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Dissecting the Role of Cytosolic Nucleic Acid Sensors in the Type I Interferon Response to Herpes Simplex Virus-1 and other Ligands: A Dissertation

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DISSECTING THE ROLE OF CYTOSOLIC NUCLEIC ACID SENSORS IN THE TYPE I INTERFERON RESPONSE TO HERPES SIMPLEX VIRUS-1 AND OTHER LIGANDS

A Dissertation Presented

By

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Thompson, Mikayla, R., Sharma, Shruti, Jensen, Søren, Atianand, Maninjay, Fitzgerald, Katherine, A., Kurt-Jones, Evelyn, A. (2014) “Interferon Gamma Inducible protein (IFI)16 transcriptionally regulates IFN-α and other interferon stimulated genes and controls the Interferon response to both DNA and RNA viruses.” *J. Biological Chem.* Submitted, under revision.

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List of Abbreviations

AIM2	Absent in melanoma-2
ALR	AIM2-like receptors
ASC	Apopotosis-associated speck-like protein containing CARD
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
CARD	Caspase activation and recruitment domain
c-di-AMP	Cyclic diadenosine monophosphate
c-di-GMP	Cyclic diguanosine monophosphate
CIA	Collagen Induced Arthritis
cGAS	Cyclic GMP-AMP Synthase
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
CNS	Central Nervous Sysytem
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DDX	DEAD (Asp-Glu-Ala-Asp) box polypeptide
DHX	DEAH (Asp-Glu-Ala-His) box polypeptide
DMXAA	5,6-Dimethylxanthenone-4-acetic acid
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein-Barr virus
ECMV	Encephalomyocarditis virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
HCMV	Human cytomegalovirus
HEK	Human embryonic kidney
HHV	Human herpes virus
HIN-200	Hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
ICP0	HHV infected cell polypeptide 0
IFI	Gamma-interferon-inducible protein
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
ISD	Interferon stimulatory DNA
ISG	Interferon stimulated gene
ISRE	Interferon-sensitive response element
JAK	Janus Kinase
JNK	c-Jun N-terminal kinases
KSHV	Kaposi sarcoma-associated herpesvirus

LCMV	Lymphocytic choriomeningitis
LGP2	laborator of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
Mal	MyD88-adapter-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signaling protein
MCMV	Murine cytomegalovirus
MDA5	Melanoma differentiation-associated gene-5
MHC	Major Histocompatibility Complex
MMTV	Mouse mammary tumor virus
MNDA	Myeloid nuclear differentiation antigen
MyD88	Myeloid differentiation primary response gene 88
NALP	NACTH, LRR and PYD domain-containing protein
NDV	Newcastle disease virus
NEMO	NF-kB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NLRP	NOD like receptors
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PDC	Plasmacytoid dendritic cell
PML	Promyelocytic leukemia protein
Poly(dA:dT)	Polydeoxyadenylic acid : polythymidylic acid
Poly(I:C)	Polyriboinosinic acid : polyribocytidylic acid
PRD	Positive Regulatory Domain
PRR	Pattern Recognition Receptor
PS	Phosphorothioate
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
RD	Repressor domain
RIG-I	Retinoic acid-inducible gene-I
RIP	Receptor-interacting serine/threonine-protein kinase 1
RLR	Rig-I-like receptors
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLE	Systemic Lupus Erythematosus
ssDNA	single-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
SV	Sendai virus
TBK1	TANK-binding kinase 1
TGF	Transforming growth factor

TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VSV	Vesicular stomatitis virus
VACV	Vaccinia Virus
VZV	Varicella-zoster virus

Abstract

The innate immune system provides the first line of defense against infection. Pathogens are detected though a variety of Pattern Recognition Receptors (PRRs), which activate downstream signaling cascades. Effector molecules such as cytokines and chemokines are released upon activation and aid in cell recruitment, control of pathogen replication, and coordination of the adaptive immune response. Nucleic acids that are released into the cytosol during viral and bacterial infection are recognized through a special class of PRRs, coined cytosolic nucleic acid sensors. Upon recognition, these receptors induce the production of type I interferons and other cytokines to aid in pathogen clearance. Although many cytosolic nucleic acid sensors have been discovered, it is unclear how they work in concert to mediate these responses.

The Interferon Gamma Inducible protein (IFI)16 and its proposed mouse orthologue IFI204 are cytosolic DNA sensors that have been linked to the detection of cytosolic DNA during infection with Herpes Simplex Virus (HSV-1). IFI16 binds dsDNA that has been released into the cytosol during viral infection and engages the adaptor molecule Stimulator of Interferon Genes (STING) leading to TANK binding kinase-1 (TBK1) dependent phosphorylation of interferon regulatory factor 3 (IRF3) and transcription of type I interferons and interferon stimulated genes. In addition to its role as a sensor, in chapter two of this thesis we describe a broader role for IFI16 in the regulation of the type I IFN response to RNA and DNA viruses in anti-viral immunity. In an effort to better understand the role of IFI16 in coordinating type I IFN gene regulation, we

generated cell lines with stable knockdown of IFI16 and examined responses to DNA and RNA viruses as well as other inducers of IFN such as cyclic-di-nucleotides. As expected, stable knockdown of IFI16 led to a severely attenuated type I IFN response to cytosolic DNA ligands and DNA viruses. In contrast, expression of the NF-κB regulated cytokines such as IL-6 and IL-1β were unaffected in IFI16 knockdown cells, suggesting that the role of IFI16 in sensing these triggers was unique to the type I IFN pathway. Surprisingly, we also found that knockdown of IFI16 led to a severe attenuation of expression of IFN-α and IFN stimulated genes such as RIG-I in response to cyclic GMP-AMP (cGAMP), a second messenger produced in response to cGAS, as well as RNA ligands and viruses. Analysis of IFI16 knockdown cells revealed compromised occupancy of RNA polymerase II on the IFN-α promoter in IFI16 knockdown cells suggesting that transcription of ISGs is dependent on IFI16. Since IFI16 knockdown compromised not only DNA virus driven pathways, we propose additional regulatory roles outside of DNA sensing. Collectively, these results indicate that IFI16 plays a role in the regulation of type I IFN gene transcription and production in response to both RNA and DNA viruses.

The role of IFI16/IFI204 has been studied extensively *in vitro*, however the role of the receptors *in vivo* has yet to be determined. In chapter three of this thesis, we developed a mouse deficient in IFI204 to explore the role of IFI204 in *in vivo* immune responses to viruses. We investigated the ability of IFI204 deficient cells to induce type I interferons and other cytokines in response to a panel of DNA and RNA ligands *in vitro*. IFI204 deficient BMDMs displayed a

partial defect in type I interferon induction in response to both DNA and RNA ligands and viruses as compared to WT mice. We also observed that this phenotype is time dependent, since there was no change in type I interferon induction after 12 hours post infection as compared to earlier time points. In contrast to these results, expression of the NF-κB regulated cytokines IL-6 and IL-1β were unaffected in IFI16 knockdown cells. These results suggest that IFI204 plays a partial role in the induction of type I interferons in response to both DNA and RNA ligands. Additionally, IFI204 may work in tandem with other receptors in a sequential manner to amplify the type I interferon response. We also studied the involvement of IFI204 in an *in vivo* model of HSV-1 infection. IFI204 knockout mice produce less brain and serum IFN-β, IL-6, and IL-1β 72 hours post intraperitoneal infection with HSV-1. Furthermore, IFI204 -/- mice are more susceptible to HSV-1 infection as compared to WT mice. These data indicate that IFI204 mediates the response to HSV-1 *in vivo* by inducing the production of cytokines that are necessary for the control of viral infection.

Preface to Chapter 1

This chapter has appeared in the following publications/manuscripts:

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- Mikayla Thompson and John Kaminski worked collectively on section 1.1.
- John Kaminski wrote section 1.2 with edits by Mikayla Thompson.
- Mikayla Thompson wrote sections 1.3-1.6 with edits by John Kaminski.
- Evelyn Kurt-Jones and Kate Fitzgerald made edits to the entire chapter.

Chapter 1: Introduction

1.1 Introduction

The innate immune system is crucial for regulation of early detection and clearance of invading pathogens. This response is governed by a specialized group of cells including macrophages, dendritic cells, mast cells, eosinophils, basophils, neutrophils, and natural killer cells(1). Activation of these leukocytes through Pattern Recognition Receptors (PRRs) leads to the production of effectors such as type I interferons and pro-inflammatory cytokines and chemokines, which aid in the clearance of infectious agents. This innate detection acts as a primer for the long term, memory response governed by the adaptive immune system. In contrast to the innate immune system, the adaptive immune system is governed by a different set of cells including B cells, T cells, and γδ.T cells(1). Although the adaptive immune response is slower to form, it is essential for the long-term antigen specific memory response.

Deficiencies in the innate immune system can lead to an onset of many diseases and reoccurring infections due to the inability of the host to initiate a proper immune response. Alternatively, hyper responses of the innate immune system can lead to uncontrollable inflammation, tissue damage, and autoimmune disease. Thus it is important that these responses are tightly regulated to ensure proper detection and clearance of invading pathogens without leading to collateral damage to the host. A greater understanding of how innate immunity defends against various microbes will further the development of safe and effective therapeutic strategies against infectious and inflammatory disease.

1.2 Pattern Recognition Receptors

Cells of the innate immune system utilize pattern recognition receptors (PRRs) to identify viral pathogens by engaging pathogen-associated molecular patterns (PAMPs). Once thought to be moieties found only on pathogens, our understanding of PAMPs (pathogen associated molecular patterns) has expanded to include not only classical PAMPS such as lipopolysaccharides found on bacteria but also nucleic acids. Nucleic acid sensing has emerged as a major component of the immune systems anti-microbial arsenal. A diverse range of pathogens are sensed via recognition of their genomes or nucleic acids which accumulate during their replication. Nowhere is this more prevalent than in viral detection. PRRs respond to signatures present in viruses such as 5' triphosphate RNA, which is not normally found in host RNA or to nucleic acids such as viral DNA which is exposed to sensors localized in the cytoplasm, a compartment normally void of DNA.

Of the PRRs, the Toll-like receptors (TLRs) are perhaps the most extensively studied. TLRs are type 1 transmembrane proteins that traffic between the plasma membrane and endosomal vesicles. They are primarily responsible for detecting PAMPs in the extracellular environment. Those located on the plasma membrane are usually specific for hydrophobic lipids and proteins while those found in endosomes detect nucleic acids. This segregation appears intentional allowing innate cells to respond to components of the viral envelope such as fusion machinery at their surface. In contrast, nucleic acids are detected in the endosome where many viruses uncoat their genomes and enter the

cytoplasm. Upon reaching the cytoplasm, viral components are subject to the scrutiny of the retinoic acid-inducble gene I-like receptors (RLRs), the nucleotide oligomerization domain-like receptors (NLRs), and cytosolic DNA sensors such as members of the AIM2 family or cGAS. Similar to TLRs, RLRs and DNA sensors regulate transcription factors essential for the production of interferons and cytokines. In contrast NLRs and AIM2 are mainly responsible for the maturation of IL-1β and IL-18 through the activation of caspase-1. Interestingly AIM2 acts as a 'check point,' regulating the activation and release of these potent effectors. In addition to the production of proinflammatory molecules, many classes of PRRs mobilize the adaptive immune response by increasing expression of MHC class II and inducing expression of the costimulatory molecules CD40, CD80 and CD86.

1.2.1 Toll Like Receptors

The Toll protein was first recognized for its role in dorsal-ventral patterning of drosophila embryos. Later studies found it to be important for the adult fly's immune response to bacterial and fungal infections fueling the search for mammalian homologs. To date, 10 TLRs have been identified in humans, 13 in mice with TLRs 1-9 common to both. TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the plasma membrane while TLR3, TLR7, TLR8, and TLR9 are endosomal. All TLRs share a common architecture consisting of extracellular leucine-rich repeats and a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain(2). These receptors signal as dimers, differentially recruiting the adaptor proteins Mal (MyD88 adapter-like), also called TIRAP (TIR domain-containing

adaptor protein) and MyD88 (Myeloid differentiation primary response gene 88) and/or TRIF (TIR-domain-containing adaptor inducing IFNβ) and TRAM (Trif-related adaptor molecule)(2). Adaptors initiate signal cascades culminating in the activation of nuclear factor kappa b (NF-κB), mitogen-activated protein kinase (MAPK) and interferon regulatory factors 1, 3, 5 and 7 (IRF-3, -5 and -7)(3). Together these transcription factors not only drive expression of interferons, cytokines and chemokines but also influence cellular maturation and survival.

TLR Signaling

With the exception of TLR3, all TLRs recruit myeloid differentiation primary response gene 88 (MyD88) upon activation. In the case of TLR2 and TLR4, the Mal/TIRAP protein acts as a bridging adapter to recruit MyD88 to the activated receptor(4). MyD88's death domain associates with and activates IL-1R-associated kinase 1 (IRAK-1) and/or IRAK-2. IRAK-4 also transiently interacts with this complex and is thought to phosphorylate IRAK-1. IRAK-1 is subsequently released and engages TNFα receptor-associated factor 6 (TRAF6). Activated TRAF6 is capable of K63-linked polyubiquitination of itself and other proteins. It interacts with NF-κB essential modulator (NEMO, also known as IKKγ), another of its ubiquitination targets, as well as TGF-β-activated kinase-1 (TAK1) and the TAK1 binding proteins (TAB1, TAB2 and TAB3). NEMO forms a complex with IKKα and IKKβ which are the catalytic kinases responsible for phosphorylating IκB. IκB binds to and sequesters NF-κB in the cytoplasm.

Following phosphorylation, IκB is ubiquitinated and finally degraded by the proteasome releasing NF-κB to enter the nucleus and induce gene expression. Studies indicate that TAK1 plays an essential role in both the NF-κB and MAPK pathways by phosphorylating IKKβ and c-Jun N-terminal kinase (JNK) respectively(5,6).

TLR3 is incapable of recruiting MyD88 and instead interacts with the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF). TRIF can directly bind TRAF6 and induce NF-κB in a manner similar to MyD88. In contrast to MyD88, TRIF is also able to recruit the protein receptor interacting protein-1 (RIP-1). RIP-1 synergizes with TRAF6 resulting in more potent NF-κB activation. A third protein recruited to TRIF is TRAF3. TRAF3 associates with TANK binding kinase-1 (TBK1) and IKKi and is essential for the production of type I interferon. TBK1 and IKKi mediate this production by phosphorylating interferon regulatory factor-3 (IRF3) and IRF7. This allows them to dimerize and enter the nucleus where they cooperate with NF-κB and activator protein 1 (AP-1) to bring about target gene transcription. TLR4 can recruit TRIF through the adaptor TRIF-related adaptor molecule (TRAM) and can therefore signal through either pathway.

A number of primary immunodeficiencies in humans are the result of defects in the innate signal pathways described above. For instance, one study of children with nonfunctional MyD88 proteins found they were predisposed to recurrent life-threatening pyogenic bacterial infections(7). A similar phenotype has been reported in patients with IRAK-4 deficiency(8). A study of two

unrelated children with defects in UNC-93B1, a protein thought to be involved in trafficking TLR3, TLR7, TLR8 and TLR9 to the endosome, found an increased susceptibility to encephalitic herpes simplex virus-1 infection(9). PBMCs and fibroblasts derived from these children demonstrated a reduced type I interferon response to HSV-1 challenge and a concomitant enhancement in viral replication(10).

TLR Expression and Activity

The inflammatory response evoked by viral PAMPs depends on a variety of factors. Firstly cellular expression of TLRs differs between innate cell types. Human macrophages are known to express high levels of TLR2 and TLR4 while plasmacytoid dendritic cells (pDCs) mainly express TLR7 and TLR9(11). Expression patterns also vary between species, where TLR9 is restricted to a few cell types in humans it is widely distributed in mice. Furthermore expression of certain downstream signaling molecules fluctuates between innate cell types. For example, pDCs are unique in that they constitutively express the transcription factor IRF7 allowing them to quickly produce high levels of type I IFNs in response to viral infection while other cell types such as macrophages may respond in a more delayed manner(12,13). Thus the response to identical viral PAMPs may differ between cell types both in the nature of effector molecules produced and the kinetics of the response. Virally encoded proteins that subvert or distort the TLR response often further complicate this picture. In the

subsequent sections we discuss the TLRs individually, detailing the viruses they detect and wherever possible the specific viral products sensed.

TLR4

The TLR4-mediated response to LPS is well known for its critical role in innate immune control of Gram-negative bacterial infection. It was also the first TLR shown to respond to a viral pathogen. In 2000, Kurt-Jones et al. reported the interaction between the fusion (F) protein of respiratory syncytial virus (RSV) and TLR4(14). The importance of TLR4 in human viral disease and RSV pathogenesis has been documented in genetic studies. In humans, inheritances of two different single nucleotide polymorphisms (SNPs) in the ectodomain of TLR4 are associated with reduced responses to both LPS and RSV F. A highly significant association was found between RSV infection in high-risk infants and inheritance of hyporesponsive TLR4 SNPs(15). This was confirmed in a separate study that likewise found a significant association between these same TLR4 SNPs and severity of RSV disease in infants(16).

Initial studies linking TLR4 expression to RSV pathogenesis were done in the TLR4-deficient mouse strain C57BL6/ScNCr (which has a deletion of the gene region containing TLR4) as well as in C3H/HeJ mice (non-signaling point mutation of TLR4)(9,14). These studies found that RSV activated NF-κB in a TLR4-dependent manner at early time points of infection(17). The original RSV infection studies with ScNCr mice were controversial as it was suggested that the failure to control RSV was due to a defect in IL-12R signaling(18). However, this

discrepancy between the different studies was due in part to confusion about the mouse nomenclature since the ScNCr mice used in the initial studies (but misidentified as ScCR in the paper(14)) have normal IL-12R(19) while the SccR mice used in the second study were IL-12R-deficient(18). More recent work using targeted TLR4 knockouts (with normal IL-12R) have confirmed the role of TLR4 in controlling RSV replication independent of IL-12R, but interestingly these studies have also revealed an even more important role for TLR2 in limiting RSV replication(20). The purified F protein of RSV induced IL-6 production in a dose-dependent manner in human peripheral blood mononuclear cells (PBMCs) and wild type mouse macrophages alike. However, this response was lost in TLR4 deficient and TLR4 knockout macrophages(14,20). Studies by Vogel and colleagues have shown that the ability of TLR4 to be triggered by RSV F is critical to prevent RSV-induced pathology. Indeed, the formalin-inactivated RSV vaccine which caused exacerbated disease in clinical trials and was found to contain a denatured, non-stimulatory F protein. The disease enhancing activity of the formalin-inactivated RSV vaccine could be reversed by the addition of MPL, a non-toxic lipid A TLR4 agonist(21). Disease severity is also correlated with the absence of “alternatively activated” (AA) macrophages that play a crucial role in tissue repair(22). Taken together with the human and mouse genetics, these studies suggest that TLR4-F protein interactions may protect the host from severe RSV disease by mitigating or reprogramming the host response to promote AA-macrophages and thus promote healing(23).

