-/-}$ or $IRF3^{-/-}7^{-/-}$ MEFs after 3 hours of 3.0nM poly I:C treatment. However, IRF9 nuclear translocation appears to increase in $IRF3^{-/-}$ and $IRF3^{-/-}7^{-/-}$ MEFs in comparison to WT MEFs; the increased nuclear translocation in $IRF3^{-/-}$ was found to be significant (figure 8).

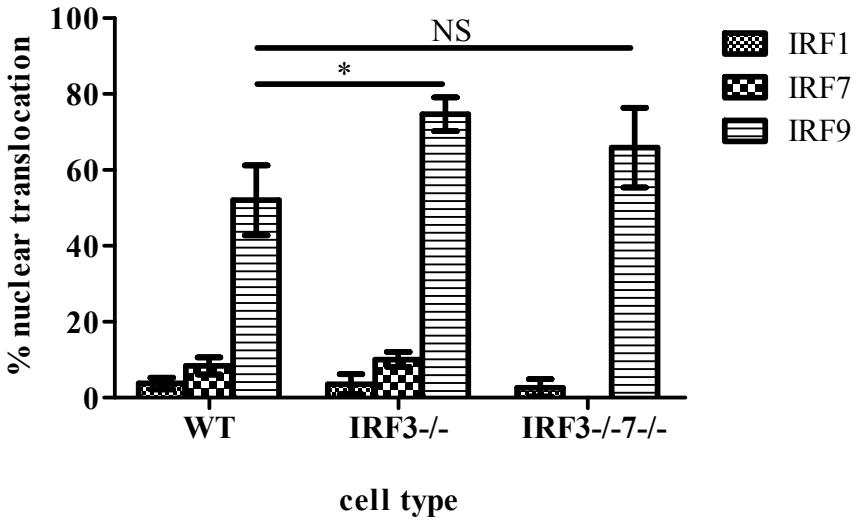


Figure 8. Nuclear translocation of the proteins IRF1, IRF7, and IRF9 in WT, IRF3$^{-/-}$ and IRF3$^{-/-}$7$^{-/-}$ MEFs following a 3.0nM poly I:C treatment for 3 hours. Nuclear translocation was assessed by immunofluorescence microscopy and is graphed as a percent of mock-treated cells. Data represent the mean of 3 individual replicates ± SEM. *, p<0.05.

As IFNs and ISGs were not detected in the absence of IRF3 and IRF9 (DeWitte-Orr et al., 2009) and increased nuclear translocation of IRF9 was observed in IRF3$^{-/-}$ and IRF3$^{-/-}$7$^{-/-}$ MEFs, IRF9 was investigated for its ability to compensate for IRF3 in IFNβ production. Oligonucleotide pull down assays were performed in WT, IRF3$^{-/-}$ and IRF3$^{-/-}$ 7$^{-/-}$ MEFs to investigate a panel of IRFs that positively regulate IFNβ production.

IRF3 bound to the short VRE of the mIFNβ promoter in WT MEFs served as a control for this experiment (figure 9a). As a positive control for the antibodies used, unbound protein extracts were loaded and probed for IRF1, IRF3, IRF7, and IRF9. In poly I:C-stimulated WT MEFs, binding of IRF1, IRF7, or IRF9 to the promoter was not observed (figure 9b-d). In the absence of IRF3, faint amounts of IRF1 and IRF7 were bound to the promoter in both mock-treated and poly I:C-treated extracts (figure 9b, c). In

contrast, IRF9 was very strongly bound to the VRE in poly I:C-treated IRF3$^{-/-}$ and IRF3$^{-/-}$ 7$^{-/-}$ MEFs (figure d). Taken together, these data suggest that IRF9 is an important mediator of the antiviral response in the absence of IRF3.

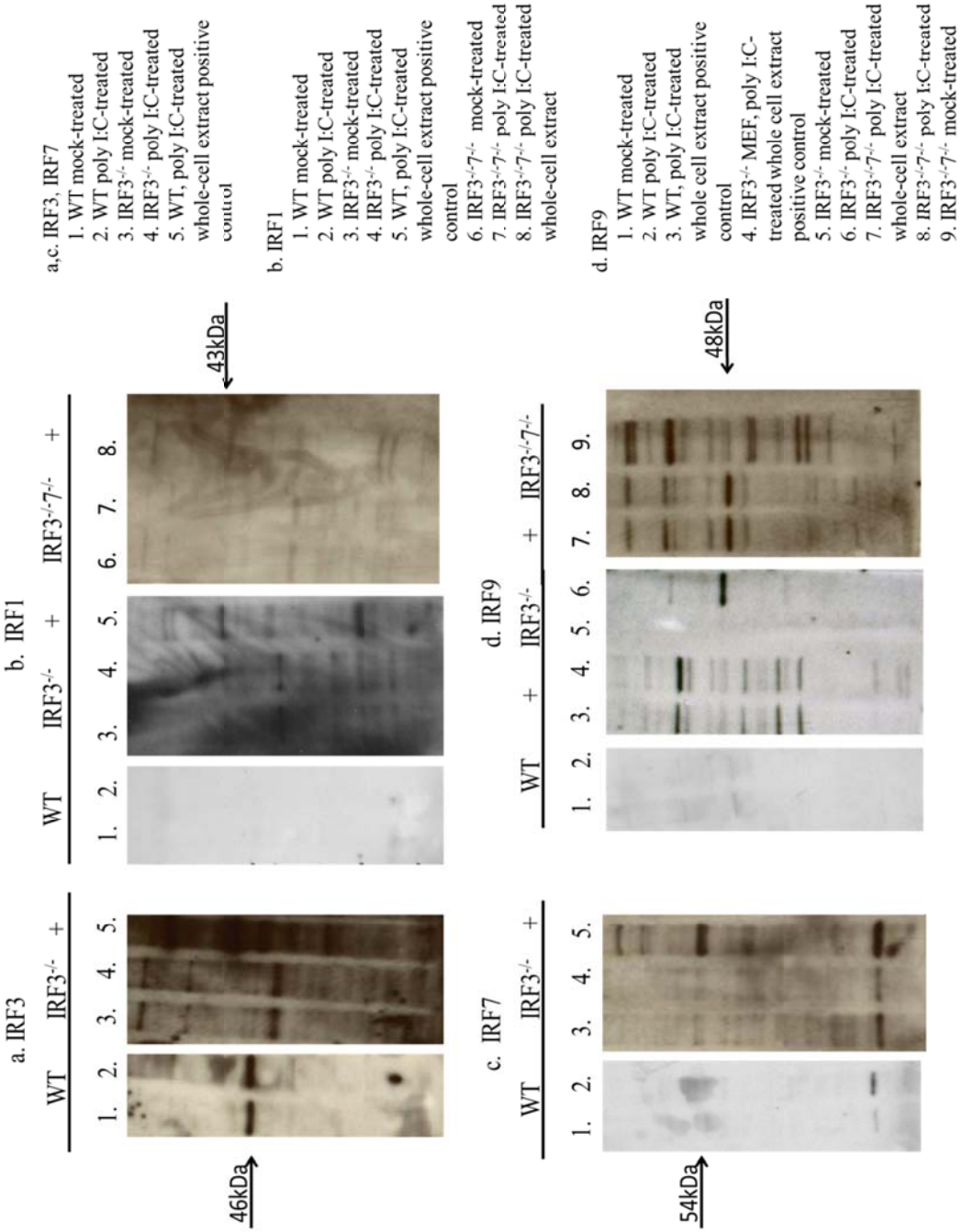


Figure 9. IRF antibody panel screen on proteins bound to the short VRE of the mIFNβ promoter in WT, IRF3$^{-/-}$ and IRF3$^{-/-}$7$^{-/-}$ MEFs. Extracts from the indicated MEFs were either mock-treated or treated with 3.0nM poly I:C for 3 hours and incubated with a DNA oligomer corresponding to the mIFNβ promoter. IRFs were probed to identify those bound to the oligomer.

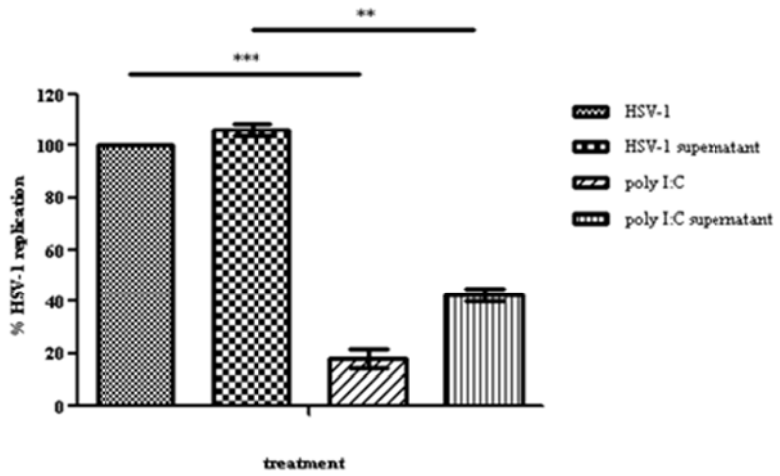
CHAPTER 4. NITRIC OXIDE CONTRIBUTES TO ANTIVIRAL PROTECTION IN THE ABSENCE OF IRF3 AND IFNs

The data presented in this chapter were submitted on July 21, 2011 to PLoS ONE as a full-length paper entitled "*Nitric Oxide Provides Potent Innate Antiviral Protection in Primary Fibroblasts in the Absence of a Type I Interferon System*".