TLR4 is also important for infections by the retrovirus mouse mammary tumor virus (MMTV). MMTV was shown to activate NF-κB and induce B220 and CD69 lymphocyte activation markers in B cells from wild type but not C3H/HeJ or congenic BALB/c (C.C3H $Tlr4^{lps-d}$) lines(24). TLR4 activation, attributed to the envelope (Env) protein, was found to stimulate production of IL-10(25). Surprisingly induction of TLR4 signaling appears to benefit MMTV. First it activates quiescent B cells encouraging cell division, which is necessary for viral genome integration in the host chromosome. Secondly it promotes secretion of IL-10, an immunosuppressive cytokine which helps the virus persist indefinitely(24).

TLR2

Functional TLR2 exists as a heterodimer with either TLR1 or TLR6 on the plasma membrane of both innate and adaptive immune cells. It can be activated by lipoteichoic acid, a common component of gram-positive bacteria, as well as GPI anchors of parasitic protozoan such as *Plasmodium falciparum*. The TLR2/TLR6 heterodimer has recently been shown to play a role in the innate immune response to RSV. Macrophages from mice deficient in TLR2 or TLR6 responded to RSV with lower levels of TNFα, IL-6, CCL2 (MCP-1) and CCL5 (RANTES) than their wild type counterparts. When TLR2 or TLR6 knockout mice were challenged intranasally with RSV they had elevated peak viral titers and lower numbers of neutrophils and activated DC in their lungs(20). Thus TLR2/TLR6 signaling likely contributes to both innate immune cell recruitment

and viral clearance *in vivo* during RSV infection(20). In human PBMCs, TLR2 contributes to IL-8 and MCP-1 production in response to Epstein-Barr virus (EBV)(26). A TLR2/TLR1-mediated proinflammatory response to the related human cytomegalovirus (HCMV) has also been reported. One study found TLR2 deficient mouse macrophages had significantly reduced IL-6 and IL-8 production in response to UV-inactivated HCMV(27). Furthermore expression of TLR2 and CD14 was required for maximal NF-κB activation and IL-8 secretion in HEK293 cells exposed to HCMV. Envelope glycoproteins B and H were later shown to coimmunoprecipitate with TLR2 and TLR1 and are theorized to be the HCMV PAMPs stimulating TLR2(28).

Lymphocytic Choriomeningitis (LCMV) is a non-cytolytic virus that can cause fatal encephalitis in mice. Wild type glial cells infected with LCMV produce TNFα, CCL2 and CCL5, a response that is abolished in cells derived from TLR2 deficient mice(29). TLR2 also induces MHC class-I and class-II, CD40 and CD86 expression in microglia challenged with LCMV, implicating this pathway in the induction of adaptive immunity(29). In LCMV infection, where much of the CNS damage is caused by the immune response itself, it remains to be determined if TLR2 signaling is protective or pathological. Interestingly TLR2 is important for type I IFN induction during LCMV infection but the mechanism is unclear(30). Although TLR2 is normally not associated with type I IFN induction, a recent study from Barton and colleagues demonstrated that on inflammatory monocytes, TLR2 regulates induction of type I interferon in response to viral but not bacterial ligands(31).

Surprisingly, it appears TLR2 can play either a protective or detrimental role in disease caused by herpes simplex virus (HSV) depending on the context of the infection. Studies using an intraperitoneal infection model found TLR2 deficient neonates were protected from lethal HSV-1 encephalitis compared to wild type mice(32). Despite having similar viral loads, the TLR2 knockouts demonstrated improved survival, attenuated symptoms and reduced CNS inflammatory lesions. In contrast TLR2 was shown to work synergistically with TLR9 to promote survival in an intranasal HSV-1 infection model(33). In addition, TLR2 has been shown to be beneficial in both intraperitoneal and intravaginal HSV-2 infection models(34). TLR2's role in murine HSV infection models may be influenced by factors such as the size of the viral inoculum, the route of administration and the age of the subject. HSV induced two distinct responses; a TLR2-dependent inflammatory cytokine response and a TLR9 and/or non-TLR-dependent type I IFN response. A strong IFN response is necessary to control early virus replication (IFNAR-deficient mice quickly succumb to infection) and prevent spread from the genital tract to the brain (34). Once in the brain, however, inflammation is linked to increased mortality(32).

Measles virus (MV) is another infection in which TLR2 signaling may have both favorable and unfavorable effects. Challenging mice with live or UV-inactivated wild type MV induces IL-6 production and CD150 surface expression in mouse macrophages; a response that is impaired in TLR2-deficient cells(35). Intriguingly CD150 is required for entry of wild type MV into monocytes thus immune activation through TLR2 may in fact benefit the virus by conferring

susceptibility. This study identified MV haemagglutinin (HA) protein as the viral PAMP triggering TLR2 activation(35). MV vaccine strains carrying a single asparagine to tyrosine substitution in the HA protein lacked the ability to activate TLR2.

TLR3

With the exception of neutrophils and pDCs, TLR3 is widely expressed in innate immune cells where it is localized to the endosomal compartment(36,37). In 2001, Alexopoulou et al. demonstrated that activation of TLR3 signaling by the double stranded RNA analog poly(I:C) contributed to the production of type I IFN and cytokines in macrophages. Moreover, genomic dsRNA isolated from reovirus was found to activate wild type but not TLR3 deficient splenocytes. The idea that TLR3 could respond to dsRNA, a common viral PAMP, lead to intense speculation about its role in the host response to numerous infections. Counterintuitively, a later study found no difference in the survival, viral titers or pathology of TLR3 deficient mice following reovirus challenge(38). The authors suggested that during *in vivo* infection, TLR3 may not encounter reovirus dsRNA or that levels may be too low to efficiently activate TLR3(38). This study also reported indistinguishable immune responses to LCMV, VSV and MCMV infection in TLR3 deficient and wild type mice(38). However, other evidence exists suggesting that TLR3 does in fact play a role in controlling MCMV as some studies observed blunted type I IFN and IL-12 production accompanied by higher viral loads in the spleens of mice lacking TLR3(39,40). Despite this, only TLR9

deficient mice had significantly decreased survival compared to wild type suggesting TLR9 is more crucial than TLR3 in MCMV infections(39). A recent study also implicates TLR3 in immune suppression of the related herpes virus HSV-1. Patients with TLR3 dominant negative mutations were found to be more susceptible to herpes simplex encephalitis, a rare but devastating manifestation of HSV-1 infection(41). The presumed ligand for TLR3 in infections with DNA viruses is dsRNA generated during bidirectional transcription of opposing DNA strands. TLR3 signaling also reduces lethality of encephalomyocarditis virus (EMCV), a ssRNA virus that directly damages heart tissue(42). TLR3 deficient mice challenged with EMCV had decreased levels of TNFα, IL-6 and IL-1β mRNA in cardiac tissue and a corresponding reduction in inflammatory infiltrate at 3 days post infection(42). Without TLR3 signaling, EMCV replicated to higher levels in the heart resulting in more rapid and extensive mortality in knockouts(42).

Although this study indicates that the TLR3-mediated inflammatory response is beneficial in EMCV infections; TLR3 signaling appears to be detrimental in a number of other viral infections. For instance, TLR3 deficient mice were protected compared to their wild type counterparts when challenged with a lethal dose of West Nile Virus (WNV)(43). This study found that TLR3 driven production of inflammatory cytokines compromised the blood-brain barrier facilitating WNV entry. This resulted in higher viral loads in the CNS and worsened neuropathology. Likewise, TLR3 was shown to play a pathologic role in infections with Punta Toro Virus (PTV)(44). Wild type mice had drastically

reduced survival and increased hepatic injury compared to TLR3 deficient mice following PTV challenge. Despite having similar serum and hepatic viral loads, wild type mice had elevated levels of IL-6, IFNγ, CCL2 and CCL5, suggesting these proinflammatory molecules may mediate much of the damage observed(44). Interestingly, although TLR3 signaling increases inflammation and reduces Influenza A virus (IAV) lung titers, it causes a paradoxical decrease in survival. Thus in IAV infections, lethality appears to be more dependent on TLR3 signaling than direct virus-induced injury.

TLR7 and TLR8

TLR7 and TLR8 are two closely related receptors that, like TLR3, act in the endosome. Human TLR7 and TLR8 were first shown to respond to the imidazoquinoline-like compound resiquimod (R-848) a synthetic drug recognized for its antiviral and antitumor activity(45,46). We now know that nearly any long single-stranded RNA (ssRNA) is capable of activating TLR7 and TLR8(47). Despite this, differences do exist between these receptors. For example, short dsRNAs containing certain motifs preferentially activate TLR7(48,49). Futhermore, synthetic agonists specific to TLR7 or TLR8 differentially activate innate immune cells leading to distinct cytokine profiles(50). In 2004, Diebold et al. showed that TLR7 mediates IFNα production by pDCs in response to live or heat-inactivated influenza virus(51). This TLR7 response could be elicited simply by exposure to purified genomic ssRNA and was completely abrogated by chloroquine, an inhibitor of endolysosomal acidification(51). Thus the authors

proposed a model, now known as the exogenous pathway, whereby pDCs endocytose and degrade a portion of incoming influenza virions, allowing TLR7 to engage exposed genomic RNA. A similar TLR7-dependent type I interferon response was observed when pDCs were challenged with vesicular stomatitis virus (VSV)(52). Under normal circumstances both influenza and VSV require endocytosis for viral entry. However using a recombinant strain of VSV (VSV-RSV-F), capable of fusing to the plasma membrane, Lund et al. demonstrated that VSV activated TLR7 regardless of the route of viral entry. TLR7 is also responsible for pDC production of IFNα in response to Sendai virus (SV); another ssRNA virus which enters at the plasma membrane(53). Interestingly studies of SV using human U937 and murine RAW 264.7 myeloid lines found only a partial role for TLR signaling in cytokine and chemokine production(54). Recent evidence suggests the cytosolic RLR receptors are chiefly responsible for the cytokine and interferon response to SV in myeloid cell types other than pDCs(55).

One important observation gleaned from studies using SV and VSV was that, in contrast to influenza, UV-inactivation of these virions abolished TLR7 activation(53). From this work a second model of TLR7 activation known as the endogenous pathway was proposed. According to this theory ssRNA intermediates produced during SV and VSV infection are transferred from the cytoplasm to the endosome by means of autophagy(53). Thus, to elicit a TLR7 response by this route, cells must be exposed to live, replication competent virus. This model is supported by studies showing that selective inhibitors of autophagy

and mice deficient in autophagic pathways lack a TLR7 mediated response to SV and VSV(53). Recent studies have implicated TLR7 and TLR8 in the response to human immunodeficiency virus (HIV). ssRNA derived from the HIV genome caused murine pDCs and macrophages and human PBMCs to produce IFNα, IL-6 and TNFα(56). In mice this activity was TLR7-dependent while in humans it appears to rely on TLR8 suggesting that HIV receptors may be species-specific. A study by Wang et al. found IFNα production by human and mouse pDCs responding to Coxsackievirus B (CVB) was also dependent on TLR7(57). Interestingly this response required the presence of CVB-specific antibodies as well as functional Fc Receptor complexes on the pDC surface. Thus they proposed a mechanism whereby opsonized CBV is delivered to the endosome via FcR and once internalized viral RNA is detected by TLR7(57). This observation suggests previous exposure to CVB can influence subsequent innate responses furthering our understanding of the complex interplay between adaptive and innate immunity.

TLR9

In both humans and mice, TLR9 is highly expressed in pDCs, innate cells renowned for their ability to rapidly produce large amounts of type I interferon(11). TLR9 responds to the unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs in viral and bacterial DNA(58). Not surprisingly TLR9 has been shown to play a crucial role in infections caused by a number of

DNA viruses. For instance, TLR9 deficient mice infected with MCMV have a drastically increased mortality compared to their wild type counterparts. This hypersensitivity is likely due to the blunted type I IFN and IL-12 response and reduced NK cell activation which results in an elevated MCMV load(39). In EBV infection, production of type I IFN, IL-6 and IL-8 by pDCs is largely dependent on TLR9(26). This is in contrast to monocytes where TLR2 synergizes with TLR9 to orchestrate the cytokine response to EBV(26). TLR9 signaling also plays a role in the interferon response to HSV types I and II. One study found IFNα production by mouse pDCs in response to HSV-2 was completely dependent on TLR9 and independent of viral replication(59). Using cholorquine it was shown that this recognition required endosomal maturation and could be evoked simply by exposure to purified HSV-2 DNA(59). Furthermore, following *in vivo* HSV-2 challenge, IFNα was only detectable in the serum of mice with intact TLR9. A similar role for TLR9 was described in the response to HSV-1 by splenic pDCs. However, this study also described a delayed IFNα response by conventional dendritic cells (cDCs) and macrophages that was both TLR9 and MyD88-independent but required exposure to replication competent virus. The TLR9-independent IFN response is likely due to cytoplasmic RLRs and may explain why one study using TLR9 deficient mice identified no *in vivo* defects in HSV-1 control(60). Alternatively TLR9 signaling may be more important in certain manifestations of HSV-1 induced disease. A recent study showed TLR9 deficient mice did have higher rates of mortality and viral replication when challenged

intranasally with HSV-1(33). Thus TLR9's precise role in HSV pathogenesis and the relative contributions of other PRRs requires further investigation.

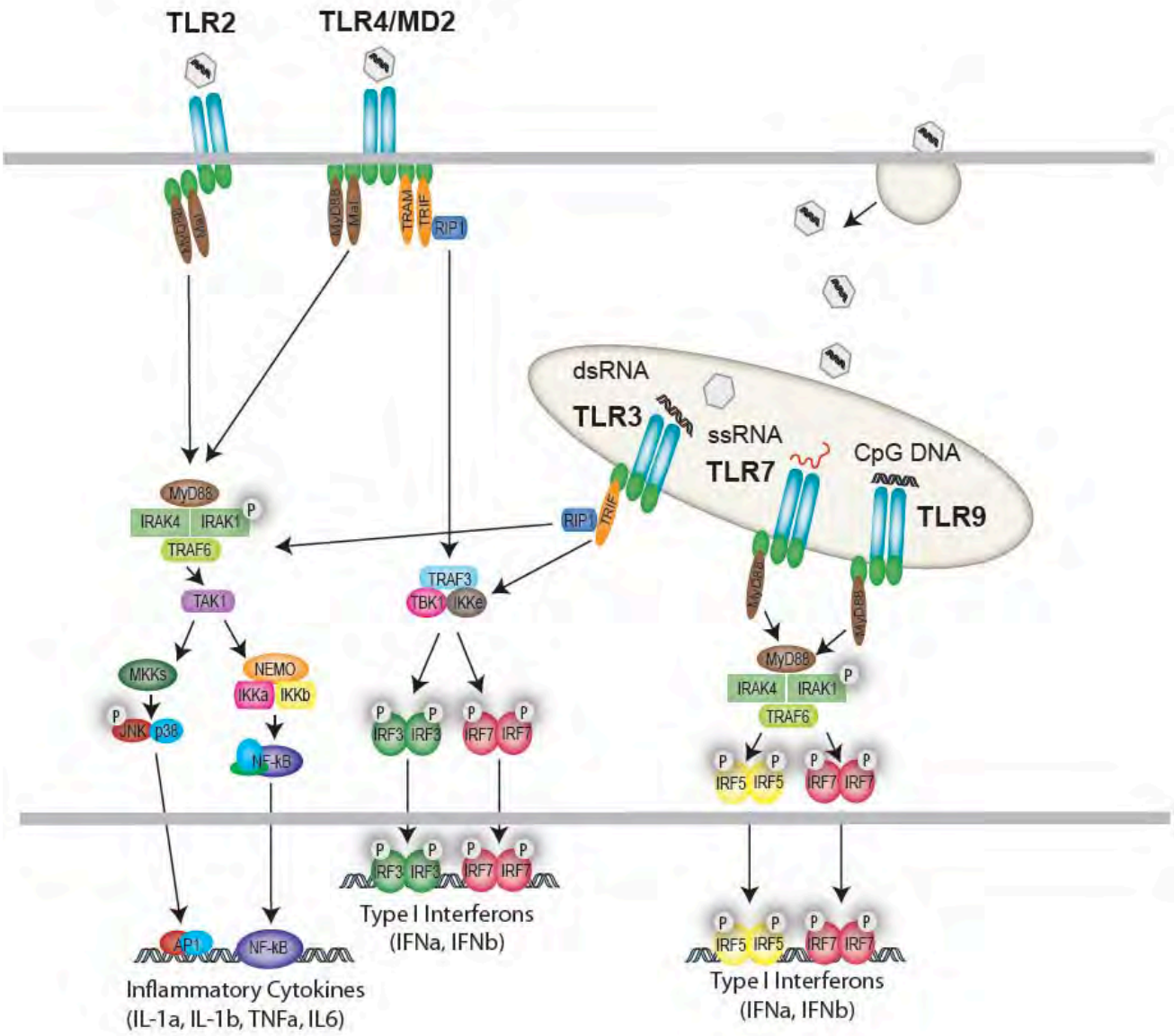


Figure 1.1. Cell surface and endosomal recognition of viruses by TLRs. TLR2 responds to a variety of viruses resulting in activation of a MyD88-dependent NF-κB and MAPK pathway. TLR4, responding to viral proteins (eg. RSV F-protein) activates both a MyD88-dependent and MyD88-independent response. The MyD88-dependent response leads to transcriptional regulation of inflammatory cytokines, while the MyD88-independent response is regulated via TRAM/TRIF and the IKK-related kinases which drive IRF3 activation and type I Interferon production. In the endosome TLR3, TLR7, TLR8 and TLR9 sense viral nucleic acids and generate either IRF3 activation (TLR3) or IRF7-driven type I IFNs (TLR7, 8 and 9).

1.3 Intracellular Cytosolic Nucleic Acid Sensors

As discussed above, the TLRs play an important role in sensing viral PAMPS that are present within the extracellular compartment, as well as in endosomes. In certain contexts, TLRs can detect viral nucleic acids generated from viruses that replicate in the cytoplasm, via an autophagy mechanism. A role for intracellular sensors in the clearance of viruses that replicate and reside within the cytosol of cells has recently emerged. Following the generation of mice lacking TLRs and examination of their susceptibility to virus infections, it became clear that additional sensing mechanisms must also exist and contribute to anti-viral defenses. The last decade or more has revealed numerous additional classes of innate sensors. Of particular relevance to anti-viral defenses was the discovery of specialized classes of cytosolic nucleic acid sensors, termed RIG-I like receptors (RLRs), which recognize intracellular RNA that is introduced to the cytosol during viral infection or that accumulates during replication. Additionally, a diverse selection of intracellular DNA sensors which recognize viral DNA within the cytosol have also emerged.

1.3.1 RIG-I Like Receptors

The RLR family is comprised of three DExD/H box RNA helicases: retinoic acid-inducible gene (RIG-I), melanoma differentiation-associated gene 5 (MDA-5), and laboratory of genetics and physiology-2 (LGP-2)(61-65). Both RIG-I and MDA-5 are comprised of tandem N-terminal caspase activation and recruitment

domains (CARDs) followed by a DExD/H box RNA helicase domain which has ATPase activity and a C-terminal repressor domain (RD). Unlike RIG-I and MDA-5, LGP-2 lacks the N-terminal CARD domains, containing only the RNA helicase domain. As such, LGP-2 was postulated to act as a negative regulator of the other RLRs (62,64). Under resting conditions, RIG-I resides in the cytoplasm in an inactive form that is auto inhibited by its regulatory domain. Upon viral infection, RIG-I undergoes a conformational change by which it dimerizes in an ATP dependent manner (64). The activated mulitmeric form of RIG-I or MDA5 then interacts with the downstream adaptor protein mitochondrial antiviral signaling protein (MAVS), also known as VISA, IPS-1, and CARDIF, via CARD-CARD interactions. MAVS is localized to the outerleaflet of the mitochondrial membrane which is an essential location to support downstream signaling. Recently, MAVS was also shown to be localized on peroxisomes, from where it induces an early antiviral response through the direct induction of a subset of anti-viral genes via the transcription factor IRF1. Upon engagement of RIG-I or MDA5 with MAVS, MAVS activates the IKK-related kinase, TBK1/IKKi, which activates IRF3/IRF7, resulting in the transcription of type I interferons. MAVS also activates NF-κB through recruitment of TRADD, FADD, caspase-8, and caspase-10 (66-70)

RNA Recognition by RLRs

The RLRs are critical components of the anti-viral defense pathway in many cell types including fibroblasts, epithelial cells, and conventional dendritic

cells(71). Initially, it was thought that both RIG-I and MDA-5 recognized the synthetic dsRNA, polyinosinic acid (polyI:C). However, studies from RIG-I and MDA-5 deficient mice determined that MDA-5 alone was responsible for interferon production by polyI:C stimulation (72). Instead, RIG-I recognizes 5'-triphosphorylated (5'-ppp), uncapped ssRNA, which is a common feature in many viral genomes. However, it is unable to recognize the capped 5'-ppp ssRNA from the host cell (73-75). These finding suggest that RIG-I uses the 5' end of a transcript to discriminate between viral and host RNA. MDA-5 distinguishes between viral and host RNA not by its 5' end, but rather by the length of the RNA sequence; long dsRNA is not naturally present in host cells and acts as a ligand of MDA-5. In addition to recognizing 5'-ppp RNA, RIG-I is also capable of recognizing short dsRNA, which is produced as a byproduct of viral replication (76).

RIG-I and MDA-5 appear to differentially recognize different classes of RNA viruses. Studies involving RIG-I deficient mice implicated RIG-I in the recognition of vesicular stomatitis virus (VSV), rabies virus, SV, Newcastle disease virus (NDV), RSV, measles virus, Influenza A and B, hepatitis C virus (HCV), Japanese encephalitis virus, and ebola virus (54,71,72,77-79). Studies from MDA-5 deficient mice show that MDA-5 is able to recognize EMCV, theiler's virus, and mengo virus (72,78). All of these viruses do not contain a 5'-ppp RNA, but are able to produce long dsRNA, providing further evidence that MDA5 discriminates between self and non-self RNA based on sequence length and not the 5'-ppp. More recently studies have shown that both Coxsackie B Virus (CVB)

and poliovirus are dependent on MDA-5 for type I IFN production (80,81). Moreover, some viruses, such as dengue, West Nile virus, and reovirus, signal through a combination of both RIG-I and MDA-5 (80,82,83).

As discussed above, LGP-2 lacks N-terminal CARD domains, and was first thought to be a negative regulator of RLR function(62,64). Initial studies found that overexpression of LGP-2 decreased the capacity of SV and NDV to induce interferon production. Evidence that LGP-2 could associate with RIG-I through mutual RD domains lead to the proposal that LGP-2 directly prevented RIG-I association and activation. Consistent with this idea, interferon signaling was found to be increased in LGP-2 deficient mice responding to polyI:C, providing evidence for negative regulation of MDA-5 as well (84). A second *in vivo* study using LGP-2 deficient mice as well as mice harboring an inactive ATPase in the DExD/H-box RNA helicase domain showed that LGP-2 acted as a positive regulator of RIG-I and MDA-5-mediated signaling after infection by RIG-I and MDA-5-specific RNA viruses. This phenotype is consistent with the possibility that LGP-2 might promote RNA accessibility, thus enabling RIG-I or MDA-5 dependent viral recognition. Further studies on these mice will no doubt clarify this upstream mechanism and the role of LGP-2 in this pathway.

DDX3

Another member of the DExD/H box RNA helicase family, DDX3 has also recently been implicated in anti-viral defenses. Schroder et al. found that the

vaccinia virus protein K7 inhibited IFNβ induction by binding to DDX3, which led to the discovery that DDX3 had a positive role in the RLR signaling pathway(85). A more recent study reported that DDX3 binds to both polyI:C and viral RNA introduced into the cytosol and associates with MAVS/IPS-1 to upregulate IFNβ production. These results led the authors to speculate that DDX3 might enhance RNA recognition, forming a complex with RIG-I and MAVS to induce interferon production (86). Further studies are required to determine whether DDX3 is a bona fide RNA sensor or a component of the RLR signaling pathway in order to fully understand the function DDX3 plays in anti-viral surveillance and signaling.

1.3.2.Cytosolic DNA Sensors

Prior to the discovery of TLR9, it was known that DNA derived from pathogens could activate fibroblasts to produce type I IFNs (87). This phenomenon was ignored or underestimated for decades and was rediscovered following the observation that transfection of pathogen-derived dsDNA activated a TLR9 negative thyroid cell line to upregulate various immunological genes(88). Akira and colleagues subsequently demonstrated that TLR9-/- MEFs, which failed to respond to CpG DNA, produced large amounts of IFN in response to transfection with synthetic B-form dsDNA or genomic DNA isolated from bacteria, viruses, and mammalian cells (88). This was similar to findings presented by the Medzhitov lab using a 45bp dsDNA region from the *Listeria monocytogenes* genome, called Immunostimulatory DNA, or ISD. Cytosolic administration of

dsDNA did not appear to utilize any known TLRs to induce interferon since cells from mice lacking both MyD88 and TRIF responded normally.

Like the cytosolic RNA recognition pathways, cytosolic DNA recognition also leads ultimately to activation of TBK1 and IRF-3 and production of type I IFNs. However, the signaling pathway linking upstream DNA sensors to TBK1 are poorly characterized. TBK1 associates with DDX3, a DEAD box RNA helicase, which regulates IFNβ transcription via IRF-3 (85,86). In addition, TBK1 interacts with the exocyst protein Sec5 in a complex that includes an endoplasmic reticulum (ER) adaptor stimulator of interferon genes (STING) (70) (89-91). STING plays a central role in the signaling pathway upstream of TBK1 following HSV infection(70). STING also interacts with the ER translocon components Sec61β and TrapB in a manner essential for regulation of cytosolic DNA-induced type I IFN production, although the mechanistic understanding of this finding is not known (89). In unstimulated cells, STING localizes to the ER and perhaps ER-associated mitochondria (91). Following stimulation with cytosolic DNA and HSV-1, STING translocates to perinuclear foci, via the Golgi (89). STING localizes partially to endosomes, particularly Sec5 positive structures (89), whilst another report has demonstrated that STING localizes to vesicular structures, which are not peroxisomes, mitochondria, endosomes or autophagosomes (92). Further work is required to clarify the precise subcellular localization of STING. What is clear is the essential role of STING in cytosolic DNA sensing pathways. A growing number of DNA sensors have now been implicated and will be outlined below.

DAI

DNA-dependent activator of IFN-regulatory factors (DAI) was among the first of the cytosolic DNA sensors to be discovered. It is composed of two binding domains for left-handed, Z form DNA, although the protein can recognize B form DNA as well. When DAI was exogenously expressed in L929 cells, it increased type I IFN production in a dose dependent manner following stimulation by both B and Z form DNA. Similarly, knockdown of DAI with siRNA impaired type I IFN production in response to DNA, the 45bp interferon stimulatory DNA (ISD) from *Listeria* and the herpesvirus, HSV-1 (93,94). The production of type I interferons by fibroblasts in response to HCMV was also found to be dependent on DAI (95,96). DAI-knockout mice were subsequently generated, and surprisingly, cells derived from DAI deficient mice respond normally to synthetic and viral dsDNA (93) (96). These results suggested that DAI might play a cell type specific, and redundant role in sensing cytoplasmic DNA, and that other sensors must also be necessary for inducing these responses. Lastly, studies have shown that DAI interacts with RIP-3 to mediate DNA virus induced necrosis. DAI knockdown or knockout cells are resistant to this pathway (97).

RNAPolIII

As discussed above, both synthetic and viral RNA trigger the production of type I IFNs via RIG-I. Although, the RLRs are sensors of RNA, some data has suggested a role for this system in detection of DNA. A somewhat surprising finding was that synthetic B-form dsDNA can also induce IFNβ production in

human cells in manner that was dependent on the RIG-I adapter molecule MAVS(98-100). These findings suggested the existence of an unknown DNA sensor that would signal via MAVS. Recently, two independent studies have provided an explanation for these findings and shown that AT-rich DNA can be transcribed by RNA polymerase III into 5'-ppp RNA, which subsequently activates RIG-I(98,101). This pathway was reported to be involved in type I IFN induction during EBV infections where the EBERs are transcribed by RNA polymerase III(102). This indirect DNA-sensing system was also reported to be involved in induction of type I IFN following HSV-1 or *Legionella* infection(98,101,103), although its role in *Legionella* remains to be confirmed.

DNA-PK/Ku70/Ku80 complex and Mre11

DNA damage repair is an important component of host response to viral infection. Furthermore, DNA damage induces type I interferon production. Studies have implicated DNA-PKCs, Ku70 and Ku80 in the induction of IFN-β in response to DNA transfection and HSV-1 and MVA infection in MEFs. This response is mediated through IRF3, but was independent of NK-κB activation(104). Another DNA damage factor, Mre11, has also been shown to be required for IFN-β production in response to transfected dsDNA in BMDCs. However, Mre11 was dispensable for immune responses to HSV-1 and Listeria infection(105).

Universal Nucleic Acid Sensors LRRFIP1, HMGBs, LSm14A

Several receptors have been identified that recognize both DNA and RNA ligands, acting as “universal nucleic acid sensors.” It has been proposed that these receptors are necessary for full immune activation (106). In addition to DAI and RNA Pol III, Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) has recently been implicated as a regulator of DNA-driven innate immune signaling. LRRFIP1 was found to bind to the drosophila homologue flightless I and play a role in actin organization during drosophila embrogenesis. In a study using *Listeria monocytogenes* to screen for potential cytosolic DNA sensing molecules, siRNA against LRRFIP1 was found to inhibit type I IFN production induced by the bacteria. The authors showed that the IFN response to VSV was dampened in these cells as well. Furthermore, knockdown of LRRFIP1 inhibited IFN production in response to polyI:C, and the synthetic DNA species, poly(dG:dC) and poly(dA:dT), implicating LRRFIP1 in the recognition of both dsRNA and both B and Z form dsDNA. Surprisingly, this function is independent of RNA Pol III. LRRFIP1 does not regulate IRF3 activation but instead appears to regulate a novel β-catenin-dependent coactivator pathway. LRRFIP1 binds RNA or DNA and leads to phosphorylation of β-Catenin, which subsequently translocates to the nucleus where it associates with the p300 acetyltransferase at the IFNβ1 promoter, leading to increased IFNβ production (103). Although LRRFIP1 has been implicated in the recognition of both *Listeria monocytogenes* and VSV, further studies are needed in order to determine its role in sensing other viruses, particularly DNA viruses.

The high mobility group box protein (HMGB1) acts as a danger signal when released from cells during necrosis. It interacts with RAGE, TLR2, and TLR4 to induce inflammation along with other cytokines. HMGB1 has been shown to bind both DNA and RNA to license activation of RIG-I and other nucleic acid sensors. Further work is needed in order to determine where in the cell HMGB1 binds its ligands and how it interacts with other sensors(107).

LSm14A has also been described as a universal nucleic acid sensor. LSm14A is a component of RNA processing bodies (P-bodies). It has been shown to bind both DNA and RNA and induce type I interferon production through IRF3 and requires STING, MAVS, and RIG I respectively. This suggests that viral recognition may take place within P-bodies and LSm14A plays a role in antiviral activation through nucleic acid sensors(108).

DDX9 and 36

Also in the family of DExD/H box RNA helicases, DHX9 and DHX36 have recently been shown to recognize and bind CpG-B and CpG-A DNA, respectively in plasmacytoid dendritic cells. Activation of DHX9 leads to IRF-7 activation and IFNα production, while activation of DHX36 leads to the activation of NF-κB and the production of IL-6 and TNFα. siRNA knockdown of DHX9 and DHX36 inhibited cytokine production in response to the DNA virus HSV-1, while response to the RNA virus influenza A was unaffected (109).

DDX41

DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41), another member of the DEAD/H helicases family, was recently identified as DNA sensor (110). siRNA knockdown of DDX41 in BMDCs and THP-1 cells led to a decrease in Type I IFN production in response to transfected DNA and HSV-1 infection (110). DDX41 was also shown to recognize the bacterial cyclic di-nucleotides cyclic di-AMP and cyclic di-GMP. Binding to these ligands leads to STING dependent activation of type I IFNs (111). It remains to be seen if DDX41 interacts with other sensors in the type I IFN signaling pathway.

IFI16

IFI16 is a human member of the HIN200 proteins. It is expressed differentially in myeloid cells and is highly inducible in response to type I and type II interferons. IFI16 is alternatively spliced into three isoforms, 16a, 16b, and 16c, which have been shown to homo or heterodimerize.(112). IFI16 and its mouse orthologue IFI204 contain two HIN200 DNA binding domains, HIN200A and HIN200B, and a pyrin domain(113). Each HIN200 domain contains two Oligonucleotide/Oligosaccharide Binding (OB) folds that bind to DNA. (114). Crystallography of the IFI16 HIN200 domains bound to dsDNA show that the DNA binding is due to electrostatic interactions between the negatively charged sugar phosphate backbone of the DNA and positively charged residues in the HIN200 domain. Thus, these interactions appear to be non-sequence specific(115).

Early experiments revealed that when IFI16 is fused to a GAL4 DNA binding domain it can act as a transcriptional repressor of CAT activity. Furthermore, in conjunction with SP1, IFI16 can repress transcription of a reporter gene containing the human cytomegalovirus promoter(116). IFI16 was also found to play a role in cell cycle regulation as it interacts with p53 and Rb(117). Due to its interaction with p53, IFI16 has also been described to play a role in a host of cancers such as breast and prostate cancer(118,119), apoptosis(120-125), and DNA damage responses(117,119,126). IFI16 has also been implicated in the pathology of SLE. Anti-IFI16 antibodies are secreted into the serum of SLE patients and serve as a biomarker for the disease(127-129).

More recently, IFI16 was identified as a cytosolic DNA sensor that recognizes viral and bacterial DNA leading to the activation of STING and downstream type I IFN production(130). IFI16 has also been shown to play a role in the recognition of HSV-1. Although the expression of IFI16 is predominately nuclear, studies have shown that IFI16 is capable of shuttling to the cytosol to detect HSV-1 DNA. This is largely attributed to acetylation of the IFI16 bipartite nuclear localization signal upon infection(131). Protesosomal degradation of the HSV-1 capsid allows for detection of the viral DNA by IFI16 in the cytoplasm(132). In contrast, other studies suggest that HSV-1 is detected by IFI16 in the nucleus, and an unknown factor is shuttled to the cytosol to activate STING and subsequent downstream pathways(133). IFI16 has also been shown to play a role in inflammasome activation in response to HSV-1(134) and to sense KSHV DNA in the nucleus and form a complex with the inflammasome

adapter molecule ASC(135,136) Most recently, IFI16 has been linked to inflammasome activation and pyroptotic death of bystander CD4 T cells during HIV infection(137-140).

Work from chapter two of this thesis indicates that IFI16 also plays a broader role in the regulation type I IFN gene transcription and production in response to both RNA and DNA viruses (Chapter 2). Our working hypothesis here is that IFI16 may act as both a sensor and in the nucleus as a regulator of gene transcription.

cGAS

Recently the second messenger, cGAMP, was discovered to activate STING and downstream type I IFN signaling in response to transfected DNA. (141,142). The discovery of cGAMP led to a greater understanding of how cyclic-di-nucleotides interact with STING. Crystallography of cGAMP with STING show that cGAMP binds at the interface of a STING dimer and then leads to a conformational change that activates STING and downstream signaling (143). Through protein purification and mass spectrometry, the enzyme responsible for synthesizing cGAMP was determined to be a nucleotidyltransferase the authors named cGMP-AMP synthase “cGAS”(144). cGAS synthesizes cGAMP from ATP and GTP upon stimulation with DNA(144). RNAi knockdown of cGAS in L929 and THP-1 cells reveal that cGAS is necessary for the production of type I IFNs in response to DNA and HSV-1 (144). Later studies in cGAS knockout cells confirmed these findings.

Multiple structrual studies found that cGAS undergoes a conformational change allowing its substrates access to its catalytic pocket in a DNA dependedant manner(141,145-148). This observation led to speculation that cGAS is the primary cytosolic DNA sensor. The cGAMP produced by cGAS contains a phosphor-di-ester linkage between 2′ -OH of GMP and 5′ -phosphate of AMP and another between 3′ -OH of AMP and 5′ phosphate of GMP. This form of cGAMP binds to STING with high affinity and leads to a more potent type I IFN response than that of bacterial cyclic-di-nucleotides (143,146,149).

cGAS has also been implicated in the recognition of HIV and other retroviruses(150). shRNA knockdown of cGAS in THP-1 cells inhibited the induction of IFN-β and phosphorylation of IRF3. This response was shown to be mediated by the reversed transcribed DNA of HIV. cGAS knockdown cells also produced less IFN-β in response to murine leukemia virus, and simian immunodeficiency virus.