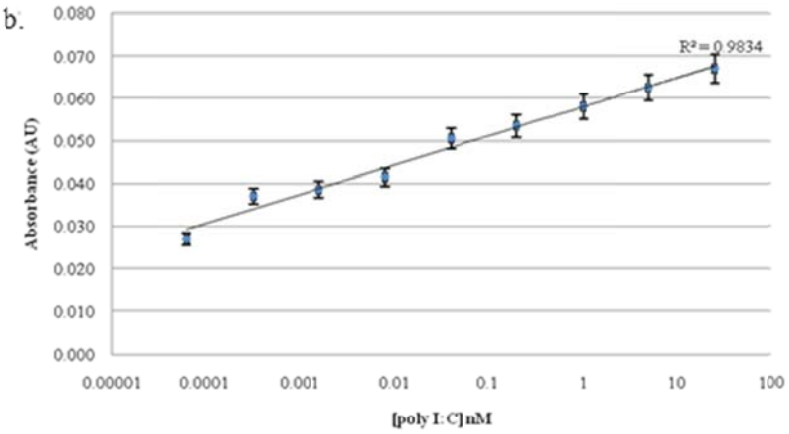
4.1 Poly I:C Induces Production of a Soluble Factor, Nitric Oxide, in the Absence of IRF3 and IRF9

Previous work showed that IRF3$^{-/-}$9$^{-/-}$MEFs, which fail to make or respond to type I IFNs, induce an antiviral response against HSV-1 and VSV following treatment with poly I:C. The antiviral response against HSV-1 in these cells was found to occur earlier and was more potent than the response against VSV (DeWitte-Orr et al., 2009). To determine whether this protection is conferred by a soluble factor, supernatants from poly I:C-treated monolayers were transferred to naïve monolayers. The transferred supernatants were able to significantly protect naïve monolayers from subsequent HSV-1 challenge (figure 10a). To confirm that residual poly I:C was not responsible for the protective effects, levels of poly I:C in the supernatants were measured relative to a standard curve of poly I:C in medium (figure 10b). The absorbance of poly I:C-treated supernatants was only slightly higher than poly I:C-deficient (mock) supernatants (figure 10c); moreover, poly I:C concentrations within this low range do not to confer resistance to HSV-1 infection in IRF3$^{-/-}$9$^{-/-}$MEFs (DeWitte-Orr et al., 2009). Thus, the soluble factor present within the supernatants was not residual poly I:C.

a.



b.



c.

Treatment	Absorbance (AU)
Mock	0.025
Poly I:C	0.029

Figure 10. A soluble factor confers resistance to HSV-1 replication in IRF3$^{-/-}$9$^{-/-}$ MEFs. Supernatants from mock- or poly I:C-treated MEFs were transferred to naïve cells and monolayers subsequently challenged with HSV-1. Antiviral protection is displayed as a percent of HSV-1 replication in comparison to untreated, infected cells (a). A standard curve of poly I:C in culture medium was generated (b). The absorbance of supernatants collected after 6 hours of mock and 8.5nM poly I:C treatment were collected and absorbance was compared to determine the amount of residual poly I:C within supernatants of treated cells (c). *, p<0.05, **, p<0.01, ***, p<0.0001; n=3.

As nitric oxide is a soluble factor and an important modulator of protection against DNA viruses such as HSV-1, we sought to determine if this molecule is involved in the antiviral response in the absence of IRF3 and IRF9. Following exposure to dsRNA for 2 hours to 7.5 hours, it was determined by Griess assay that both WT and IRF3$^{-/-}$9$^{-/-}$ MEFs made peak levels of nitric oxide, as assessed by nitrite concentration, within 5 hours of dsRNA treatment (figure 11a).

Varying concentrations of nitric oxide were added to untreated cells to confirm that nitric oxide production in IRF3$^{-/-}$9$^{-/-}$ MEFs inhibited replication of HSV-1. Nitric oxide was added with the use of the nitric oxide donor, DETA-NO, which is has a 27 hour half-life (Tanner et al., 2000). DETA-NO and the control vector, DETA, were added to cells at concentrations ranging from 0-200μM. DETA-NO used between 50-100μM was found by Griess assay to release nitric oxide to levels similar to those observed in MEFs after treatment with 8.5nM poly I:C for 5 hours (figure 11b). A concentration-dependent effect on HSV-1 replication in these cells was observed in both WT (figure 11c) and IRF3$^{-/-}$9$^{-/-}$ (figure 11d) MEFs. In both cell types, 0-25μM DETA-NO was unable to significantly protect cells from HSV-1 infection and replication. However, DETA-NO used at concentrations ranging between 50-200μM was able to limit HSV-1 replication in WT and IRF3$^{-/-}$9$^{-/-}$ MEFs. At the higher concentrations, a greater effect of DETA-NO was observed in IRF3$^{-/-}$9$^{-/-}$MEFs. The control reagent DETA did not significantly contribute to nitric oxide production or protection.

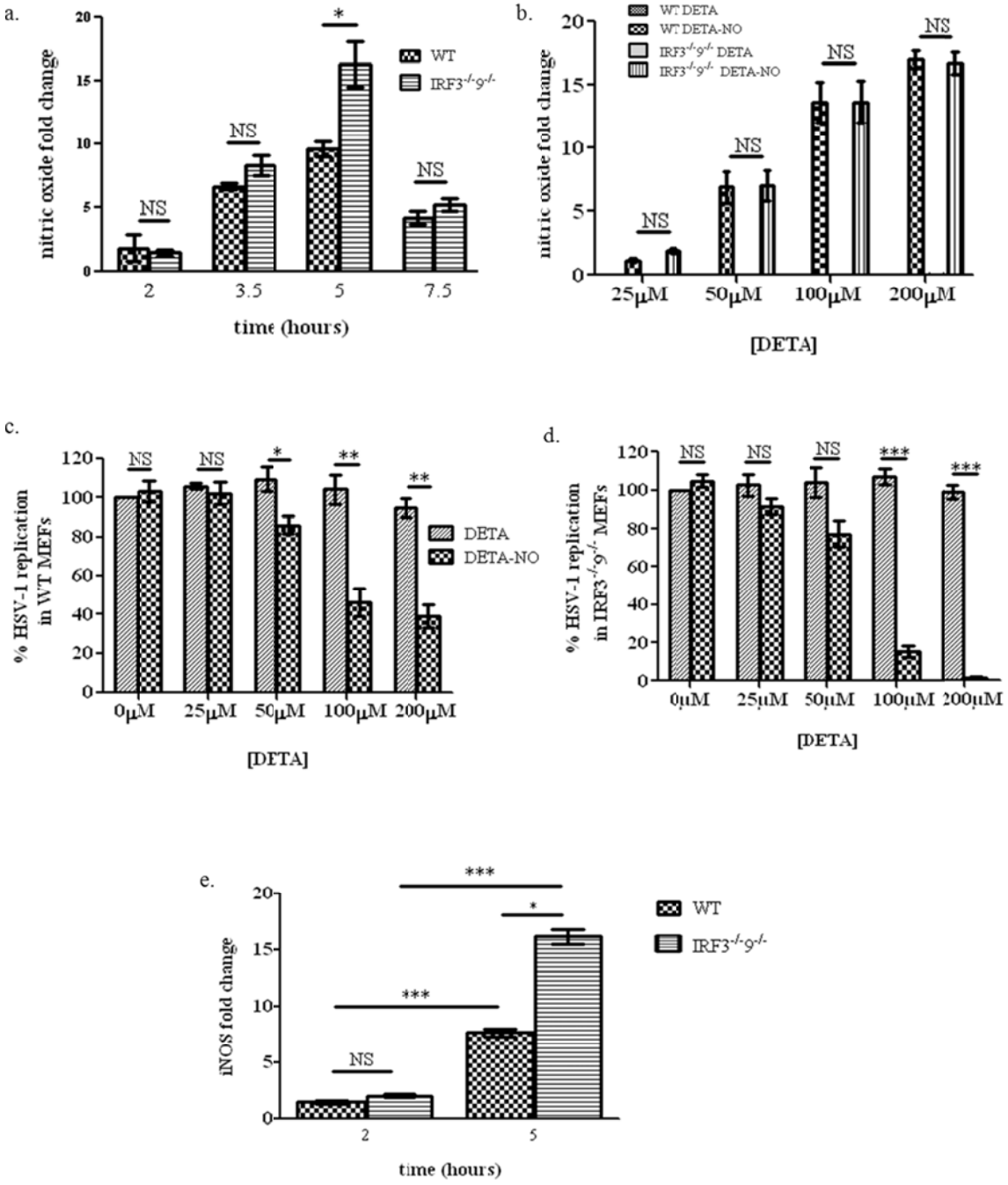


Figure 11. Nitric oxide is synthesized by iNOS in response to dsRNA in MEFs. Nitric oxide is produced following treatment with poly I:C in MEFs as determined by Griess assay (a). DETA-NO, a nitric oxide donor, induces nitric oxide production in MEFs (b). Nitric oxide produced via DETA-NO limits replication of HSV-1 in WT (c) and IRF3$^{-/-}$9$^{-/-}$ MEFs (d). DETA alone did not affect nitric oxide production and subsequent antiviral responses in either cell line. Production of iNOS, measured by

qRT-PCR, in WT and IRF3$^{-/-}$9$^{-/-}$ MEFs treated with poly I:C (e). Fold changes in transcript levels were compared relative to the housekeeping gene, GAPDH. Data are plotted as mean ± SEM, n=3; *, p<0.05, **, p<0.01, ***, p<0.0001.

4.2 Role of iNOS in the Antiviral Response in IRF3$^{-/-}$9$^{-/-}$ MEFs

Depending on the stimulus and the cell type, nitric oxide can be made by eNOS, nNOS, or iNOS. In response to viruses and viral components however, synthesis has generally been shown to be a result of iNOS induction (Akaike & Maeda, 2000). To measure the levels of eNOS and iNOS in WT and IRF3$^{-/-}$9$^{-/-}$ MEFs, qRT-PCR was employed. The levels of nNOS transcript were not measured, as this gene is relatively restricted to neuronal cells (Akaike & Maeda, 2000). Although eNOS was not detected in either cell type, iNOS was basally detected in untreated cells to levels of approximately 0.3-fold in comparison to the housekeeping gene, GAPDH. Levels of iNOS transcript increased in both WT and IRF3$^{-/-}$9$^{-/-}$MEFs (figure 11e) upon treatment with poly I:C within 2 hours. Induction further increased after 5 hours of poly I:C treatment, coincident with the timeframe in which protection against HSV-1 was observed. Levels of iNOS transcript were measured after 7.5 hours of poly I:C treatment as well and were found to have been reduced to baseline measurements (data not shown).

AMG and L-NIL are inhibitors of iNOS; these compounds were used to further investigate and confirm the involvement of iNOS in the antiviral response observed against HSV-1 in IRF3$^{-/-}$9$^{-/-}$MEFs. Efficacy of AMG and L-NIL as inhibitors of iNOS in WT and IRF3$^{-/-}$9$^{-/-}$MEFs are displayed in WT (figure 12a) and IRF3$^{-/-}$9$^{-/-}$MEFs (figure 12b). Both were found to be equally effective; there was an average reduction in iNOS induction upon treatment with poly I:C and either of the inhibitors ranging from 80-82%

in WT MEFs. In a similar fashion, inhibition of iNOS ranged from 80-87% in IRF3$^{-/-}$9$^{-/-}$ MEFs upon treatment with poly I:C and either of the two inhibitors.

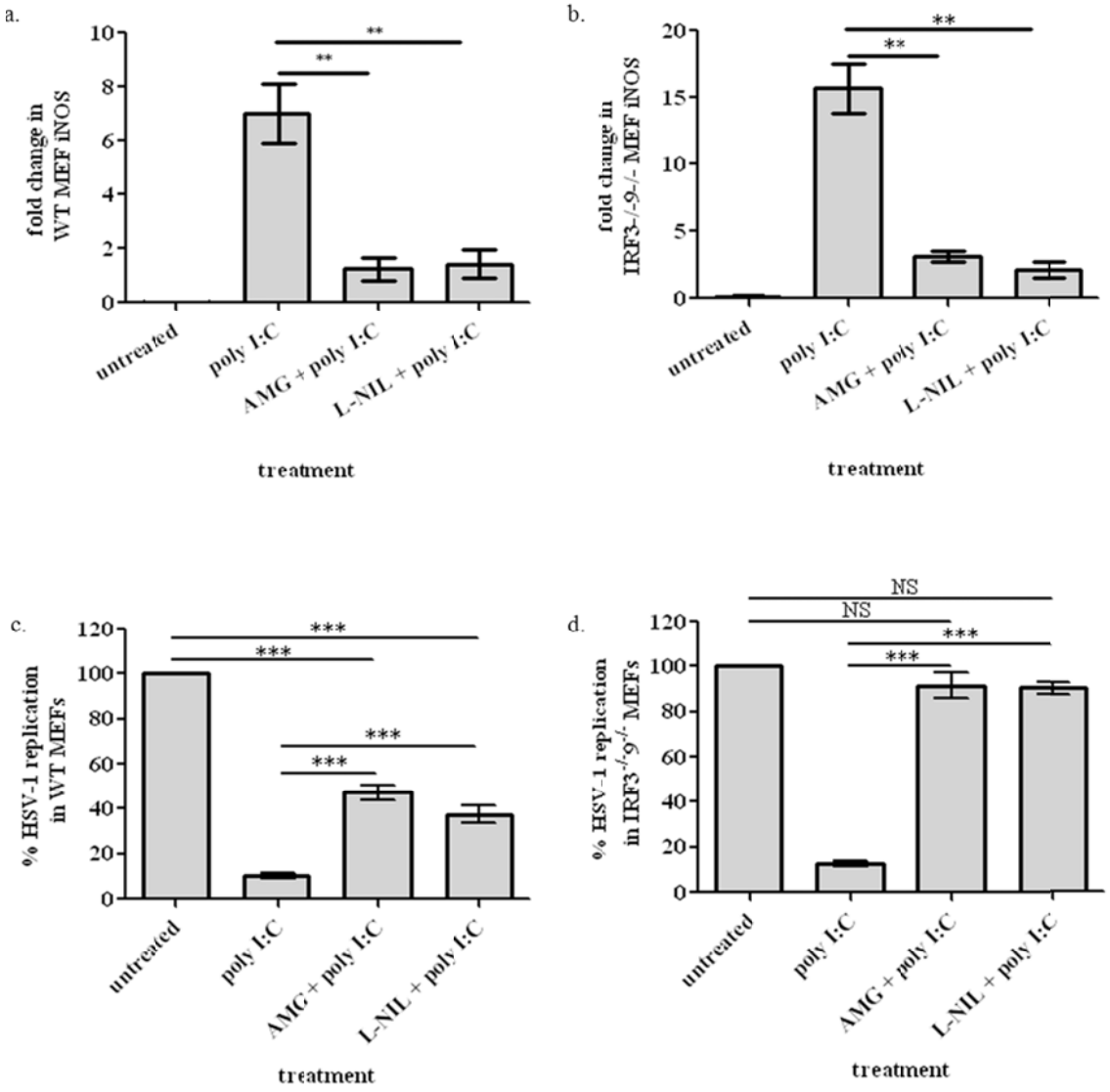


Figure 12. Nitric oxide made by iNOS is involved in the IRF3- and IFN-independent antiviral response. The efficacy of the iNOS inhibitors AMG and L-NIL to block iNOS induction was examined by qRT-PCR following poly I:C treatment of WT (a) and IRF3$^{-/-}$9$^{-/-}$ (b) MEFs. HSV-1 replication in WT (c) and IRF3$^{-/-}$9$^{-/-}$ (d) MEFs following treatment with poly I:C in the presence or absence of the iNOS inhibitors. Data are plotted as mean ± SEM, n=3. A Student's t-test was performed, *, p<0.05, **, p<0.01, ***, p<0.0001.

Consistent with our previous observations (DeWitte-Orr et al., 2009), a strong and effective antiviral response against HSV-1 was observed after treatment with poly I:C for 5-6 hours in both WT and IRF3$^{-/-}$9$^{-/-}$MEFs. Upon addition of AMG and L-NIL, HSV-1 was able to replicate to greater amounts in WT MEFs; however, these cells were still capable of partial protection against infection with HSV-1 (figure 12c), consistent with their ability to elicit IFN and ISG induction (DeWitte-Orr et al., 2009). In comparison, inhibition of iNOS in IRF3$^{-/-}$9$^{-/-}$MEFs almost fully restored replication of HSV-1 despite poly I:C treatment (figure 12d).

4.3 Protection Against HSV-1 by iNOS Induction Mediated by NF-κB and IRF1

Although the transcription factors NF-κB and IRF1 bind to the iNOS promoter to induce its transcription, these factors can signal independently of one another (Pautz et al. 2010). To confirm the role of NF-κB in the antiviral response observed in the absence of IRF3 and IFNs, NF-κB was blocked using the inhibitor Bay 11-7082, which targets the phosphorylation of IκBα. Bay 11-7082 was tested at a concentration ranging from 0μM-10μM to determine the optimal concentration to inhibit the nuclear translocation of the p65 subunit of NF-κB following treatment of MEFs with poly I:C. A concentration of 5μM was determined to be the optimal concentration (figure 13a). Inhibition of NF-κB significantly decreased the fold change in iNOS transcript expression as determined by qRT-PCR in WT (figure 13b) and IRF3$^{-/-}$9$^{-/-}$ MEFs (figure 13c). In an antiviral assay, inhibition of NF-κB with Bay 11-7082 in poly I:C-treated WT (figure 13d) and IRF3$^{-/-}$9$^{-/-}$

MEFs (figure 13e) resulted in increased HSV-1 replication; the increase in virus replication was found to be significant in IRF3$^{-/-}$9$^{-/-}$ MEFs.

To evaluate the importance of IRF1 in iNOS induction and the antiviral response in the absence of IRF3 and IFNs, an IRF1-targeting siRNA was generated. The control siRNA was a scrambled sequence of the IRF1 siRNA used to account for nonspecific effects. The efficacy of siRNA on IRF1 transcript levels was assessed by qRT-PCR (figure 14a). IRF1 transcript was significantly reduced in IRF3$^{-/-}$9$^{-/-}$ MEFs treated with IRF1-specific siRNA compared with cells treated with transfection reagent DharmaFECT (DF) alone. Although treatment with poly I:C increased levels of IRF1, these levels were significantly decreased upon addition of IRF1-targeting siRNA.

Quantitative RT-PCR analysis indicated that siRNA inhibition of IRF1 significantly reduced iNOS transcript accumulation in comparison to control siRNA (figure 14b). Coincident with these observations, HSV-1 replication was significantly increased in poly I:C-treated IRF3$^{-/-}$9$^{-/-}$ MEFs in which IRF1 levels were decreased following siRNA treatment (figure 14c). Taken together, these data suggest that both NF-κB and IRF1 contribute to iNOS induction and subsequent NO production in poly I:C-treated IRF3$^{-/-}$9$^{-/-}$MEFs.