Lastly, the generation of cGAS deficient mice provided further insight into the role cGAS plays in DNA sensing. BMDMs and BMDCs generated from cGAS-/- mice displayed a severely attenuated type I IFN response to DNA ligands as compared to WT mice. The type I Interferon response to Sendai Virus was unaffected in cGAS -/- cells (151). These mice were also more susceptible to HSV-1 infection than WT mice. cGAS-/- mice produce less IFNα/β in the serum up to 12 hours post infection. Thereafter, IFNα/β production returned back to WT levels. The cGAS -/- mice also display increased HSV-1 viral titer in the brain 72 hours post infection. Further studies are needed to determine if cGAS is

playing a redundant role or is working along with other cytosolic DNA sensors such as IFI16.

1.3.3 The Inflammasome

Although the sensing of cytoplasmic DNA is linked to the transcriptional induction of type I IFN and other pro-inflammatory cytokines, cytosolic DNA has also been shown to trigger the caspase-1-dependent maturation of the pro-inflammatory cytokines IL-1β and IL-18 (152,153). IL-1β, a close biological relative of TNFα, has a variety of effects including innate cell recruitment, activation of T-lymphocytes and induction of fever (154). IL-18 increases the cytolytic activity and IFNγ production of natural killer (NK) cells and influences neutrophil recruitment and activation (154,155). Growing evidence supports the importance of these cytokines in anti-viral defenses (156,157). Mice lacking either one of these cytokines have demonstrated enhanced susceptibility to influenza A virus and HSV-1 infections (158). Moreover, pretreating mice with IL-18 protects them from subsequent HSV-1 and VV challenge (159,160).

In contrast to type I IFNs and TNFα, the production of IL-1β is controlled at the level of transcription, translation, maturation and secretion (161,162). Many cell stimuli including TLR-ligands activate the transcription of the pro-forms of IL-1β and IL-18. Unlike most other cytokines however, these pro-cytokines lack leader sequences and are retained in the cytoplasm rather than loaded into secretory vesicles. Maturation (i.e., the cleavage) of pro-IL-1β and pro-IL-18 is catalyzed by the cysteine protease caspase-1 (formerly known as IL-1 converting

enzyme). In resting cells, caspase-1 itself is present as an inactive zymogen pro-caspase-1(163). A large ‘inflammasome protein complex‘ controls the activity of the inflammatory caspase-1 (163). Several protein complexes have been shown to form inflammasomes upon recognizing specific stimuli. NLRPs 2 to 14, which contain a C-terminal LRR-rich domain, a central nucleotide-binding NACHT oligomerization domain, and an N-terminal protein–protein interaction pyrin domain (PYD) associate with the PYD containing adaptor molecule apoptosis-associated speck-like protein (ASC; also termed pycard or TMS1) (164). ASC links the NLRP’s via its C-terminal CARD domain to the CARD domain of pro-caspase-1. This close association of pro-caspase-1 molecules is then believed to provoke self-cleavage into active caspase-1. Active caspase-1 then cleaves pro-IL-1β and pro-IL18. ASC is critical for caspase-1 activation in response to many stimuli (154,165) (163) (166,167).

AIM2

Cytosolic dsDNA also triggers an ASC dependent activation of caspase-1 resulting in the maturation and secretion of IL-1β and IL-18. These findings suggested the existence of an inflammsome complex that can be triggered by DNA. Analysis of this response in macrophages lacking members of the NLRs revealed normal caspase-1 activation in these cells. Subsequent studies from several groups revealed that this response was instead dependent on AIM2 (Absent in melanoma-2), an interferon inducible protein that belongs to the same

PYHIN family as IFI16 (153) (168-170). AIM2 recognizes cytosolic dsDNA of self and nonself origin including viral DNA via its HIN200 domain in a sequence-independent manner. Contrary to other cytosolic sensors of DNA, the recognition of DNA by AIM2 triggers the assembly of an inflammasome complex. Upon DNA binding, AIM2 likely undergoes oligomerization and associates with ASC via homotypic pyrin-pyrin domain interactions, which in turn recruits pro-caspase 1. Published data has shown that the AIM2 inflammasome is an integral component of innate sensing of DNA viruses (157). AIM2 is essential for the activation of caspase-1 and proteolytic processing of IL-1β and IL-18 in antigen presenting cells in response to infection with MCMV and VV. Furthermore, AIM2-ASC dependent IL-18 secretion and NK-cell activation is critical in the early control MCMV infection in vivo (153) (157). In addition to viruses, AIM2 has also been shown to recognize *Francisella tularensis* and as observed for DNA viruses appears to be critical in early control of *Francisella tularensis* infection in vivo. Moreoever, AIM2 as well as NLRP3 and IPAF function in a redundant manner in the recognition of *Listeria monocytogenes(157)* (169).

NLRP3

In addition to the AIM2 inflammasome, a number of recent studies have shown that mice deficient in NLRP3 are more susceptible to virus infections, particularly RNA viruses (152) (171). Loss of NLRP3 was found to attenuate the normal IL-1β and IL-18 responses to IAV and was associated with diminished

innate cell recruitment to the lung and increased pathology (171). Further studies revealed that influenza's M2 protein, a proton-specific ion channel was sufficient to trigger the NLRP3 inflammasome (172). Viral RNA has also been shown to trigger NLRP3 activation, although this is unlikely to be a direct RNA-NLRP3-interaction. The precise relationship between M2 and RNA in NLRP3 activation remains to be clarified. The NLRP3 inflammasome also plays a role in the response to adenovirus, a DNA virus (152). Peritoneal macrophages isolated from NLRP3 or ASC deficient mice exposed to adenovirus are unable to secrete mature IL-1β (152). When challenged in vivo, NLRP3 knockout mice had reduced levels of IL-1β, IL-6, CCL4 (MIP-1β) and CXCL10 (IP-10) in the liver. Recently, a viral NLR homologue was identified in the dsDNA virus, KSHV. The KSHV tegument protein ORF63 appears to be an NLR homolog that can inhibit inflammasome activation by binding to NLRP1 and NLRP3 (135). Inflammasome activation suppresses KSHV reactivation from latency, suggesting that inflammasome activation and IL-1β mediated signaling facilitates KSHV latency. These observations are consistent with a model whereby the KSHV tegument ORF63 protein might bind NLRP3 and/or NLRP1 to block the detrimental effects of inflammasome activation. More recently studies have shown that in response to gram-negative bacteria, caspase 11 can be directly activated by TRIF to license the NLRP3 inflammasome (173).

Intriguingly, a recent study has revealed a role for IFI16 in the recognition of Kaposi sarcoma-associated herpesvirus (KSHV) in endothelial cells. IFI16 is known to recognize viral DNA in the cytosol and drive type I Interferon

production, as discussed above. In endothelial cells however, IFI16 in the nucleus can sense the KSHV DNA and form a complex with the inflammasome adapter molecule ASC. These findings suggest that IFI16 can form an inflammasome complex following recognition of nuclear DNA during infection with this virus (135). Figure 2 portrays the cytosolic and nuclear receptors known to respond to viral pathogens and their downstream signal pathways.

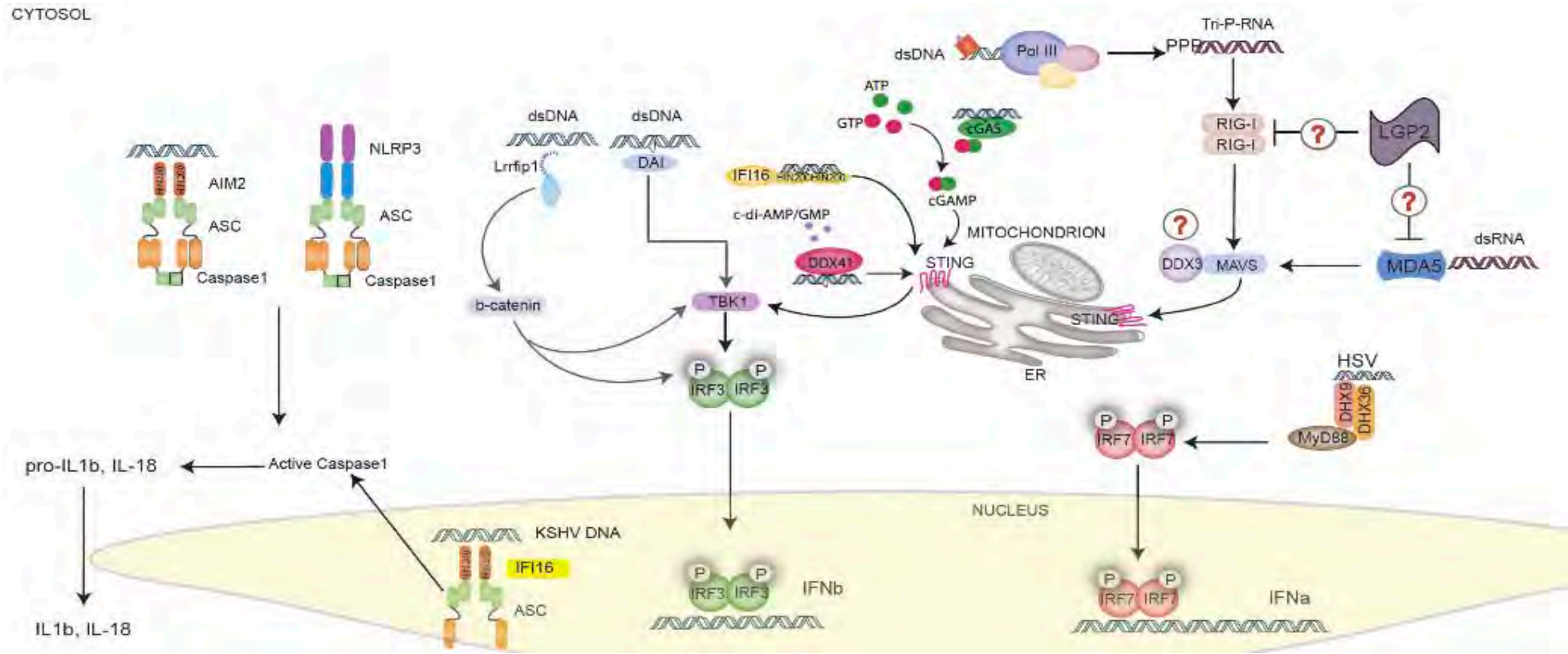


Figure 1.2. Cytosolic and Nuclear PRRs. A multitude of DNA sensors, including IFI16, cGAS, DDX41, RNA Polymerase III, DAI, LRRFIP1, and DDX9/36 recognize DNA and drive type I IFNs and cytokine production. RIG-I and MDA5 recognize RNA in the cytosol. All of these molecules converve on STING in the case of DNA or MAVS in the case of RNA. STING and MAVS then engage either the TBK1-IRF3 or the IKKb-NFkB pathways, resulting in the activation of type I IFN responses and inflammatory cytokines, respectively. AIM2 (which binds to dsDNA) and NLRP3 (which can respond to viral RNA (probably indirectly) act in the cytosol to promote the formation of a multiprotein inflammasome complex that contains the adaptor protein ASC, and caspase-1. IFI16 can also detect DNA in the nucleus during KSHV infection. Nuclear IFI16 engages ASC which then triggers caspase-1 in the cytosol. Activation of caspase-1 results in the proteolytic cleavage of pro-IL- 1β and pro-IL-18 to IL-1β and IL-18 respectively. The mature cytokines can then be released from the cell. Figure adapted from Mikayla R. Thompson, John J. Kaminski, Evelyn A. Kurt-Jones, Katherine A. Fitzgerald. 2011. *Pattern recognition receptors and the innate immune response to viral infection*. Viruses. 6:920-40.

1.4 Herpes Simplex Virus 1

Herpes Simplex Virus 1 (HSV-1) is a large, (152 kb), linear, double stranded DNA virus of the alpha herpresviridae subfamily that encodes for over 80 proteins. The viral genome is encompassed by an icosahedral capsid that is surrounded by tegument proteins and a lipid envelope. The HSV-1 genome is transcribed by host RNA Pol II in three stages: Immediate early, early, and late, over the course of 8 hours. The immediate early genes are responsible for host transcription shut off and viral evasion mechanisms, the early genes regulate viral replication, and the late genes encode for the structural components of the virion. Viral entry to the host cell is mediated by a series of glycoproteins (gC, gD, gH, gL, gB) found on the viral envelope. The viral capsid then enters the cytoplasm, where it attaches to the host nucleus and injects the viral DNA through a nuclear pore. In the nucleus, the virus undergoes a lytic infection, where the genome is replicated and viral particles are formed and bud from the host cell. The virus can also enter a latent stage of infection in the trigeminal ganglia, where it expresses Latency Associated Transcripts (LAT), which silence lytic cycle genes(174).

HSV-1 is typically associated with primary infections of the oropharynx, although it can infect the genitalia as well. The virus enters the host through skin lesions and contact through the mucosal membrane. HSV-1 primarily infects epithelial cells except for when it enters a state of latency whereby it resides dormant in the sensory neurons evading detection by the immune system. Reactivation of the virus from latency due to emotional or physical stress can

cause lesions commonly known as cold sores, which form as a result of cell lysis, apoptosis, and inflammation. The virus also has the ability to travel to the central nervous system (CNS), infecting neurons and astrocyte cell populations (175), resulting in Herpes Simplex Encephalitis (HSE), a disease that causes severe inflammation of the brain. These severe cases are rare in healthy adults but occur more frequently in immunocompromised individuals.

The innate immune response is crucial for the early recognition and control of a HSV-1 infection. Recognition of the virus is largely dependent upon signaling through various pattern recognition receptors (PRRs) including the Toll-Like Receptors (TLRs). TLR9, which recognizes dsDNA in the cytosol, has been shown to recognize HSV-1 in plasmacytoid dendritic cells (pDCs)(59,60) and splenic $CD11c^+$ DCs(60). TLR2 has also been implicated in the recognition of HSV-1 via its glycoproteins in DCs, peritoneal macrophages, (32) and microglial cells(176) in the brain. Furthermore, it has been reported that bone marrow derived dendritic cells have the capacity to recognize HSV by both TLR2 and TLR9 simultaneously (177). Lastly, as HSV-1 has a dsRNA intermediate, it is able to signal through TLR3, and has been implicated as a vital receptor in immunity against HSE(41). HSV-1 can also be recognized by cytosolic RNA helicases such as retinoic acid inducible gene (RIG-I) and melanoma differentiation associated gene-5 (MDA-5), and cytosolic DNA sensors, IFI16 and cGAS, which leads to the production of Type-1 Interferons (178). HSV-1 signaling induces type I interferon production in a MyD88 independent and

Interferon Regulating Factor (IRF7) dependent manner, which is known as the "classical pathway" (12).

Signaling through these PRRs activate the common transcription factors, IRF3, IRF7, NF-κB and AP-1, thus triggering production of type-1 interferons, which play a major role in viral clearance, as well as inflammatory cytokines such as pro-IL-1, IL-6, pro-IL-18 and chemokines such as RANTES, and MCP-1. These effectors block viral replication and recruit additional immune cells necessary to activate the adaptive immune response. More recently, it has been suggested that the inflammasome complex provides a critical role in the clearance of HSV-1 as well. The role of IL-1β in HSV-1 clearance has been clearly elucidated(179), however the specific inflammasome that mediates this response are not well defined. Preliminary data in our lab show that although NLRP3 does not respond to HSV in an HSE model *in vivo*, the NLRP3 inflammasome mediates IL-1 responses to HSV *in vitro* in macrophages and DCs. Furthermore, the NLRP12 inflammasome also plays a role in driving IL-1 maturation in HSV-1 infection *in vitro*, although how HSV-1 triggers NLRP12 activation is unclear. Furthermore, loss of NALP12 leads to increased susceptibility to HSE *in vivo*. Other studies have suggested that IFI16 forms an inflammasome in response to HSV-1(135), however conflicting studies show that IFI16 expression suppresses caspase-1 activation by the NLRP3 and AIM2 inflammasomes(134). Additional studies are necessary to determine the role the inflammasome plays in HSV-1 recognition.

1.5 Type I IFN Induction

Upon viral infection and PRR activation, type I interferons are among the most important effectors that are secreted from cells and lead to an inflammatory antiviral response that is critical in the clearance of pathogens. The production of type I IFNs initiates the transcription of many interferon stimulated genes, which further aid in viral clearance, and triggers the production of other cytokines and chemokines that enhance the inflammatory response and viral clearance. These ISGs also potentiate the production of type I IFNs, thus leading to a positive feedback loop. The importance of type I IFNs in viral infection is highly evident from studies in IFNAR deficient mice, which are susceptible to many viral infections (180,181). In addition to their role in innate immunity, type I IFNs are also important for the development of adaptive immunity. Type I IFNs up regulate MHC-I to facilitate antigen presentation and long-term memory response(182). The induction and regulation of type I IFNs are described below.

The promoter of type I IFNs contains four positive regulatory domains, PRD I, PRD II, PRD III, and PRD IV. PRD I and PRD III are activated by binding of IRFs, while PRD II and PRD IV are activated by binding of NF-κB and ATF-2/cJun respectively(183). The IFN-β promoter contains all four response elements, PRDI-IV. Therefore, IFN-β activation requires binding of the IRFs, NF-κB and ATF-2/cJun. Binding of these transcription factors to the IFN-β promoter leads to the formation of the enhancesome, which also contains the co-activator cAMP response element-binding (CREB)-binding protein (CBP) or p300, to initiate gene transcription (184). In contrast, the IFN-α promoter contains only

PRD I and PRD III. Therefore, IFN-α is regulated solely via binding of the IRFs(185-188).

To date IRF1, IRF3, IRF5, IRF7, and IRF9 have been implicated in type I IFN induction, with IRF3 and IRF7 being most crucial for IFNα/β gene induction. (188,189). As discussed above, upon viral infection, IRF3 is phosphorylated, and undergoes dimerization, and nuclear translocation. Binding of IRF3 to the IFN-β promoter induces the production of IFN-β and ISGs. IFN-β then activates the IFNAR receptor causing a positive feedback loop and amplifying the type I IFN response.