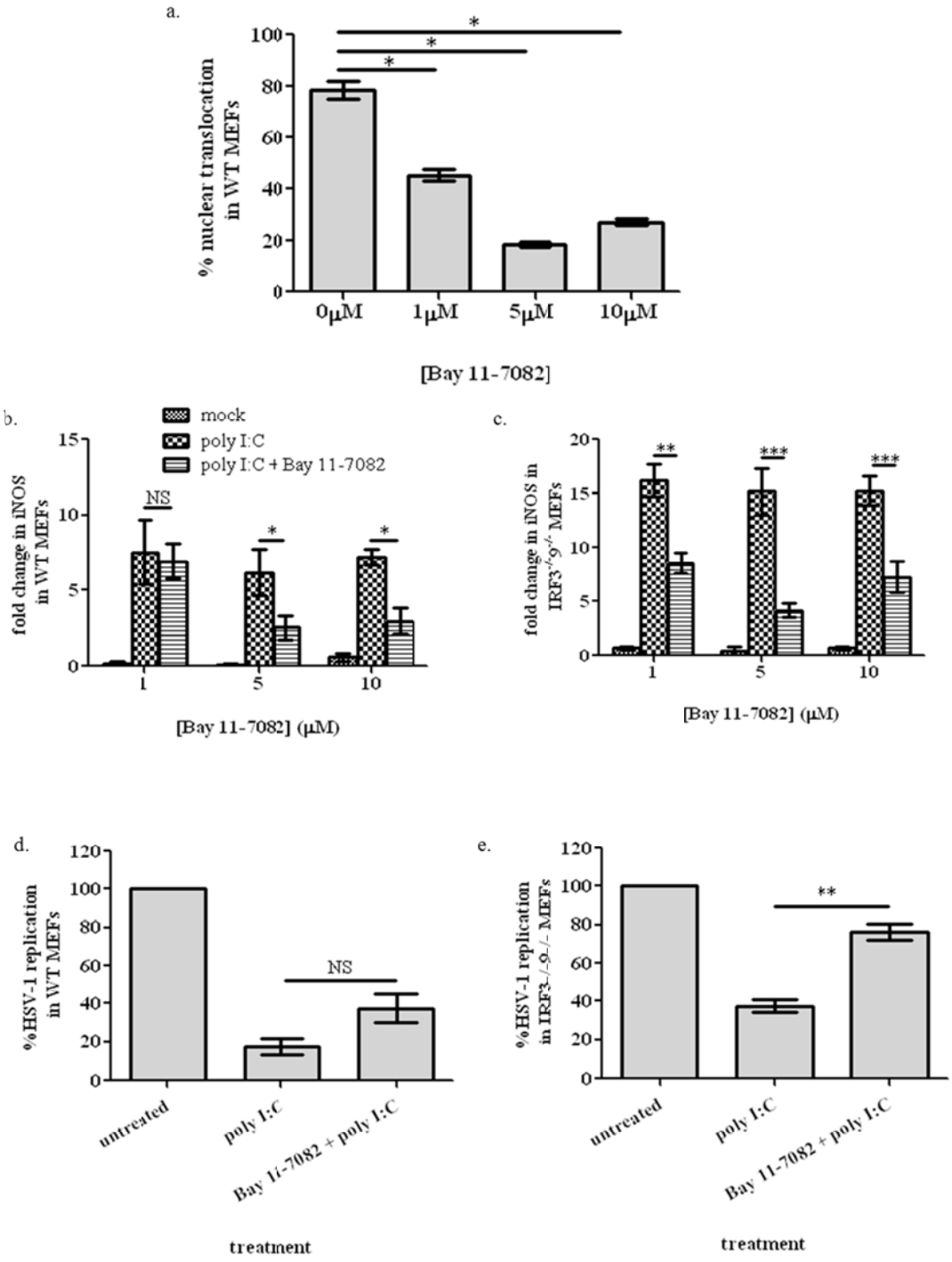


Figure 13. NF-κB contributes to the induction of iNOS and subsequent antiviral protection against HSV-1 in IRF3$^{-/-}$9$^{-/-}$ MEFs. The effective concentration of Bay 11-7082 to inhibit NF-κB translocation to the nucleus after treatment with poly I:C was determined (a). The effect of NF-κB inhibition by Bay 11-7082 on iNOS mRNA

accumulation was measured by qRT-PCR in poly I:C treated WT (b) and IRF3$^{-/-}$9$^{-/-}$ (c). HSV-1 replication in WT (d) and IRF3$^{-/-}$9$^{-/-}$ (e) MEFs following poly I:C treatment in the presence or absence of Bay 11-7082. A 1-way ANOVA with a Tukey post-test was performed to compare efficacy of a range of NF-κB inhibitor concentrations. A Student's t-test was performed to compare the antiviral response in cells that received poly I:C treatment with and without Bay 11-7082. Data are plotted as mean ± SEM, n=3; *, p<0.05, **, p<0.01, ***, p<0.0001.

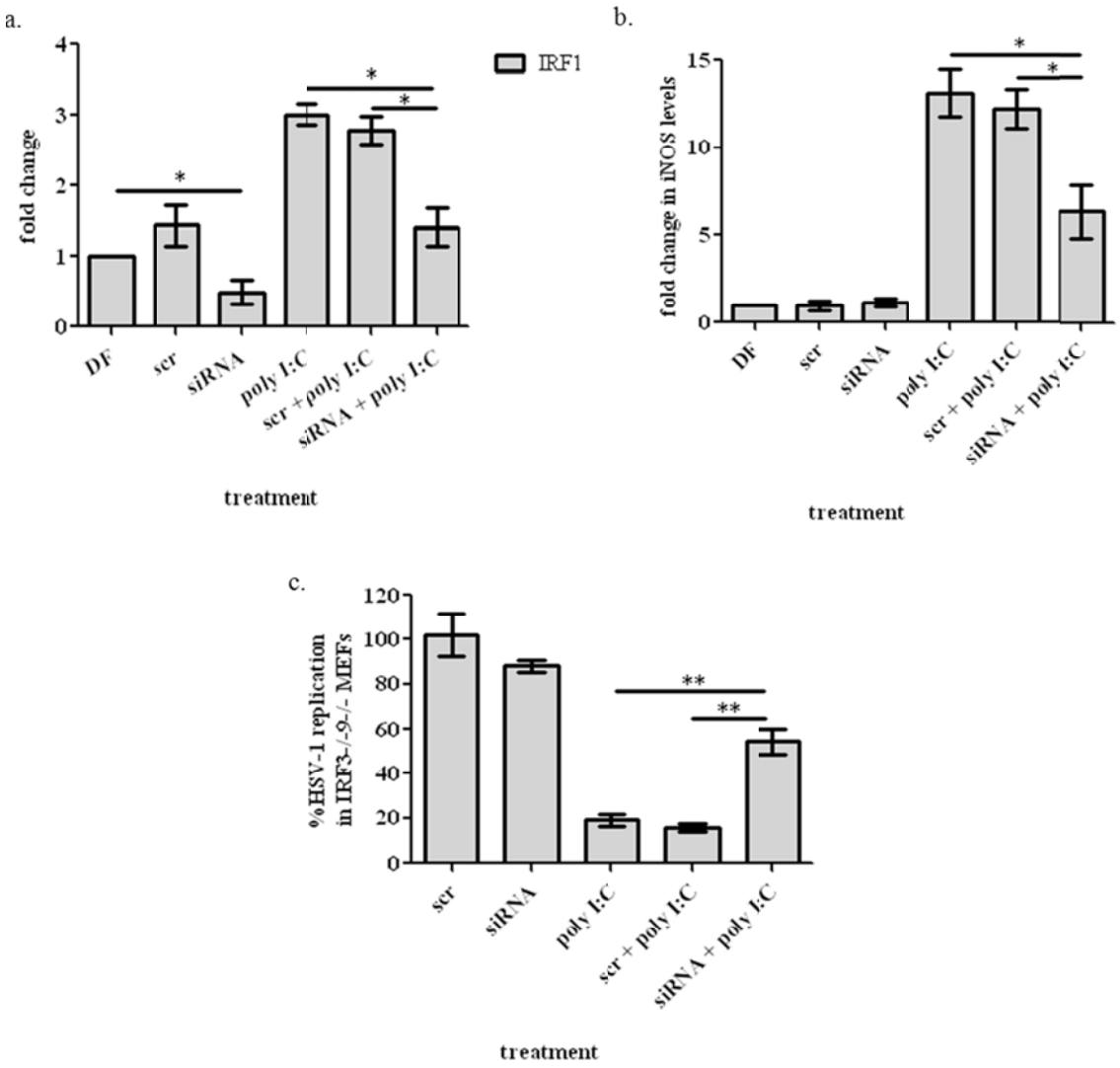


Figure 14. IRF1 contributes to iNOS induction in response to HSV-1 in MEFs. Knockdown of IRF1 by siRNA in IRF3$^{-/-}$9$^{-/-}$ MEFs in the presence or absence of poly I:C treatment (a). Fold change in iNOS mRNA accumulation (b) and HSV-1 replication (c) in IRF3$^{-/-}$9$^{-/-}$ MEFs treated with 8.5nM poly I:C in the presence or absence of siRNA against IRF1. Scr = scrambled IRF1 oligomer. Data are plotted as mean ± SEM, n=3. A Student's t-test was performed, *, p<0.05, **, p<0.01, ***, p<0.0001.

CHAPTER 5. DISCUSSION

5.1 Cellular Protection in Response to dsRNA

Considered nonprofessional immune cells, fibroblasts are amongst the first cell types involved in the line of defense against numerous pathogens. Fibroblasts are widely distributed in organisms (Pinkerton et al., 1982) and play an important role in the transition from innate to adaptive immunity (Lo et al., 1999, Buckley et al. 2001). This role is largely a result of cytokine production (Smith et al., 1997, Schilte et al., 2010), including IFNβ, which was originally termed fibroblast IFN. As such, fibroblasts are important effectors of the early innate immune response.

It is well established that early production of type I IFNs following virus infection is critical for defense against most viruses, as IFNs limit virus replication and spread (Kumar et al., 2011). Various structural components of a virus can trigger production of type I IFNs. Robust IFN production is largely associated with dsRNA, particularly in fibroblasts.

In MEFs, at least four cellular proteins recognize dsRNA and mediate production of type I IFNs: TLR-3, double-stranded RNA-dependent protein kinase (PKR), RIG-I, and MDA-5. These proteins preferentially bind dsRNA on the basis of its localization within the cell, viral origin, and/or length. Regardless of which protein binds dsRNA, the outcome of ligand recognition is signalling through the adaptors TRIF (TLR-3) or IFN promoter stimulator-1 (PKR, RIG-I, and MDA-5) to activate IRF3. Upon activation, IRF3 can directly induce ISGs in the absence of IFN production or collaborate with NF-

κB, ATF-2/cJUN, IRF7 and other transcription factors to mediate type I IFN production (Grandvaux et al., 2002).

5.2 IRF1 and IRF7 do not mediate the IRF3-Independent and IFN-Dependent Response to dsRNA

We previously showed that IRF3 plays a critical role in the antiviral response to recognition of short dsRNA molecules (DeWitte-Orr et al., 2009). However, long dsRNA molecules (3000bp and poly I:C) were capable of preventing virus replication in the presence and absence of IRF3. Furthermore, long dsRNA molecules were found to induce similar levels of ISGs and IFNs in WT and IRF3$^{-/-}$ MEFs (DeWitte-Orr et al., 2009). As IRF3 is not essential for a protective response induced by long dsRNA molecules, for objective 1 of this thesis, I investigated the IRF3-independent, IFN-dependent antiviral response. Our data suggested the existence of either a novel transcription factor with similar activity to IRF3 or involvement of another member of the IRF family in compensating for the loss of IRF3. Characterization of the IFNβ promoter over the last two decades has shown that IRF binding is critical for a functional IFNβ enhanceosome and ultimately, IFNβ transcription (Hiscott, 2007, Panne et al., 2007, Paun & Pitha, 2007).

IRF1 and IRF7 have been implicated in type I IFN production. However, I failed to detect differences in endogenous and induced expression of IRF1 and IRF7 protein following 3 hours of poly I:C treatment. Furthermore, nuclear translocation of IRF1 and IRF7 in various MEFs did not occur in response to dsRNA treatment. Consistent with these observations, IRF1 fails to restore induction of type I IFNs in the absence of IRF3

(Sato et al., 2000) and IRF7 expression in fibroblasts is largely dependent on IFNβ signalling (Sato et al., 2000, DeWitte-Orr et al., 2009).

Although IRF1$^{-/-}$3$^{-/-}$ mice were not available in time for this thesis, IRF3$^{-/-}$7$^{-/-}$ mice were available and thus, MEFs were made. While MEFs showed only partial protection in the absence of IRF3 and IRF7, IFNβ and ISGs were transcribed in response to dsRNA, albeit at lower levels than in WT and IRF3$^{-/-}$ MEFs. Thus, the diminished protective capabilities in these MEFs likely result from an impaired positive feedback loop of IFN production normally involving IRF7. This observation is especially apparent in the response to shorter dsRNA molecules.

5.3 IRF9 Compensates for IRF3 in Production of IFNβ

Particularly interesting was the inability of MEFs deficient for IRF3 and IRF9 to produce IFNs and ISGs (Sato et al., 2000, DeWitte-Orr et al., 2009). Thus, IRF9 was investigated for its ability to compensate for IFNβ production in the absence of IRF3. In comparison to other IRFs examined, IRF9 showed high levels of translocation to the nucleus in all cell types analyzed. A significant increase in nuclear translocation was observed in MEFs deficient for IRF3 in comparison to WT MEFs. While not significant, an increase in translocation was observed in MEFs deficient for both IRF3 and IRF7 as well. It is unclear at this time whether IRF9 translocation resulted directly from dsRNA treatment or indirectly from subsequent IFN-mediated signalling as a component of the ISGF3 complex (Honda & Taniguchi, 2006, Kawai & Akira, 2010). Thus, to distinguish between these two possible reasons IRF9 was shown to translocate to the nucleus,

cycloheximide should have been used, as it is a molecule well known to inhibit *de novo* protein synthesis. Nuclear translocation of IRF9 following inhibition of *de novo* protein synthesis would provide evidence that the translocation observed is a result of direct dsRNA-mediated signalling.

IRF9, but not IRF1 and IRF7, was found to bind to the IRF-containing portion of the mIFNβ promoter in $IRF3^{-/-}$ and $IRF3^{-/-}7^{-/-}$ MEFs. Of note, IRF9 did not bind to the short VRE of the mIFNβ promoter in WT MEFs, perhaps as a result of preferential or competitive binding of IRF3. Two reports in the mid-1990s showed that IRF9 is capable of binding to the mIFNβ promoter following NDV infection via a biochemical approach (Kawakami et al., 1995, Harada et al., 1996). However, upon the discovery of IRF3 and IRF7, the role of IRF9 has been studied exclusively in the context of type I IFN signal transduction. These data, however, collectively suggest that IRF9 becomes important in the absence of IRF3.

Although the findings presented in this thesis are consistent with those published (Kawakami et al., 1995, Harada et al., 1996), there are caveats associated with oligonucleotide pull down assays. In these experiments, I prepared cytoplasmic and nuclear extracts and pooled the protein extracts together. These pooled extracts were then incubated with the oligonucleotide. Thus, proteins that do not normally interact with the IFNβ promoter, due to their localization in the cytoplasm, were exposed to the DNA and may have bound nonspecifically (Melchjorsen et al., 2005). To confirm these results, a chromatin immunoprecipitation assay should be performed.

Given that viruses such as the highly successful human HSV-1 inactivate IRF3 (Melroe et al., 2004, Lin et al., 2004, Melroe et al., 2007, Paladino et al., 2010), it is not surprising that there are compensatory mechanisms to ensure viral clearance. For example, it was recently shown that IRF7 can function in place of the ISGF3 complex for ISG induction (Schmid et al., 2010). Taken together with our data, it appears that redundancy in regulators of the antiviral response, particularly in transcription factors modulating IFN production, evolved to ensure effective induction of the antiviral state.

5.4 Nitric Oxide and the IRF3- and IFN-Independent Antiviral Response

While macrophages are primary producers of nitric oxide in response to pathogens, nitric oxide production by dermal skin fibroblasts and rat embryo fibroblasts has previously been shown to play a role in wound healing and host defense in response to bacterial PAMPs and inflammatory cytokines (Lavnikova et al., 1995, Witte et al., 2000). With regards to the viral PAMP dsRNA, studies have shown induction of iNOS in human astroglia and bronchial epithelial cells (Uetani et al., 2001, Auch et al., 2004).

It is now appreciated that IFNs can be made in the absence of IRF3 and that ISGs can be produced in the absence of IFNs (Grandvaux, 2002, Noyce et al., 2006, DeWitte-Orr et al., 2009). However, it is unclear what innate antiviral response exists in the absence of IRF3, IFN and ISGs. Viruses encode multiple strategies to evade host innate responses, thus it is not surprising that most viruses are able to inactivate or degrade IRF3 and disable the IFN signal transduction cascade. Thus, I set out to study the mechanism by which an antiviral response against HSV-1 occurred in the absence of IRF3, IFNs and

ISGs, particularly since this response was earlier and more robust than the cellular response against VSV, a small RNA virus that is exquisitely sensitive to the host IFN response (DeWitte-Orr et al., 2009). I first determined that the antiviral factor responsible for controlling HSV-1 replication was a soluble factor. Coincidentally, nitric oxide acts as a soluble antiviral factor that is more potent against DNA viruses in comparison to RNA viruses. I then found that nitric oxide is rapidly produced by MEFs in response to the dsRNA mimetic poly I:C and serves to inhibit HSV-1 replication. The antiviral activity of poly I:C-induced nitric oxide was confirmed by the addition of nitric oxide to MEFs using DETA-NO. Of interest, in WT fibroblasts, nitric oxide is a minor contributor to the antiviral response, likely due to IRF3 and IFN activity. However, in IRF3$^{-/-}$9$^{-/-}$ MEFs, the nitric oxide pathway appears to be the dominant antiviral pathway. These data suggest that nitric oxide is an important antiviral molecule in the absence of IFN, ISGs and other cytokines.