Type I IFN feedback loop is mediated by binding of IFNα/β to the IFNAR receptor and subsequent activation of JAK/STAT pathway. IFNAR is comprised of two subunits, IFNAR1, which associates with tyrosine kinase 2 (TYK2), and IFNAR2, which associates with JAK1. Upon binding of IFNα/β, the receptor dimerization promotes phosphorylation of TYK2 and JAK1 and then phosphorylation of STAT1 and STAT2 and recruitment of IRF9. The heterotrimeric complex of STAT1, STAT2, and IRF9, termed ISGF3, translocates to the nucleus, and binds ISREs, leading to the production of IRF7, IFN-α, and many ISGs(190-192). Over 400 ISGs, including viperin and RIG-I have been identified, all of which are inducible by type I IFNs.

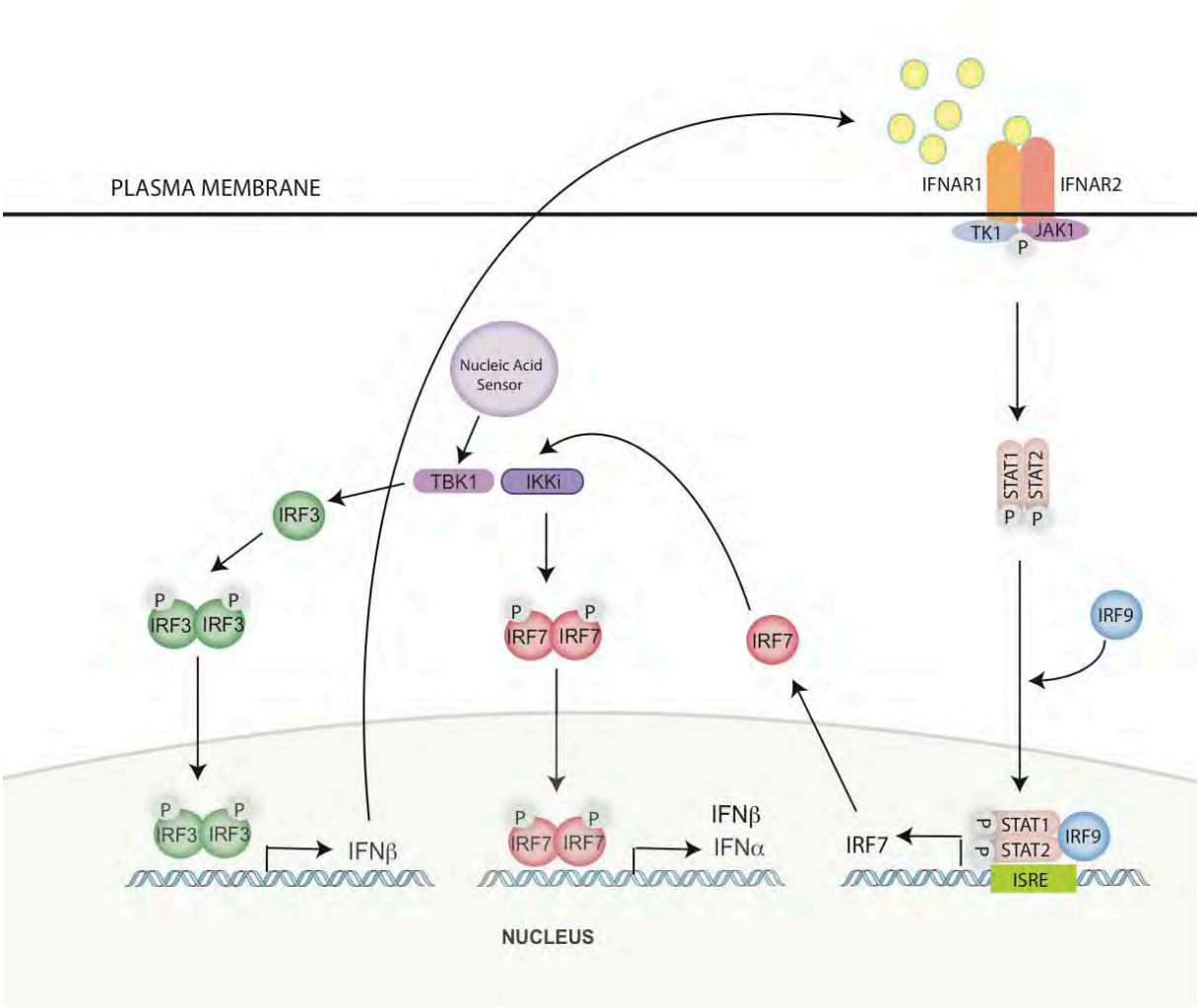


Figure 1.3 Type I IFN signaling**.** Binding of nucleic acids to cytosolic sensors leads to the phosphorylation of IRF3 and the production if IFN-β. IFN-β then activates the IFNAR receptor, which then promotes IFNAR1/IFNAR2 dimerization, phosphorylation of TYK2 and JAK1, and phosphorylation of STAT1 and STAT2. After being activated, STAT1 and STAT2 heterodimers recruit IRF9 to form the transcriptional complex, ISGF3, which translocates into the nucleus to activate ISGs, including IRF7. IRF7 then turns on IFNα/β and other ISGs leading to the positive feedback loop and amplification of antiviral responses. Figure adapted from Mikayla R. Thompson, John J. Kaminski, Evelyn A. Kurt-Jones, Katherine A. Fitzgerald. 2011. *Pattern recognition receptors and the innate immune response to viral infection*. Viruses. 6:920-40.

1.6 Dissertation Objectives

This dissertation has two main objectives: (1) To Determine the role IFI16 plays in the innate immune response to cytosolic nucleic acids, not only by detection of dsDNA, but also in the nucleus by transcriptional regulation of ISGs that are critical in the type I IFN pathway. (2) Define the role of cGAS and IFI16 in anti-viral immunity using mice deficient in cGAS and mice with a targeted deletion of the proposed mouse orthologue of IFI16, IFI204.

1.1 *Delineate the role of IFI16 in sensing RNA and DNA virus infection in vitro and determine the downstream signaling components necessary for this response*. We tested the ability of RNA and DNA viruses and ligands to induce type I IFNs in IFI16 shRNA knockdown THP-1 cells and determined the downstream signaling components necessary for this response by western blot. We demonstrated that IFI16 is necessary for type I interferon production in response both DNA and RNA ligands in THP-1 and U2OS cells.

1.2 *Determine the impact of IFI16 knockdown on immune response gene expression.* Using nanostring analysis, we demonstrate that IFI16 plays a role in the transcriptional regulation of type I IFNs and interferon stimulated genes.

1.3 *Assess the ability of IFI16 to bind to promoter regions of genes whose expression is dependent on IFI16*. By ChIP analysis, we demonstrate that IFI16 knockdown THP-1 cells display less RNA Pol II recruitment to the IFN-α promoter.

2.1 *Determine the role of IFI16 in anti-viral immunity using cells from mice with a targeted deletion of the proposed mouse orthologue of IFI16, IFI204.* We determined that IFNα/β production in response to DNA and RNA ligands is partially dependent on IFI204 in primary mouse macrophages. However, this response is independent of NF-κB activation and cell death.

2.2 *Determine the role of cGAS in anti-viral immunity using cells from cGAS deficient mice*. In contrast to IFI204 deficient cells, primary mouse cells deficient in cGAS were limited in their ability to produce type I IFNs in response to a broad range of DNA ligands. We also implicate cGAS in detection of Malarial DNA.

2.3 *Determine the role of IFI204 in HSV-1 infection in vivo.* We demonstrate that IFI204 plays a role in mediating the host response to HSV-1 infection *in vivo*. IF204 deficient mice display decreased IL-6, IL-1β, and IFN-β levels in the serum and brains of infected mice as compared to WT infected mice.

Preface to Chapter 2

This chapter has appeared in the following publications/manuscripts:

Mikayla R. Thompson, Shruti Sharma, Søren B. Jensen, Maninjay Atianand, Katherine A. Fitzgerald, and Evelyn Kurt-Jones. 2014. *Interferon Gamma Inducible protein (IFI)16 transcriptionally regulates IFN-α and other interferon stimulated genes and controls the Interferon response to both DNA and RNA viruses*. J. Biological Chem. Submitted, under revision.

- Mikayla Thompson performed all experiments.
- Maninjay Atianand provided expertise for ChIP experiments.
- Søren Jensen made the initial IFI16 CDS knockdown cell line.
- Shruti Sharma provided scientific advice
- Mikayla Thompson wrote chapter 2, with edits made by Kate Fitzgerald and Evelyn Kurt-Jones

Chapter 2: Interferon Gamma Inducible protein (IFI)16 transcriptionally regulates IFN-α and other interferon stimulated genes and controls the Interferon response to both DNA and RNA viruses

Abstract

The Interferon Gamma Inducible protein (IFI)16 has recently been linked to the detection of cytosolic DNA during infection with herpes simplex virus and HIV. IFI16 binds dsDNA via HIN200 domains and engages the adaptor molecule Stimulator of Interferon genes (STING) leading to TANK binding kinase-1 (TBK1) dependent phosphorylation of interferon regulatory factor 3 (IRF3) and transcription of type I interferons (IFN) and related genes. In an effort to better understand the role of IFI16 in coordinating type I IFN gene regulation, we generated cell lines with stable knockdown of IFI16 and examined responses to DNA and RNA viruses as well as other inducers of IFN such as cyclic-di-nucleotides. As expected stable knockdown of IFI16 led to a severely attenuated type I IFN response to cytosolic DNA ligands and DNA viruses. In contrast, expression of the NF-κB regulated cytokines such as IL-6 and IL-1β were unaffected in IFI16 knockdown cells, suggesting that the role of IFI16 in sensing these triggers was unique to the type I IFN pathway. Surprisingly, we also found that knockdown of IFI16 led to a severe attenuation of IFN-α and IFN stimulated genes such as RIG-I in response to cyclic GMP-AMP (cGAMP), a second messenger produced in response to cGAS as well as RNA ligands and viruses. Analysis of IFI16 knockdown cells revealed compromised occupancy of RNA

polymerase II on the IFN-α promoter in IFI16-knockdown cells suggesting that transcription of ISGs is dependent on IFI16. These results indicate a broader role for IFI16 in the regulation of the type I IFN response to RNA and DNA viruses in anti-viral immunity.

Introduction

The innate immune system is crucial for regulation of early detection and clearance of invading pathogens. The innate detection of pathogens acts as a primer for the long term, memory response governed by the adaptive immune system. Innate immunity is triggered by a panel of germline encoded pattern recognition receptors that sense foreign pathogens and trigger downstream signaling. This leads to the production of effector proteins such as type I interferons (IFNs), pro-inflammatory cytokines, and chemokines, which are important mediators of this response.

To date, several germline encoded pattern recognition receptors have been described. The Toll Like Receptors (TLRs), which are present on both the cell surface and within endosomal compartments, are perhaps the most widely known and extensively studied. The TLRs recognize conserved pathogen associated molecular patterns (PAMP) such as bacterial LPS as well as danger associated molecular patterns (DAMPS), such as host cell DNA released by damaged cells during cellular stress. During viral infection, members of the TLR family such as TLR3 and TLR7/8 recognize dsRNA and ssRNA respectively. Additionally, in plasmacytoid dendritic cells, TLR9 is responsible for the

recognition of CpG DNA, leading to the production of type I IFNs (58,193). The IFN response to nucleic acids is not exclusively mediated by TLRs, however. RNA and DNA that access or accumulate in the cytosol during viral and bacterial infection are also potent activators of the innate immune response (94,153,194). RNA and DNA elicits TLR-independent responses, particularly those leading to robust induction of type I IFNs. This observation led to the discovery of several cytosolic nucleic acid sensors that sense RNA and DNA in the absence of TLRs and couple pathogen recognition to immune activation.

In the case of RNA, the DExD/H box RNA helicases termed RIG-I like receptors (RLRs) respond to cytosolic RNA. This family is comprised of RIG-I, MDA5, and LGP2. RIG-I, recognizes 5'ppp ssRNA, a moiety commonly found on uncapped negative sense RNA viruses. MDA5, recognizes long dsRNA, a byproduct of positive sense RNA viral replication (72-74,195). A third member, LGP2 has been postulated to act as a negative regulator of RIG-I function but has also been shown to promote MDA5 signaling (61). Growing evidence also supports the existence of multiple cytosolic DNA sensing receptors, which engage STING, leading to TBK-1 dependent phosphorylation of IRF3 and transcription of type I IFN genes (70,89). Through less well-understood pathways, these sensors also elicit NF-κB activation and transcription of NF-κB-dependent genes.

Our lab has been involved in implicating two members of the PYHIN protein family in the recognition of DNA. The first of these AIM2, which is highly conserved in mice and humans, binds DNA via a HIN200 domain and forms a

mulitprotein inflammasome complex with ASC and caspase-1. Activation of caspase-1 results in the maturation and secretion of pro-IL-1β and pro-IL-18 as well as pyroptotic cell death (153,168,170,196,197). A second PYHIN protein IFI16 as well as a murine PYHIN family member IFI204 (also called p204), have also been shown to bind DNA via HIN200 domain(s). IFI16 was initially described as a transcriptional regulator. IFI16 contains a transcriptional regulatory domain and has been shown to bind and transcriptionally repress p53 (116,198,199). It has also been shown to interact with SP-1-like factors to block human cytomegalovirus replication (200,201). In a series of biochemical and loss of function studies, IFI16 was shown to associate with STING and coordinate IRF3 and NF-κB signaling in response to Herpes Simplex Virus-1 and intracellular delivery of dsDNA. Although IFI16 is predominately nuclear, the recognition of DNA by IFI16 was proposed to occur in the cytosol and coordinate the transcription of type I IFNs and associated genes (130). Knockdown of IFI16 or IFI204 (in corresponding mouse cells) by RNAi lead to a decrease in IFN-β production in response to various synthetic DNA ligands and viruses. Orzalli et al have shown that nuclear localization of IFI16 is important in immunity to HSV-1 in human foreskin fibroblasts, permissive cells where HSV-1 undergoes productive infection. Nuclear localized IFI16 is capable of recognizing viral dsDNA and activating IRF3 in response to HSV-1 infection (133). Conrady et al have shown that knockdown of IFI204 in corneal epithelium leads to resistance of HSV-1 infection (202). Other studies have shown that in endothelial cells, IFI16 forms an inflammasome with ASC to produce IL-1β in response to human Kaposi Sarcoma

Virus (KSHV) (135). Most recently, IFI16 has been linked to inflammasome activation and pyroptotic death of bystander CD4 T cells during HIV infection(138-140,203).

Since the initial discovery of IFI16 and IFI204, compelling recent evidence from both human and mouse cells using both RNAi, TALEN knockdown approaches and gene knockouts has convincingly demonstrated the importance of a DNA sensing enzyme called cyclic GMP-AMP synthase (cGAS) in the cytosolic response to dsDNA. The Chen lab was the first to identify cGAS, which binds DNA in the presence of ATP and GTP leading to the generation of a second messenger cGAMP. cGAMP then binds to STING and leads to IRF3 activation. This is true for responses to viruses such as HSV-1 and HIV (140,142,144,150). Given the compelling insight into DNA sensing obtained from the studies of the cGAS-cGAMP pathway, further work is needed in order to fully elucidate the mechanism by which IFI16 contributes to the immune response to cytosolic dsDNA and DNA viruses.

Here we elucidate the role that IFI16 plays in the induction of type I IFNs by examining responses to DNA as well as DNA viruses. Consistent with published studies, we find a critical role for IFI16 in coordinating the induction of type I IFNs and IFN stimulated genes in response to cytosolic DNA as well as DNA viruses. In contrast, we found that the induction of NF-κB dependent genes such as IL-1 and IL-6 however was unaffected in cells with reduced expression of IFI16. Surprisingly, we also reveal that knockdown of IFI16 attenuates IFN/ISG responses to RNA viruses. The role of IFI16 in sensing RNA viruses was also

expanded to demonstrate compromised IFN-α and ISG expression in response to synthetic ligands, which engage the RIG-I pathway. Analysis of RNA polymerase II recruitment to the IFN promoter identifies compromised Pol II association, indicating that the effects on ISGs were at the level of gene transcription. Collectively, these studies describe a regulatory role for IFI16 in the induction of IFN stimulatory genes and subsequent IFN production in response to a broader array of IFN inducers than previously anticipated, expanding the function of IFI16 beyond strictly sensing of microbial DNA.

Results

DNA induced type I interferon production is dependent on IFI16.

The type I IFN response induced by DNA in the human THP-1 monocytic cell line or mouse RAW 264.7 cells is dependent on IFI16 or its proposed mouse orthologue IFI204(130). siRNA studies have shown that transient knockdown of IFI16 in THP-1 cells leads to a decrease in IFN production and reduced IRF3 and NF-κB activation in response to the DNA virus HSV-1 as well as to DNA ligands. In order to provide a more in depth characterization of the DNA response through IFI16, we created a stable IFI16 knockdown in the human myeloid cell line THP-1 via lentiviral transduction of shRNA in a pLKO.1 vector. We also created stable IFI16 knockdown in a second human cell line, U2OS. Two different shRNA plasmids were used; one targeting the coding sequence of IFI16 (IFI16 shRNA CDS-KD), and one targeting the 3'UTR of IFI16 (IFI16 shRNA 3'UTR-KD). The empty pLKO.1 vector was used as a transduction control. In all IFI16 shRNA

transduced cell lines we achieved at least 50 percent reduction in IFI16 levels of both mRNA and protein (Figure 2.1).

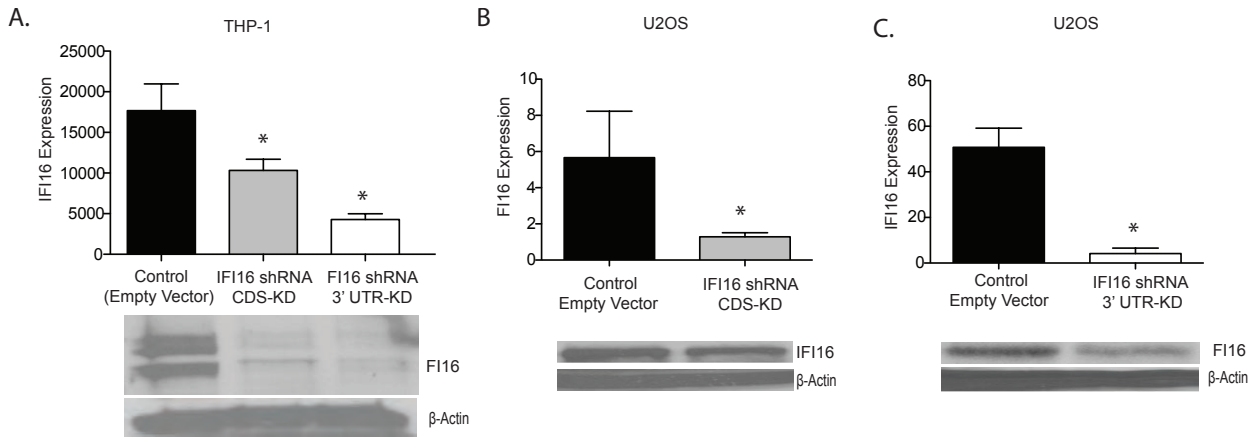


Figure 2.1. Stable knockdown of IFI16 by lentiviral transduction. A. THP-1 or B and C. U2OS cells were targeted with shRNA against the coding sequence of IFI16 (IFI16 shRNA CDS-KD), the 3'UTR of IFI16 (IFI16 shRNA 3'UTR-KD), or empty vector control plasmid by lentiviral transduction. Stable clones were selected and monitored for IFI16 expression by immunoblotting and q-RT-PCR. (* $p<0.05$ assessed by Two-Tailed t-Test compared to empty vector control, data are represented as mean + S.E. Data represents 3 biological replicates).