Previous studies have shown that HSV-1 is susceptible to the effects of nitric oxide *in vivo* in mice and rats (Croen, 1993, Bi & Reis, 1995, Zaki et al., 2005). Although nitric oxide is synthesized during the host response to pathogen invasion, its precise role remains unclear. Despite its antiviral activity, nitric oxide is not always beneficial, as it can promote the pathogenesis of HSV-1 by damaging cells in host tissues, thus aiding infection (Fujii et al., 1999, Akaike & Maeda, 2000). It is unknown at this time whether the antiviral protection provided by nitric oxide *in vivo* is exerted in the form of cytotoxicity as a result of nitrative stress or by an alternative mechanism. *In vitro*, treatment of primary fibroblasts with dsRNA or DETA-NO did not elicit noticeable

cytotoxic effects, suggesting that the level of nitric oxide production that is sufficient to block virus replication is not linked to cytotoxicity.

5.5 Role of NF-κB and IRF1 in Mediating the Anti-HSV-1 Response in the Absence of IRF3 and IFNs

It was found in this thesis the importance of iNOS as the enzyme by which nitric oxide is synthesized. This is not surprising, as iNOS induction in response to virus infection, as well as viral components, is well known (MacLean et al., 1998, Zaragoza et al., 1999, Lee et al., 2009). During viral infection, nitric oxide production by iNOS is induced by cytokines such as IFNγ; however, virus infection can upregulate iNOS independently of such cytokines (Akaike & Maeda, 2000). While MEFs can respond to IFNγ, they do not make it in response to poly I:C, suggesting that in fibroblasts, iNOS is induced independent of IFNγ.

The iNOS gene locus has low homology between human, rat and mouse sequences. As a result, the transcription factors involved in iNOS induction are species and cell type dependent (Chu et al., 1998, Pautz et al., 2010). For example, regulation of nitric oxide production by iNOS in humans has been shown to be dependent on activator protein 1 (AP-1), but a binding site for this transcription factor is not present on the mouse iNOS promoter (Chu et al., 1998). NF-κB and IRF1 are most commonly published as regulators of iNOS expression in various species (LePage et al., 1996, Spitsin et al., 1997, Farlik et al., 2010). Furthermore, virus infection leads to the activation of NF-κB and IRF1 (Kamijo et al., 1994, Honda & Taniguchi, 2006). Here I found that regulation of iNOS expression is largely dependent on both NF-κB and IRF1. These data are

consistent with other reports indicating that both transcription factors are important for iNOS expression in mice in response to dsRNA (Blair et al., 2002, Pautz et al., 2010).

5.6 Caveats Associated with the use of Poly I:C

These studies utilized poly I:C to investigate type I IFN responses to dsRNA. Poly I:C has been used extensively for over half a century to study dsRNA-induced signalling pathways (DeWitte-Orr & Mossman, 2010), and although valuable information has been uncovered, it is not a perfect substitute for viral dsRNA or virus infection and may introduce bias into evaluation of the antiviral pathway (Gantier & Williams, 2007, Milev-Milovanovic et al., 2009, DeWitte-Orr & Mossman, 2010). Poly I:C is composed of stretches of complementary homopolymers of inosine and cytidine forming dsRNA-like motifs. Because it is composed of stretched of ribonucleotides annealed together and forms dsRNA motifs of varied size, and because it relies on inosine, a relatively rare ribonucleotide, poly I:C is not a perfect substitute for viral dsRNA. Despite this caveat, poly I:C was chosen for these studies, as it is a well-defined ligand that triggers IFN production in a wide variety of cells. Use of poly I:C also avoids inconsistent responses due to variation between infections (Milev-Milovanovic et al., 2009). However, dsRNA-mediated antiviral responses will more accurately be understood once native viral dsRNA is investigated within the context of a live viral infection.

CHAPTER 6. CONCLUSIONS

Overall, these data show that IRF9 serves as an alternative antiviral factor in the absence of IRF3 and nitric oxide serves as an alternative antiviral factor in the absence of the IRF3- and IFN-mediated signalling pathway. In terms of the IRF3-independent antiviral response, it can be concluded that IRF9 binding to the IRF binding site of the mIFNβ promoter leads to induction of IFNβ, which subsequently initiates a secondary arm of signalling mediated by IFNβ leading to antiviral immunity as a result of ISG production. I have also shown that inhibition of iNOS, and thus, nitric oxide, in the absence of both IRF3 and IFNs leads to a significant increase in HSV-1 replication that is comparable to the untreated, infected control. While it can be said that the nitric oxide pathway is important, involvement of other pathways or factors cannot be ruled out. Furthermore, the factors involved in the antiviral response against VSV after 24 hours of poly I:C pretreatment (DeWitte-Orr et al., 2009) are still unknown. As levels of nitric oxide began to decline within 7.5 hours of treatment with poly I:C, it is unlikely that it is involved in the antiviral response observed against VSV within this timeframe. These data emphasize the intricacies of the host response to different pathogens and underscore the requirement of the host to have multiple strategies to counteract the immune evasion properties of viruses.

CHAPTER 7. REFERENCES

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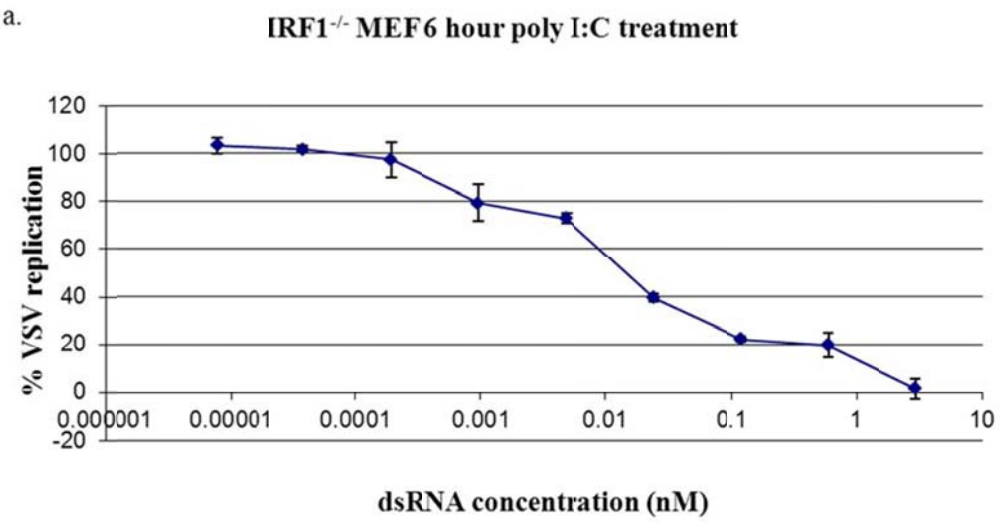
CHAPTER 8. APPENDIX I

8.1 Antiviral Protection in MEFs Deficient for IRF1, IRF7 or IRF9

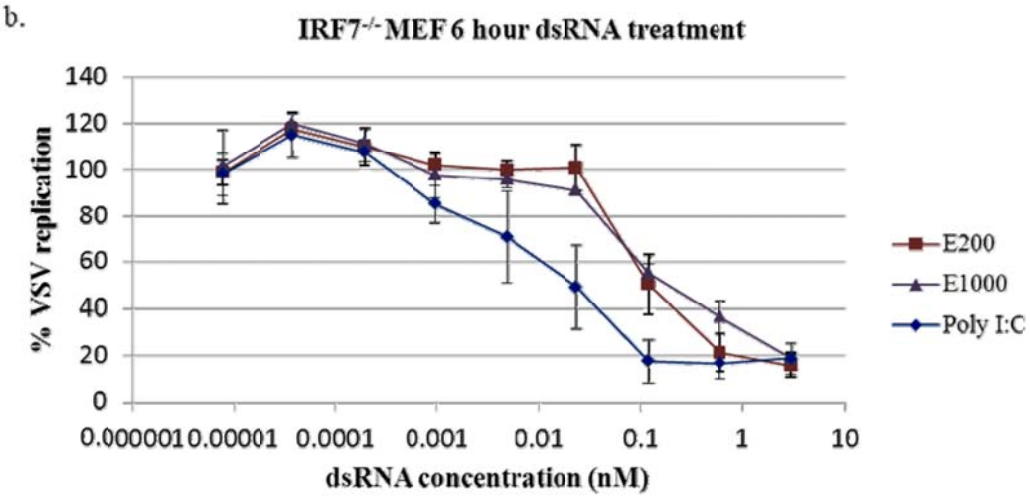
Several antiviral assays were performed with various knockout MEF cultures to assess the relative importance of IRF1, IRF7, and IRF9, all of which positively regulate IFNβ production, to antiviral protection. Cells deficient for IRF1, IRF7 or IRF9 were treated with dsRNA for either 6 or 24 hours and subsequently challenged with VSVgfp or HSV-1gfp to determine if these cells were protected against infection. These antiviral assays were meant to supplement those performed previously and to serve as a control for double knockout MEFs discussed in our previous publication (DeWitte-Orr et al., 2009) as well as in this thesis (Chapter 3).

Protection was observed within 6 hours in $IRF1^{-/-}$ MEFs pretreated with poly I:C (figure A-1a). In $IRF7^{-/-}$ MEFs treated with E200, E1000, or with poly I:C for 6 hours, a robust antiviral response was observed when the cells were challenged with VSVgfp (figure A-1b); full protection was observed in one experiment after 24 hours of treatment (figure A-1c). As IRF3 is present, full protection was expected in both these cultures; furthermore, complete protection in cells deficient for either IRF1 or IRF7 has been reported previously (Matsuyama et al., 1993, Steinberg et al., 2009).