We next determined if the IFI16 knockdown cells were hindered in their ability to produce type I IFNs in response to DNA ligands. We challenged the THP-1 and U2OS IFI16-knockdown cells with poly(dA:dT), or HSV 60mer (a double stranded DNA sequences derived from the Herpes Simplex Virus-1 genome), or infected with HSV-1 7134 (a mutant strain of the virus that lacks the immunosuppressive ICP0 gene), for 6 hours and then collected RNA from the samples. IFN-α and IFN-β levels were measured by q-RT-PCR and normalized to HPRT. Levels of IFN-β mRNA were decreased in IFI16 knockdown THP-1 cells as compared to empty vector control cells in response to poly(dA:dT), HSV 60mer and HSV-1 (Figures 2.2a, b, and c respectively). We also monitored these responses in U2OS cells as an independent control knockdown cell line. Similar to THP-1 cells, levels of IFN-β mRNA were decreased in IFI16-knockdown U2OS cells in response to poly(dA:dT) and HSV 60mer compared to empty vector controls (Figure 2.2d and e). More interestingly, levels of IFN-α were more drastically reduced in U2OS IFI16-knockdown cells as compared to the empty vector control cells in response to poly(dA:dT) (Figure 2.2f). This result is consistent with the production of IFN-α being downstream of IFN-β production and activation of IRF-7. (In contrast to THP-1 cells, HSV-1 infection did not induce detectable IFN-β mRNA in either vector control or knockdown U2OS cells).

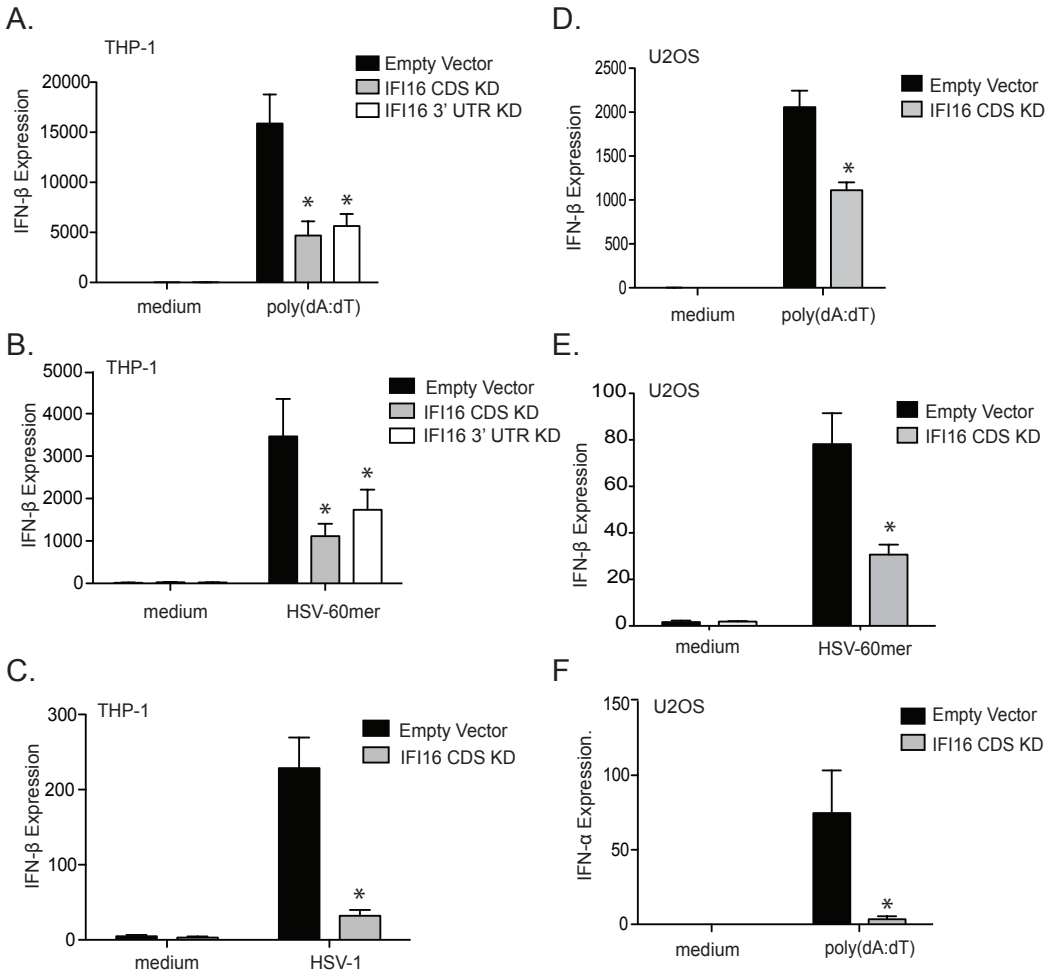


Figure 2.2. IFI16 knockdown cells display an abrogated type I IFN response to various DNA stimuli. IFI16 knockdown THP-1 cells were challenged with A. poly(dA:dT), B. HSV 60mer, or C. HSV-1 virus for 6 hours. Levels of IFN-β were measured by q-RT-PCR. IFI16 knockdown U2OS cells were challenged with D. poly(dA:dT) or E. HSV 60mer for 6 hours and IFN-β levels were measured by q-RT-PCR. F. IFI16 knockdown U20S cells were challenged with poly(dA:dT) and IFN-α levels were measured by q-RT-PCR and normalized to HPRT. (* $p<0.05$ assessed by Two-Tailed T-Test compared to empty vector control, data are represented as mean + S.E. Data represents 3 biological replicates).

IFI16 dependent interferon production occurs independently of NF-κB

In addition to reduced IFN-α mRNA levels, we also noticed a marked decrease in IFN-α production by ELISA in response to poly(dA:dT) in both THP-1 and U2OS IFI16 knockdown cells compared to empty vector controls (Figure 2.3a and b). RANTES production, which is dependent on IFN signaling, was also decreased in U2OS IFI16 knockdown cells in response to poly(dA:dT), HSV 60mer, and VACV 70mer (a double stranded DNA sequences derived from the Vaccinia Virus genome) (Figure 2.3c).

In contrast to the lower levels of type I IFN production, NF-κB related cytokines, IL-6 and IL-1β were unchanged or increased in THP-1 IFI16 knockdown cells that were treated with poly(dA:dT), HSV 60mer or HSV-1 for 12 hours (Figure 2.3d and e). An increase in proinflammatory cytokines may suggest a compensation for the lack of IFN production in these cells. We did not detect differences in phospho-IκBα protein expression in response to poly(dA:dT), HSV 60mer or HSV-1 between IFI16 knockdown and control cells (data not shown). Further, we did not detect a significant difference in cell viability between the empty vector control and IFI16 knockdown cells under our experimental conditions (Figure 2.3f).

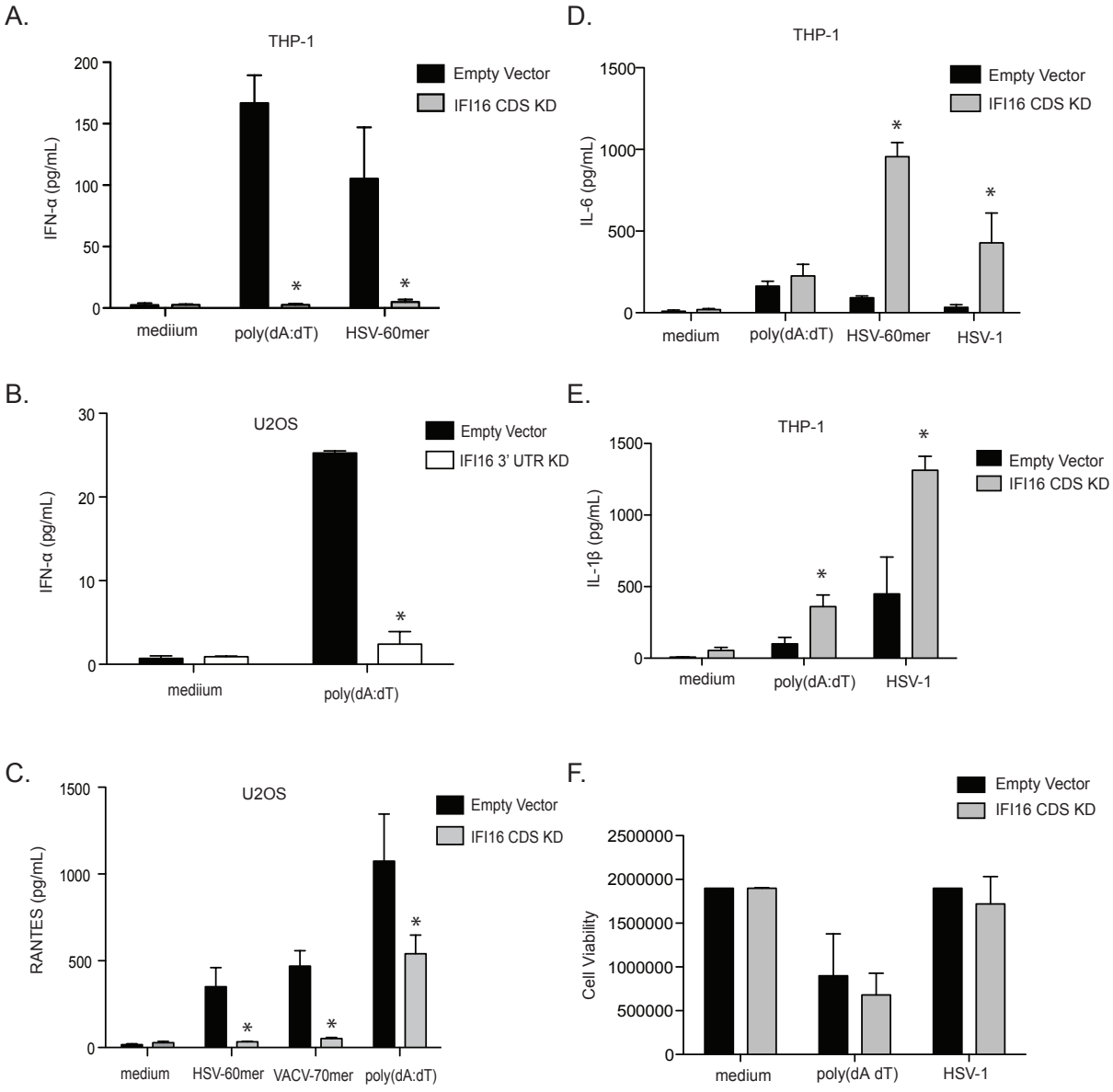


Figure 2.3. IFI16 dependent IFN production occurs independently of NF-κB. A, B. IFI16 knockdown THP-1 and U2OS cells were challenged with poly(dA:dT), VACV 70mer or HSV 60mer for 12 hours. Levels of IFN-α or C. RANTES were measured by ELISA . IFI16 knockdown THP-1 cells were challenged with poly(dA:dT), HSV 60mer or infected with HSV-1 for 12 hours. Levels of D. IL-6 and E. IL-1β were measured by ELISA. F. IFI16 knockdown cells were challenged with poly(dA:dT) or infected with HSV-1 for 6 hours. Cells were stained with calcein for 1 hour and viability was determined by uptake of calcein stain and FITC fluorescence. (* $p<0.05$ assessed by Two-Tailed T-Test compared to empty vector control, data are represented as mean + S.E. Data represents 3 biological replicates). Bars without * are not significant.

To explore whether IFI16 is necessary and sufficient for the production of type I IFNs we performed an addback experiment in which IFI16 was ectopically expressed in the knockdown cell lines. We transduced both CDS and 3'UTR IFI16 knockdown cells with the pRGP-IFI16 retroviral vector to create a transient addback of IFI16. While we did achieve expression of the IFI16 transgene in the CDS knockdown cells, the expression levels were modest (Figure 2.4a). This is not surprising as the shRNA in this cell line could target the expression vector as well as the endogenous gene. We did however achieve much higher expression of IFI16 transgene in the 3'UTR knockdown cell line (Figure 2.4c.) The IFI16 addback cells were challenged with poly(dA:dT), HSV 60mer, VACV 70mer or ISD (immunostimulatory DNA sequence (130)), or infected the cells with HSV-1 or Sendai Virus (Figure 2.4b and data not shown). Despite the modest IFI16 rescue in the CDS knockdown, we did see a partial restoration of IFN-α production in the addback cell line as compared to the knockdown cells without addback. The levels of IFN production were consistent with the amount of IFI16 expression in the addback cell line. We also saw almost a complete restoration of IFN-β production in the 3'UTR addback cell line (Figure 2.4d), also consistent with the amount of IFI16 expression in the addback cell line. These results suggest that IFI16 is necessary for the production of type I IFNs, and the phenotype that we see is not due to off target effects of the shRNA used to knockdown IFI16.

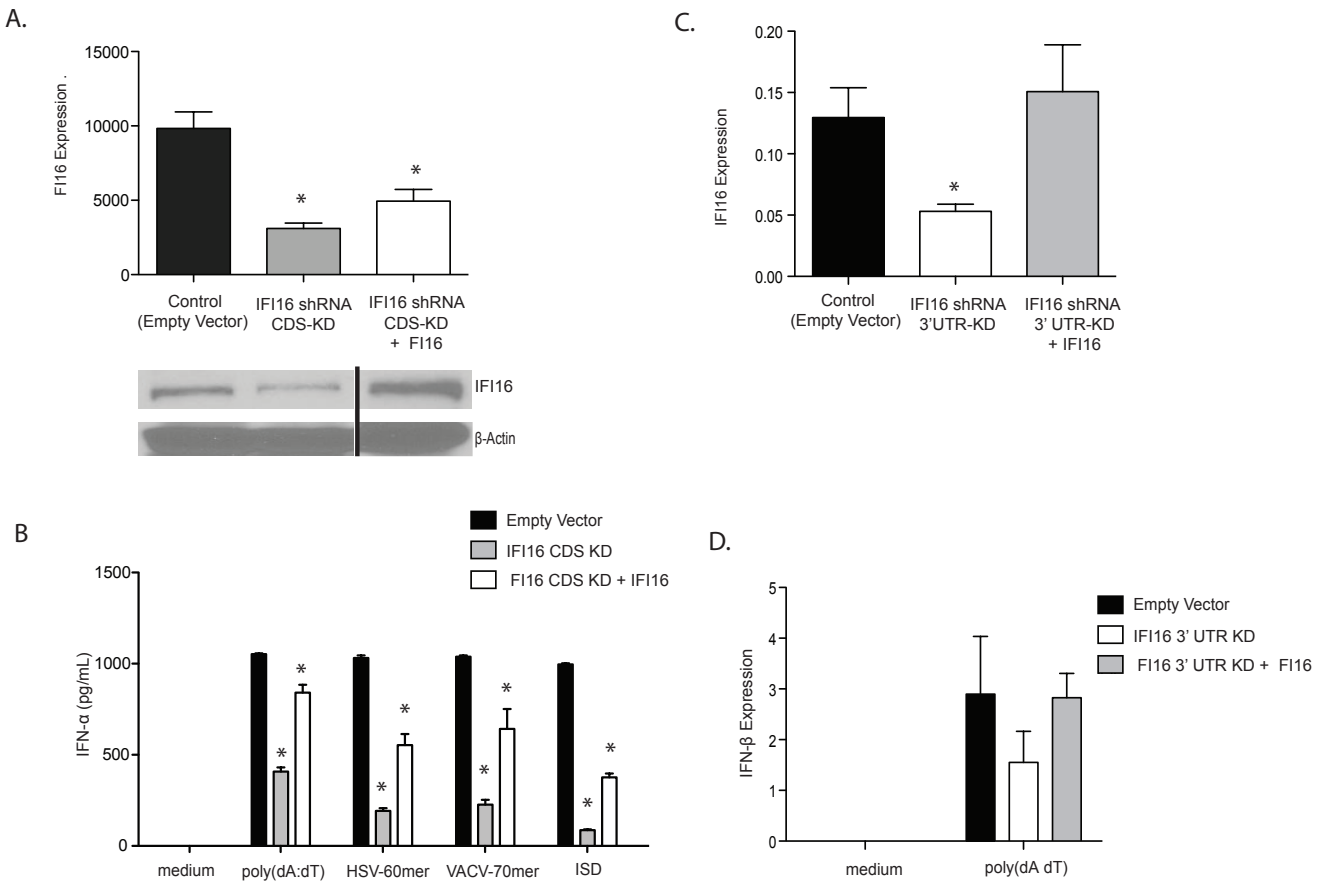


Figure 2.4. IFI16 Addback rescues the type I IFN phenotype. A. IFI16 CDS or C. 3'UTR knockdown THP-1 cells were transduced with IFI16 plasmid to create a stable addback cell line. Levels of IFI16 were monitored by q-RT-PCR or by immunoblot. B, D. Cells were transfected with poly(dA:dT), HSV 60mer, ISD, VACV 70mer or infected with Sendai Virus for 6 or 12 hours for q-RT-PCR or ELISA respectively. Levels of IFN-α and IFN-β were measured by ELISA and q-RT-PCR. (* $p<0.05$ assessed by Two-Tailed T-Test compared to empty vector control or knockdown versus addback, data are represented as mean + S.E. Data represents 3 biological replicates). Bars without * are not significant.

IFI16 knockdown cells display an abrogated type I interferon response to various RNA stimuli and cGAMP.

IFI16 is known to sense infection with DNA viruses such as HSV-1 but is not known to be involved in the detection of RNA viruses such as Sendai virus. Previous studies by Unterholzner et al. have shown that siRNA knockdown of p204 in RAW cells or IFI16 in THP-1 cells does not have an effect on Sendai Virus induced type I IFN production. In contrast to these studies of transient IFI16 knockdown, we found that stable IFI16 knockdown cells display a decrease in type I IFN production in response to Sendai virus. IFI16 knockdown reduced Sendai-induced IFN-β in THP-1 cells (Figure 2.5c) and both IFN-β and IFN-α in U2OS cells (Figure 2.5e and f). Furthermore, the inhibitory effect of IFI16 knockdown on IFN responses was not limited to Sendai virus signaling. We also saw defects in responses to *Listeria monocytogenes*, cyclic di-AMP (a second messenger that is secreted by some bacteria, including *Listeria*, and binds to and activates STING), human metapneumovirus (HMPV) (a RNA virus that signals through RIG-I), and 5'pppRNA (a synthetic RNA that signals through RIG-I (Figure 2.5a, b, and d). We also detected a decrease in the response to EMCV, an RNA virus that signals through MDA5 (data not shown). In contrast to the IFN response, levels of IL-6 and IL-1β in response to Sendai Virus were not affected by IFI16 knockdown (data not shown).

We also monitored the ability of IFI16 knockdown cells to respond to cGAMP, since cGAMP is proposed to work at a level downstream of DNA sensing by engaging STING directly. Empty vector control and IFI16 KD THP-1

cells were stimulated with 2’5’ linkage cGAMP overnight and supernatant was tested for IFN-α production by ELISA. Similar to the DNA and RNA ligands, IFI16 knockdown cells produced less IFN-α than the empty vector control cells (Figure 2.5g). We also looked at protein levels of cGAS by western blot and found that cGAS protein levels were normal in the IFI16 knockdown cells as compared to the control cells, even after stimulation with type I IFN and Sendai Virus (Figure 2.5h).

These results indicate that IFI16 not only plays a role in the detection of Sendai Virus and signaling through RIG-I but also has a global effect on type I IFN production in response to a diverse panel of innate triggers. Furthermore, since cGAS/cGAMP signaling should bypass the need for IFI16 as a DNA sensor, and directly activate STING, these results provide further evidence that although IFI16 can sense DNA leading to STING activation, IFI16 also regulates ISG expression independent of its role in DNA recognition.

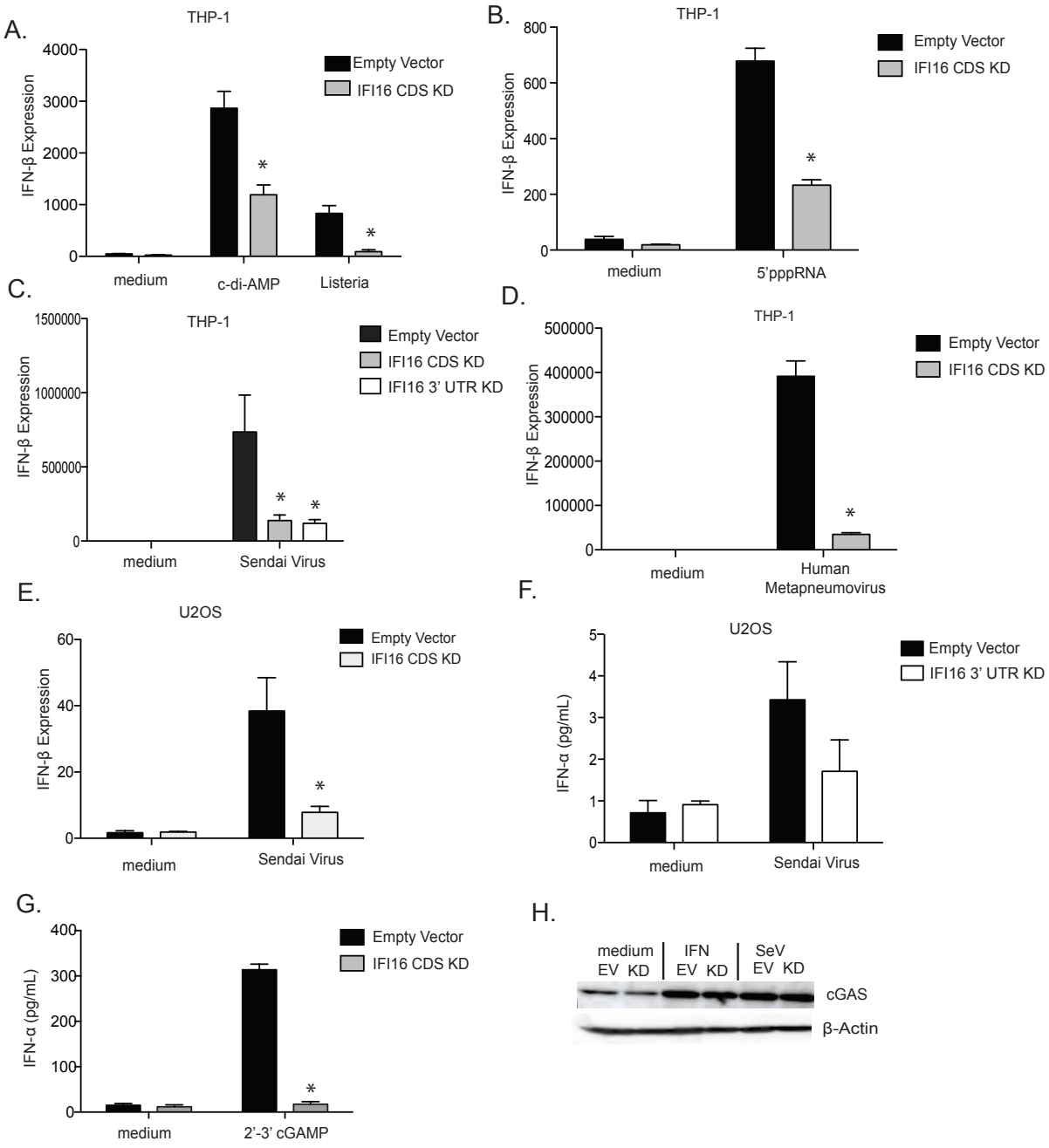


Figure 2.5. IFI16 knockdown cells display an abrogated type I IFN response to various RNA stimuli. A. IFI16 Stable knockdown THP-1 cells were challenged with cyclic-di-AMP, *Listeria monocytogenes*, B. 5'pppRNA, C. Sendai Virus, D. HMPV, or G. 2'-3' cGAMP for 12 hours. E., F. U2OS cells were stimulated with Sendai Virus for 6 or 12 hours and IFN-β levels were determined by q-RT-PCR or IFN-α levels were measured by ELISA respectively. H. Empty vector (EV) and IFI16 knockdown (KD) cells were challenged with type-1 IFN of Sendai Virus for 2 hours and monitored for cGAS expression by western blot. (* $p<0.05$ assessed by Two-Tailed T-Test compared to empty vector control, data are represented as mean + S.E. Data represents 3 biological replicates). Bars without * are not significant.

IFI16 has a global effect on ISG expression

Since IFI16 knockdown cells display a dramatic decrease in IFN production to multiple triggers, and because we saw defects in response to both RNA and DNA stimulation, we next determined where IFI16 was acting in the type I IFN pathway. Western blot analysis demonstrated that expression levels of STING and TBK1 remained unchanged in IFI16 knockdown compared to control cells, however there was a decrease in activation of TBK1 by both RNA or DNA stimulants in IFI16 knockdown cells. Levels of phosphorylated TBK1 were lower in IFI16 knockdown THP1 cells compared to empty vector control cells challenged with poly(dA:dT) or HSV 60mer (Figure 2.6a). Similarly, levels of phosphorylated IRF3 were also decreased. These results are consistent with IFI16 acting as a DNA sensor controlling TBK1 dependent IRF3 activation and type I IFN induction.

We next wanted to determine what effect IFI16 had on global expression of a panel of immune genes including IFN stimulated genes (ISGs). We treated empty vector control and IFI16 knockdown cells with poly(dA:dT) or Sendai Virus for 6 hours and collected RNA for multiplex gene expression analysis by nanostring technology. Nanostring analysis use fluorescently labeled probes that hybridize directly to target mRNA, allowing each individual mRNA to be counted in a highly sensitive manner (204). We found a decrease in the expression of many ISGs and an increase in NF-κB related cytokine gene expression (Figure 2.6b). Some immune genes such as NLRP3, MNDA, and MyD88 remained

unchanged (data not shown). More interestingly, we saw a decrease in RIG-I mRNA expression both basally (Figure 2.6c) and after treatment with LPS, poly(dA:dT), and Sendai Virus (Figure 2.6d). The reduced expression of RIG-I in knockdown cells may explain why there is a defect in Sendai virus signaling in IFI16 knockdown cells and further suggests a role for IFI16 in the transcriptional regulation of ISGs following challenge with RNA ligands or RNA virus infection in IFI16 knockdown cells.

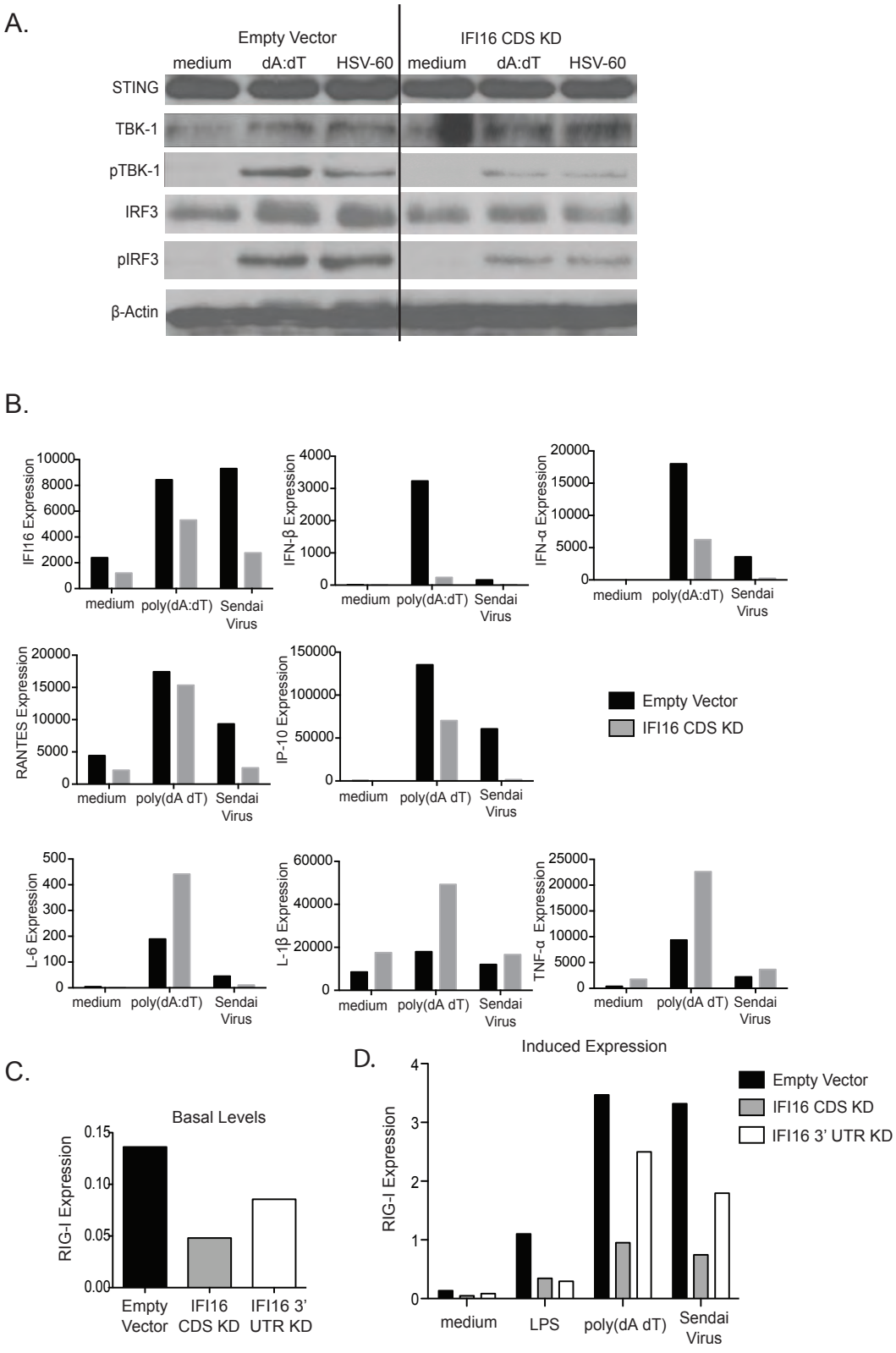


Figure 2.6. IFI16 has a global effect on ISG expression. A. IFI16 knockdown THP-1 cells were challenged with poly(dA:dT) or HSV 60mer for 6 hours and monitored for protein expression by immunoblot. B. IFI16 stable knockdown THP-1 cells were transfected with poly(dA:dT) or infected with Sendai Virus for 6 hours and RNA was collected. Graphs show selected genes from NanoString analysis. C. Basal and D stimulated levels of RIG-I as determined by NanoString analysis. Data represents one experiment.

IFI16 transcriptionally regulates IFN-α expression independently of STAT-1

RIG-I is an IFN-inducible gene that is basally expressed and further upregulated in response to type I IFN production. To determine if a decrease in type I IFN production was responsible for the lower expression levels of RIG-I in IFI16 knockdown cells, we performed an IFN addback experiment. Cells were pretreated with pan-type I IFN for 2 hours and then infected with Sendai Virus for 2, 8 or 24 hours. RNA was harvested from cells and IFN-α and RIG-I expression were monitored by q-RT-PCR. IFI16 knockdown cells were unaltered in their ability to produce IFN-α in response to exogenous pan-IFN (Figure 2.7a). Furthermore, IFI16 knockdown cells were able to produce levels of RIG-I comparable to that of empty vector control cells (Figure 2.7c). Type I IFNs signal through IFNAR and STAT-1 to induce expression of IFN-α and ISGs, such as RIG-I. Since production of IFN-α and RIG-I are normal in the IFI16 knockdown cells after pan-type I IFN stimulation, these results suggest that signaling through IFNAR and STAT-1 are unaffected in these cells.

In contrast, IFI16 knockdown cells were limited in their ability to produce IFN-α mRNA when infected with Sendai Virus, even in the presence of exogenous IFN stimulation (Figure 2.7b). Furthermore, in response to Sendai Virus, IFI16 knockdown cells are only able to induce RIG-I expression to 50 percent or less of that of control cells, even in the presence of exogenous IFN. Although the peak of RIG-I expression is at 2 hours post stimulation, even at 24 hours post stimulation, RIG-I levels in the IFI16 knockdown cells do not return to normal (Figure 2.7c). We also saw a similar phenotype for other ISGs, including

viperin and levels of IRF3 and IRF7 (data not shown). Both basal and IFN induced levels of IL-6 were unchanged or increased in response to exogenous IFN and Sendai Virus in the IFI16 knockdown cells which demonstrates that the phenotype is specific to the type-1 IFN pathway (Figure 2.7d). Taken together, these results suggest that IFI16 knockdown cells are capable of responding to exogenous IFN through the IFNAR/STAT-1 pathway to induce initial RIG-I expression. However, in response to Sendai Virus infection, IFI16 knockdown cells are incapable of amplifying the type I IFN response which controls long-term IFN-α production, and maintains levels of RIG-I expression. More importantly, levels of IFN-α in the IFI16 knockdown cells are dramatically reduced and never return to normal after Sendai Virus infection. These results led us to believe that IFI16 may impact the transcription of IFN-α, which subsequently results in reduced expression of ISGs.

We next monitored STAT-1 expression and activation in these cells. We stimulated cells with type I IFN or infected with Sendai Virus for 2, 8, and 24 hours and collected total cell lysate for immunoblot analysis. Basal levels of STAT-1 were decreased in the IFI16 knockdown cells, which is consistent with IFI16 playing a role in the tonic singling of IFN-α. In response to exogenous IFN and Sendai Virus, the levels of STAT-1 are similar in both the knockdown and control cell lines at 2 hours, suggesting that the initial addition of IFN is enough to induce a response through IFNAR and STAT-1. However, consistent with the inability of the knockdown cells to continuously produce type I IFNs, STAT-1 levels in the IFI16 knockdown cells decrease over time (Figure 2.7e). These

results further support the claim that the defect in IFI16 knockdown cells is not due to their inability to respond to IFN but due to the fact the IFI16 has a plays a role in the regulation of IFN-α expression and IFN stimulatory gene expression.

In order to determine if IFI16 is playing a role in the transcription of IFN-α we next performed a chromatin immunoprecipitation assay in which we monitored RNA Pol II recruitment to the IFN-α, IL-6, and TNF-α promoters. Empty Vector control and IFI16 knockdown cells were stimulated with Sendai Virus for 4 hours and cells were crosslinked with formaldehyde. Chromatin was immunoprecipitated with antibodies directed against RNA Pol II or IgG1 control. Primers for the region immediately upstream of the transcription start site of IFN-α, IL-6, or TNF-α were used to detect Pol II recruitment to these regions via q-RT-PCR. We found that IFI16 knockdown cells have less basal RNA Pol II recruited to the IFN-α promoter than the empty vector control cells. Consistent with our work mentioned above, RNA Pol II recruitment to both IL-6 and TNF-α promoter was higher in the IFI16 knockdown cells (Figure 2.7f). These results suggest that IFI16 plays a role in controlling RNA Pol II recruitment to the promoter of IFN-α.

Based on our results and previous IFI16 siRNA knockdown studies, we suggest that there are multiple mechanisms by which IFI16 can induce type I IFN. IFI16 can act as an intracellular sensor, whereby in response to DNA, IRF3 is activated and type I IFNs and ISGs are produced. Here we show that in the absence of IFI16, DNA activation through TLRs can compensate to induce a pro-inflammatory response. RNA viruses signal through RIG-I to also induce IRF3

activation leading to the production of type I IFNs and ISGs. However, IFI16 also acts at the transcriptional level, whereby it regulates the transcription of IFN-α and ISGs, by facilitating Pol II placement at the promoter of the target gene, thus allowing for the production of genes that regulate type I IFN production. Lastly, type I IFNs can signal through the IFNAR receptor leading to the activation of STAT-1 and type I IFN production. IFI16 operates independently of this pathway (Figure 2.7g).