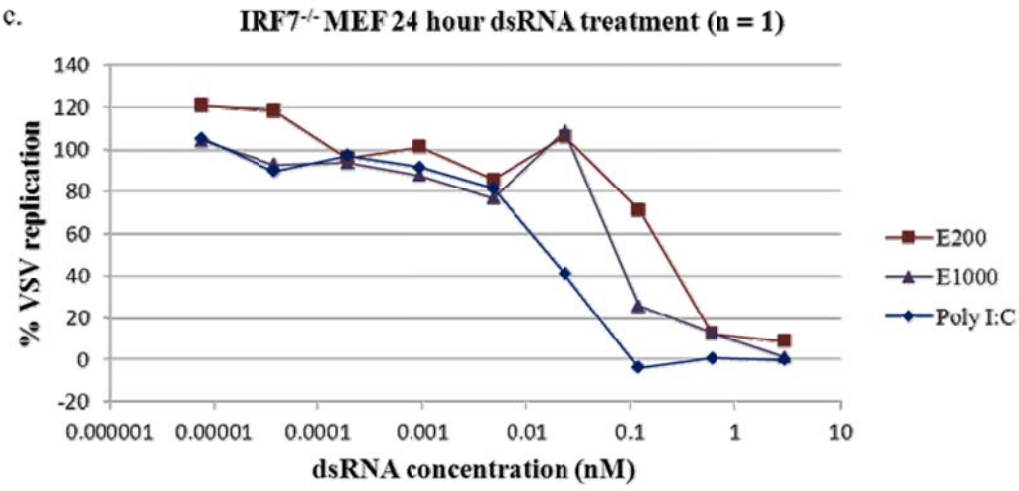
a.


IRF1-/- MEF 6 hour poly I:C treatment
% VSV replication
120
100
80
60
40
20
0
-20
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)

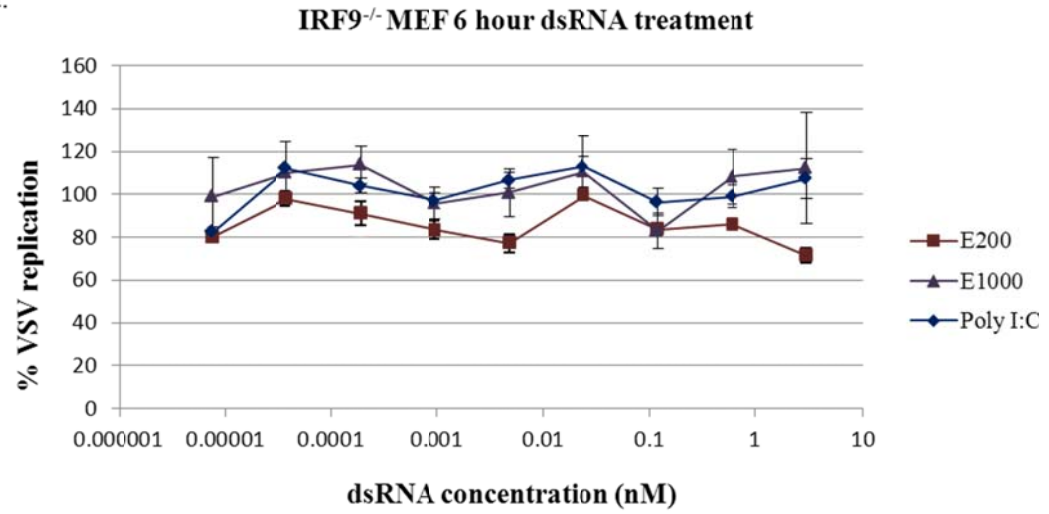
b.


IRF7-/- MEF 6 hour dsRNA treatment
% VSV replication
140
120
100
80
60
40
20
0
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)
E200
E1000
Poly I:C

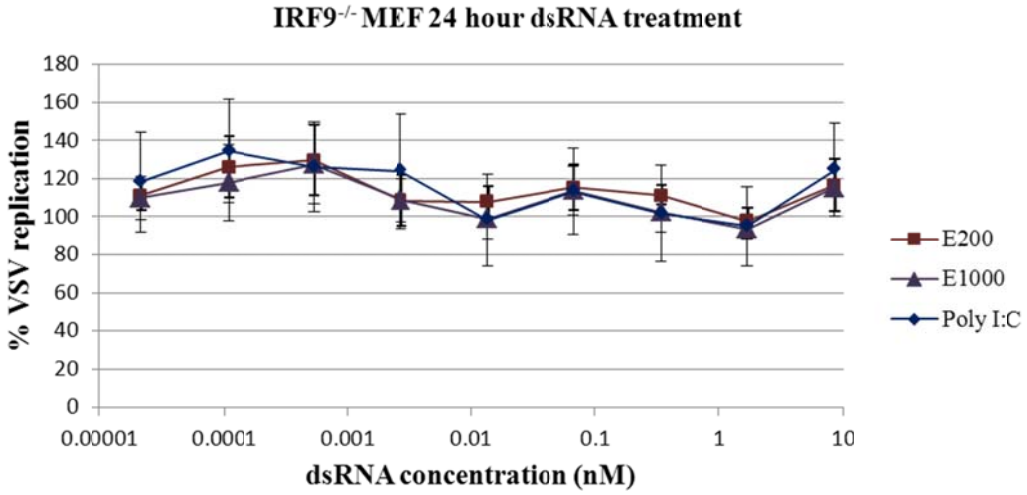
c.


IRF7-/- MEF 24 hour dsRNA treatment (n = 1)
% VSV replication
140
120
100
80
60
40
20
0
-20
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)
E200
E1000
Poly I:C

d.


IRF9-/- MEF 6 hour dsRNA treatment
% VSV replication
160
140
120
100
80
60
40
20
0
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)
E200
E1000
Poly I:C

e.


IRF9-/- MEF 24 hour dsRNA treatment
% VSV replication
180
160
140
120
100
80
60
40
20
0
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)
E200
E1000
Poly I:C

f.

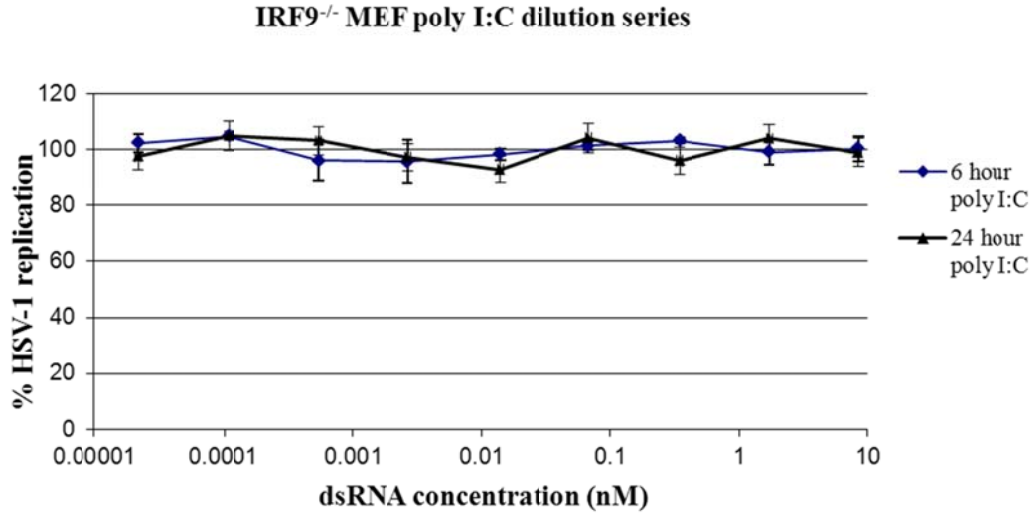

IRF9-/- MEF poly I:C dilution series
% HSV-1 replication
120
100
80
60
40
20
0
0.00001
0.0001
0.001
0.01
0.1
1
10
dsRNA concentration (nM)
6 hour poly I:C
24 hour poly I:C

Figure A-1. Antiviral protection in MEFs deficient for IRF1, IRF7, or IRF9. Antiviral protection in IRF1$^{-/-}$ following 6 hours of poly I:C treatment was quantified (a). Protection in IRF7$^{-/-}$ MEFs after 6 hours (b) and 24 hours (c) of dsRNA treatment was quantified as well following a VSVgfp challenge at a MOI of 0.1. MEFs deficient for IRF9 were also treated with dsRNA for either 6 hours (d) or 24 hours (e) and subsequently challenged with VSVgfp. When no protection was observed in these cells, IRF9-deficient MEFs were treated with poly I:C for either 6 hour or 24 hours and challenged with HSV-1gfp at an MOI of 0.1 (f). Fluorescence from mock-treated cells was subtracted from all treatment groups and was compared to the untreated, infected cells taken as a percent of virus replication. Data are expressed as the mean ± SEM; n=3.

No protection was observed against VSV in MEFs deficient for IRF9 regardless of dsRNA length or pretreatment time (figure A-1d and A-1e). These observations were unexpected given that an antiviral response was observed previously in IRF3$^{-/-}$9$^{-/-}$ MEFs (DeWitte-Orr et al., 2009), however, abolished ISG induction and a lack of subsequent protection has previously been observed in MEFs deficient for IRF9 in response to VSV and encephalomyocarditis virus (Kimura et al., 1996, Sato et al., 1998, Marie et al., 1998). It was hypothesized that a response against HSV-1 may be observed, as the response observed against HSV-1 in IRF3$^{-/-}$9$^{-/-}$ MEFs was much more robust and occurred at an earlier time than the response against VSV (DeWitte-Orr et al., 2009). However, no protection was observed against HSV-1 (figure A-1f).

The EC_{50} of curves generated after 6 hours or 24 hours of treatment was calculated using GraphPad Prism 5.0 (table A-I). As there was limited or no protection in most of the treatment groups, EC_{50} could not be determined. However in several of the cultures treated with poly I:C, EC_{50} could be determined and was found to be quite similar in IRF1$^{-/-}$, IRF7$^{-/-}$ and IRF3$^{-/-}$7$^{-/-}$ MEFs. It was therefore more prudent to compare

the length of time it took for an antiviral response to occur. As in the absence of IRF3 (figure 6b; DeWitte-Orr et al., 2009), a potent antiviral response after pretreatment with poly I:C occurred within 6 hours in the absence of IRF1 or IRF7 (figure A-Ia, b). This took 24 hours to occur to similar levels in the absence of both IRF3 and IRF7 (figure 6d), reinforcing the importance of the presence of both of these IRFs together for effective control of virus replication.

Table A-I. EC_{50} values based on the antiviral curves presented in figure 6. Values were determined for each dsRNA treatment in IRF1$^{-/-}$, IRF7$^{-/-}$, IRF3$^{-/-}$7$^{-/-}$ and IRF9$^{-/-}$ MEFs using GraphPad Prism 5.0 and are presented as the mean of three replicates. Groups for which EC_{50} could not be determined are indicated as "ND", whereas a dash (--) indicated an antiviral assay was not performed using the specified length of dsRNA.

IRF	E200	E1000	NS3000	Poly I:C
IRF1$^{-/-}$	ND	ND	--	0.77nM
IRF7$^{-/-}$ (6 hours)	1.23nM	1.27nM	--	0.79nM
IRF3$^{-/-}$7$^{-/-}$ (24 hours)	ND	--	ND	0.65nM
IRF9$^{-/-}$ (6 hours)	ND	ND	--	ND

To confirm IFNβ production in MEFs deficient for IRF1, IRF7 or IRF9, ISG induction was measured by qRT-PCR (figure A-2). As the transcription factors required for IFNβ production on the basis of the prototypic antiviral response were present in these cells, ISG induction was a good indication that type I IFNs were made in MEFs deficient for IRF1 or IRF7. However, ISG induction in MEFs deficient for IRF9 was limited. It is

likely that as all components classically important for IFNβ production are present in IRF9$^{-/-}$ MEFs, IFNβ is upregulated. However, due to the absence of IRF9, an essential component of the ISGF3 complex (Honda & Taniguchi, 2006, Kawai & Akira, 2010), IFNβ-mediated ISG induction could not occur. Thus, viral clearance was impaired as a result.

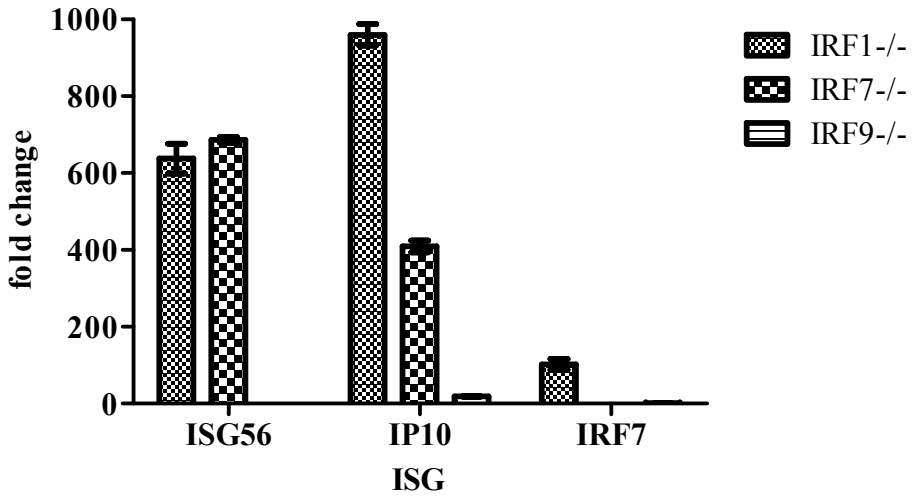


Figure A-2. ISG production in knockout MEF cultures. ISGs were measured by qRT-PCR in IRF1$^{-/-}$, IRF7$^{-/-}$ and IRF9$^{-/-}$ MEFs following 6 hours of 3.0nM poly I:C treatment. Data represent the mean ± SEM; n=3.

Despite the likely impairment of IFNβ signalling in IRF9$^{-/-}$ MEFs, IRF3 is capable of directly stimulating the production of several ISGs for viral clearance independently of IFNβ (Grandvaux et al., 2002). IRF3 can directly regulate ISG56 (Grandvaux et al., 2002) and IP10 (Nakaya et al., 2001), however, ISG56 was minimally produced and IP10 induction was not enough to protect from VSV or HSV-1 infection and replication (figure A-2). It may be that all endogenous IRF3 is being used to produce IFNs, and thus, does

not make ISGs. It may also be that in MEFs, IRF3 only regulates ISG expression directly upon impairment of IFNβ production.

8.2 IRF9 and Nitric Oxide Production

Apart from IRF3-mediated induction of ISGs, in this thesis, I have shown that nitric oxide becomes an important antiviral mechanism in the absence of IRF3 and IRF9 production. Thus, the lack of protection observed in IRF9-deficient MEFs was particularly interesting, as nitric oxide production was expected to be a mechanism used to protect MEFs from virus infection, particularly HSV-1. The fold change in nitric oxide production and iNOS induction in IRF9$^{-/-}$ MEFs was comparable to that detected in WT MEFs (figure A-3), in which nitric oxide was not necessary for antiviral protection. Thus, it may be that nitric oxide is only engaged as a viable antiviral mechanism in the absence of IFNβ and perhaps ISGs. Further investigation is necessary beginning with measurement of iNOS and nitric oxide in WT, IRF9$^{-/-}$ and IRF3$^{-/-}$9$^{-/-}$ MEFs following a range of poly I:C doses. Subsequently, iNOS and nitric oxide should be measured after addition IFNβ or nitric oxide to the system.

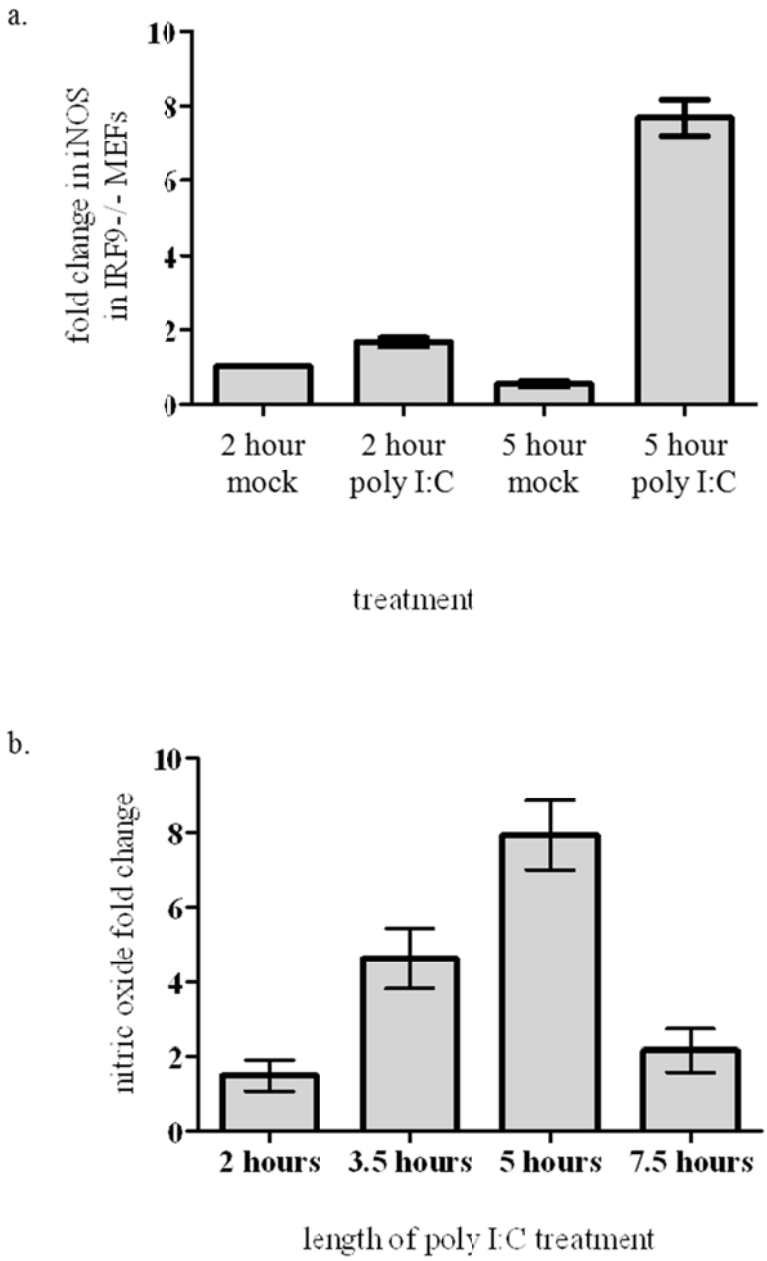


Figure A-3. In IRF9$^{-/-}$ MEFs, qRT-PCR was performed to identify increases in iNOS induction in response to poly I:C (a). A Griess assay was also performed as described to determine if nitric oxide was being produced (b). Data are representative of three independent experiments. Upon comparison to levels of iNOS and nitric oxide detected in WT and IRF3$^{-/-}$9$^{-/-}$ MEFs, this culture produced these antiviral factors to levels similar to that observed in WT MEFs. Data represent the mean ± SEM; n=3