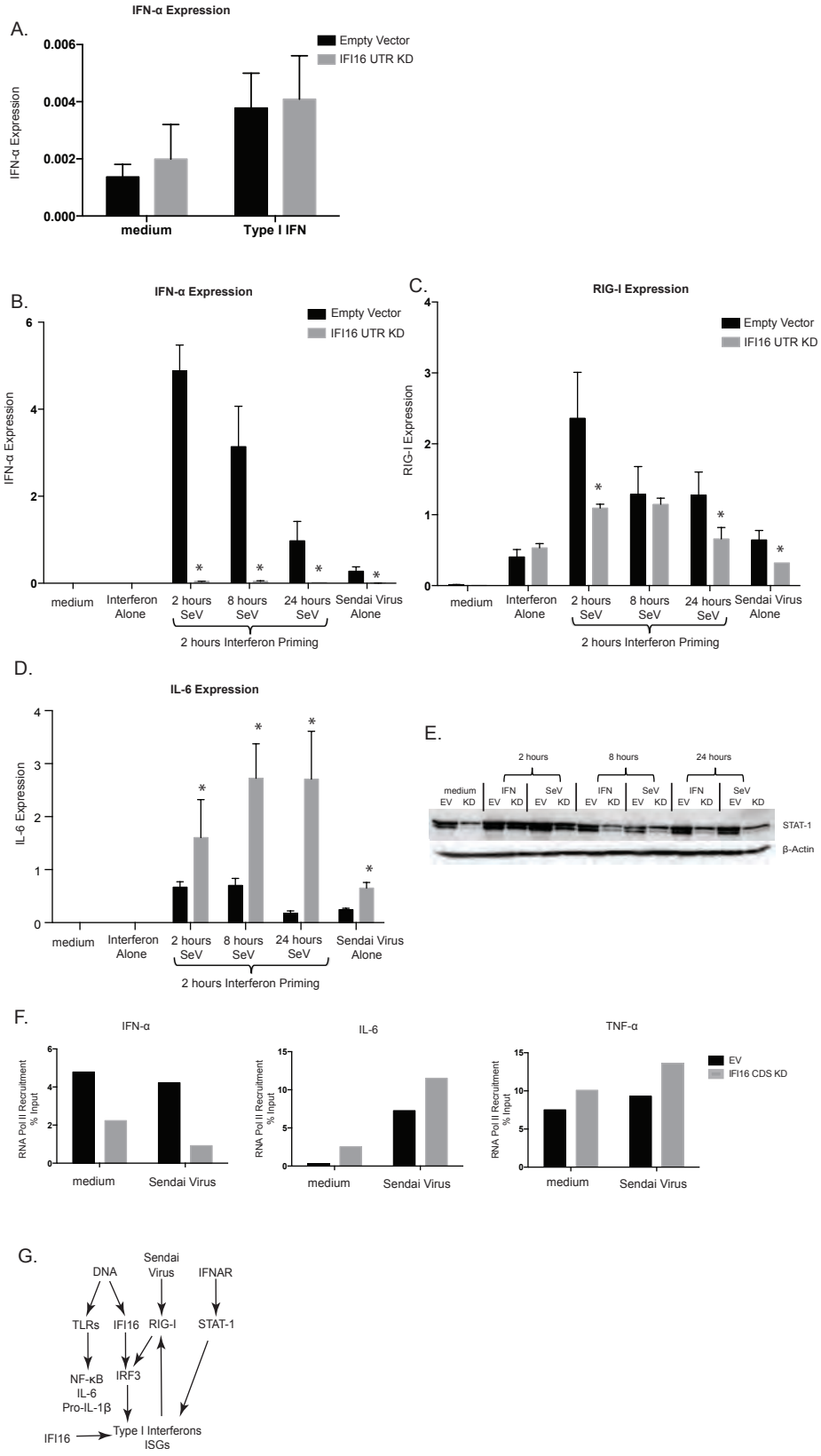


Figure 2.7. IFI16 transcriptionally regulates IFN-α expression independently of STAT-1. Pan Type I IFN was added to cells for 2 hours. A. IFN-α expression was measured by q-RT-PCR. Pan Type I IFN was added to cells 2 hours before stimulation with Sendai Virus (SeV) for 2, 8, or 24 hours. B. IFN-α, C. RIG-I, and D. IL-6 expression were measured by q-RT-PCR. E. Empty vector (EV) and IFI16 knockdown (KD) cells were stimulated with Type I IFN or Sendai Virus for 2, 8, and 24 hours and monitored for total STAT-1 expression by immunoblot. F. Cells were stimulated with Sendai Virus for 4 hours and ChIP Assay was performed. RNA Pol II recruitment to IFN-α, IL-6, and TNF-α was determined by q-RT-PCR. Data is represented as %input minus IgG background. Data is representative of 3 experiments. G. Proposed signaling pathway for IFI16. (* $p<0.05$ assessed by Two-Tailed T-Test compared to empty vector control, data are represented as mean + S.E. Data represents 3 biological replicates). Bars without * are not significant.

Discussion

Here we expand our understanding of IFI16's role. We demonstrate a broader role for IFI16 in the transcriptional regulation of ISGs in response to multiple stimuli. In keeping with previous work by Unterholzner et al, IFI16 knockdown leads to an abrogated type I IFN production when cells were stimulated with either synthetic DNA or viral DNA. However, in contrast to previous work, we also see a defect in type I IFN production in response to RNA ligands, including a defective IFN response to Sendai Virus which was not seen in the original studies. The observed impact of IFI16 deficiency on the IFN response may be attributed to the fact that we used a stable knockdown of IFI16 whereas previous studies used only transient siRNA knockdown. Complete or chronic abrogation of IFI16 protein expression may be necessary to see the full effects of IFI16 deficiency on both DNA and RNA viral ligands. Consistent with these results, when IFI16 is added back to the knockdown cell lines, we see levels of IFN-β restored towards normal production (comparable to empty vector contol cells) when stimulated with various DNA and RNA ligands, suggesting that IFI16 is necessary for these responses.

In addition to a type I IFN response, viral nucleic acids trigger the production of NF-kB-dependent inflammatory cytokines. In contrast to a defect in type I IFN production, the NF-κB response is unchanged or elevated in IFI16 knockdown cells. We see an increase in both IL-6 and IL-1β production in response to both DNA and RNA ligands. We did not see any changes in levels of phospho-IκBα (data not shown) post-challenge indicating that there is no defect

in NF-κB signaling and the impact of IFI16 deficiency on the antiviral response is specific to the type I IFN pathway. However, the increase in inflammatory cytokine production suggests that there may be a shift to a pro-inflammatory phenotype in IFI16 knockdown cells to compensate for a lack of a type I IFN response that is the primary defense when exposed to cytosolic nucleic acids.

In addition to a cytosolic nucleic acid sensing role for IFI16, we also see a role for IFI16 in the transcriptional regulation of IFN-α gene expression as well. Both basal and stimulated levels of RIG-I, a major driver of the IFN response to RNA ligands, are decreased in IFI16 knockdown cells. We also saw a similar defect in other ISGs, including IRF7 and Viperin (data not shown). We postulate that the lower levels of RIG-I in IFI16 knockdown cells are due to the fact that RIG-I is itself an ISG, meaning that type I IFN production leads to the upregulation of the RIG-I gene. When type I IFN is added to cells, expression levels of RIG-I return to normal. In contrast, stimulation with Sendai Virus did not restore expression of RIG-I in IFI16 knockdown cells but did enhance RIG-I levels in control cells. These results suggest that the low level of RIG-I expression is not due to an inability of IFI16 knockdown cells to respond to IFN, but due to the fact that IFI16 plays a role in regulating the production of IFN-α. We also see a decrease in type I IFN production when IFI16 knockdown cells are stimulated with 2'-3' cGAMP. This result provides further evidence that IFI16 has plays a role in the regulation of IFN-α as cGAMP bypasses DNA sensing by IFI16 and activates STING.

To date there has been confilicting studies in determining the role for IFI16 in innate immunity. Part of the perceived conflicts may be due to the dual cellular location of IFI16. IFI16 is both nuclear and cytosolic and the function of this protein may differ by location. The cytosolic location of IFI16 is important for the DNA sensing function of the protein. Studies have shown that acetylation of IFI16 which inhibits the nuclear localization of the protein, allows IFI16 to be exposed to cytosolic viral DNA during HSV infection for interaction with STING and subsequent downstream activation of the type I IFN pathway (131). Furthermore, it was shown that the HSV-1 capsid can be ubiquitinated and degraded by the proteosome, leading to leaked DNA that is then recognized by IFI16 in the cytosol (132). However, several studies have shown a nuclear role for IFI16 during virus infection as well. Thus IFI16 is capable of forming an inflammasome with ASC in the nucleus of epithelial cells in response to KSHV (135) or in CD4 T cells in the case of bystander cells to HIV infected T cells (138,140). Other work shows that IFI16-dependent recognition of HSV-1 occurs in the nucleus of infected human foreskin fibroblasts (133). In our current studies using THP-1 and U2OS cells, we noted that IFI16 is predominantly nuclear and knockdown of IFI16 occurred in the nucleus (data not shown). The nuclear location of IFI16 along with the effect of IFI16 knockdown on IFN and ISG gene expression provide evidence for a regulatory role for the gene in addition to its role as a cytosolic sensor.

We propose that IFI16 acts to position RNA Pol II in a complex with other transcription factors at the IFN-α promoter in order to regulate activation of

transcription of type I IFN production. Previous studies have shown that IFI16 is capable of acting as a transcriptional repressor. Before IFI16 was implicated in innate sensing of DNA, much of the work focused on IFI16 as a transcriptional regulator in cancer cells. It is well known that IFI16 is capable of binding double stranded DNA through its HIN200 domains (130,205). Studies show that when IFI16 is fused to the GAL4 DNA binding domain and transfected into HeLa cells with GAL4-tk-CAT, there was a dose-dependent decrease in CAT activity, suggesting transcriptional repression. This activity is dependent on the HIN200 domain of IFI16 (116). Negative transcriptional regulation is also seen when IFI16 is transfected into HeLa cells with a reporter fused with the promoter of HCMV(116). IFI16 was also shown to repress transcription of CMV DNA polymerase when bound in a complex with SP1 and IR-1 promoter element (200,201). A possible cross talk between transcription regulation and viral nucleic acid sensing with IFI16 may prove to be an important addition to antiviral innate immunity whereby IFI16 not only recognizes an infection to trigger the production of type I IFNs but also promotes the transcription of important antiviral effectors.

There is also evidence that IFI16 plays a role in the cell cycle by negative regulation of p53. IFI16 was found to be bound directly to p53 (199). This interaction inhibits binding of p53 to the p21 promoter, leading to cell cycle arrest (198). Although we do not see an effect on cell death in the IFI16 knockdown cells, these studies provide further evidence that IFI16 is capable of acting in a regulatory manner. In our studies, we reveal a positive regulatory role for IFI16 on ISGs.

Further work needs to be done in order to determine the mechanism by which IFI16 mediates the expression of IFN-α. ChIP analysis of IFI16 would be ideal, however a suitable antibody for these studies is not yet available. We predict that IFI16 may bind directly to the promoter region of IFN-α, possibly in complex with other transcription factors or co-regulators, thus regulating its expression. In addition, ChIP assays coupled to deep-sequencing would provide further information on what promoter regions IFI16 binds and some insight as to what other factors IFI16 may interact with that bind in that region. In the present study, we have performed a ChIP assay in which RNA Polymerase II (Pol II) recruitment to the IFN-α promoter in both empty vector and IFI16 knockdown cells was assessed. Interestingly we found that there was less Pol II recruitment to the IFN-α regulatory region in the IFI16 knockdown cells basally, consistent with the defect in IFN-α transcription. Previous studies have shown that serine 5 phosphorylated Pol II is bound and inactive on some promoters at a basal state. Upon stimulation, Pol II is phosphorylated on serine 2 leading to active transcription (206-209). IFI16 knockdown cells may recruit less Pol II to the IFN-α promoter basally, leading to a defect in IFN-α production and, therefore, a defect in basal levels of ISGs. Stimulation is not enough to overcome the absence of Pol II basally in these cells, thus leading to a continued defect in type I IFN production.

There are many conflicting results in the literature surrounding the cytosolic sensing of nucleic acids. Many sensors, including IFI16, DDX41, and cGAS, and even direct binding to STING have been implicated in the sensing of

cytosolic DNA. More work needs to be done in order to clearly elucidate the contribution of IFI16 to these events and to determine if these proteins are working together, playing redundant roles or functioning in cell type specific manners. This work offers a broader understanding of the role IFI16 plays in tight regulation of the IFN/ISG pathway.

Materials and Methods

Reagents and Antibodies

LPS and poly(dA:dT) were obtained from Sigma-Aldrich (St. Louis, MO). 5'ppp-RNA was from Invivogen (San Diego, CA). HSV 60mer, VACV 70mer, and ISD oligonucleotides were synthesized as described in (130), Cyclic-di-GMP was from Biolog (Hayward, CA). cGAMP 2'-3' was from Veit Hornung (U. Bonn, Germany). *L. monocytogenes* (clinical isolate 10403s) was from V. Boyartchuk (NTNY, Trondheim, Norway). HSV-1 (7134) was a gift from D. Knipe (Harvard Medical School, MA). Sendai virus (SeV, Cantrell strain) was purchased from Charles River Laboratories (Wilmington, MA). The trypsin-independent HMPV isolate A1 (NL\1\001) was from MedImmune (Gaithersburg, MD) and was propagated in Vero cells cultured in IMDM containing 4% BSA and trypsin (210,211). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Genejuice was from Novagen (Madison, WI). Universal Type I IFN was from PBL IFN Source (Piscataway, NJ).

ShRNA-Mediated Silencing

Lentiviral shRNA sequences targeting IFI16 in the pLKO.1 TRC cloning vector were purchased from Sigma-Aldrich (St. Louis, MO). The IFI16 silencing sequences were from the MISSION TRC-Hs 1.0 (Human) as follows: TRCN0000019079 (CDS), and TRCN0000364735 (3'UTR). The production of viral particles and transduction of target cells were conducted according to the following protocols:

(http://www.broad.mit.edu/genome_bio/trc/publicProtocols.html). Lentiviral particles were produced in 293T HEK cells transfected with 3 µg shRNA along with 4 µg pSPAX and pMD2 for 48 hours. Viral supernatant was collected, filtered, and then added to target THP-1 cells. THP-1 cells with shRNA knockdown were selected by puromycin 48 hours later. Knockdown efficiency in stable knockdown cell lines was assessed q-RT-PCR with the following primers: IFI16-F 5′-CCGTTCATGACCAGCATAGG -3′; IFI16-R 5′-TCAGTCTTGGTTTCAACGTGGT -3’

IFI16 Addback

IFI16 coding sequence was cloned into the pRGP retroviral vector and the production of viral particles and transduction of target cells were conducted according to the protocols referenced above. Addback cells were selected by puromycin.

Cell Culture, Stimulation and ELISA

THP-1 cells were differentiated with 0.5 µM PMA overnight prior to stimulation. For stimulations, poly(dA:dT) (1 µg/ml), ISD (3 µM) HSV 60mer (3 µM) VAVC 70mer (3 µM) ci-di-AMP (3 µM), 5’ppp RNA (600ng/mL), cGAMP 2’-5’ (3 µM) were transfected into the cells with lipofectamine in accordance with the manufacturer's instructions. Cells were infected with HMPV or HSV-1 viruses at multiplicities of infection (MOI) of 10. Cells were infected with Sendai virus at 200 IU/ml. Cells were stimulated with Pan Type-1 IFN at 1000 U/mL for 2, 8, or

24 hours. For bacterial infection, cells were challenged with *L. monocytogenes* at an MOI of 5 for 1 hr. Infected cells were then washed twice and medium containing gentamicin (100 μg/ml) was added to kill extracellular bacteria. Knockdown and control cells were challenged with stimulants or microbes for 6 hrs (for RNA analysis and immunoblot analysis) or 12 hours (for protein analysis by ELISA). Cytokine and IFN levels in culture supernatants were assayed for IL-1β and IL-6 (BD Biosciences, Franklin Lakes, NJ) and IFN-α (Mabtech, Mariemont, OH) by sandwich ELISA.

Nanostring and RT-QPCR experiments

Cells were treated for 6 hours and RNA was purified with a quick RNA mini prep kit (Zymo Research, Irvine, CA). RNA transcript counting was performed on total RNA hybridized to a custom gene expression CodeSet and analyzed on an nCounter Digital Analyzer. Counts were normalized to internal spike-in and endogenous controls per Nanostring Technologies' specifications. A pseudo count was added to all values such that the smallest value in the dataset was equal to 1.

cDNA was synthesized, and quantitative RT-PCR analysis was performed as described (212,213). Primers used include: HPRT-F 5'-ATCAGACTGAAGAGCTATTGTAATGA-3', HPRT-R 5'-TGGCTTATATCCAACACTTCGTG-3', IFN-α-F 5'-CACACAGGCTTCCAGGCATTC-3'

IFN-α-R 5’-TCTTCAGCACAAAGGACTCATCTG-3’, RIG-I-F 5’-CTGGACCCTACCTACATCCTG-3’, RIG-I-R 5’-GGCATCCAAAAAGCCACGG-3’ IFN-β-F 5’-GTCTCCTCCAAATTGCTCTC-3’, IFN-β-R 5’-ACAGGAGCTTCTGACACTGA-3’

Gene expression is shown as a ratio of gene copy number per 100 copies of HPRT ± SD.

Immunoblotting

Cells were washed twice with PBS and lysed using a 1% NP-40 buffer. Immunoblotting was performed as described (157). Anti-murine IFI16 was from Abcam, anti-rabbit STAT-1 was from Santa Cruz, anti-rabbit phospho-STAT-1, anti-rabbit phospho-IRF3, anti-rabbit TBK1, and anti-rabbit phospho-TBK1 are from Cell Signaling, anti-murine IRF3 is from BD Biosciences, anti-murine STING was a gift from Glen Barber (University of Miami Health System), anti cGAS is from Sigma.

Cell Viability Assay

Calcein AM stain was obtained from R&D Systems. Cells were stimulated as described above and cell viability was assayed according to the following protocol http://www.funakoshi.co.jp/data/datasheet/RSD/4892-010-K.pdf Cells were washed in PBS and incubated in calcein AM stain for 30 min at 37°C. Calcein AM stain was then washed off with PBS and cells were monitored for FITC fluorescence.

Chromatin Immunoprecipitation

8.0×10^6 empty vector control and IFI16 Knockdown THP-1 cells were stimulated with 200 HAU Sendai Virus for 4 hours, washed with PBS and fixed with 1% formaldehyde for 5 min at RT. Formaldehyde fixation was stopped with the addition of 1.25 M glycine for 5min at RT. The nuclear pellet was sonicated using Bioruptor UCD-200 (Diagenode Inc., Sparta, NJ) to shear the DNA to obtain fragments ranging from 200-500 bp in size. 5µg/IP of sheared chromatin was immunoprecipitated with 2µg anti-RNA pol II (Active Motif; Clone 4H8) or IgG1 isotype control (Imgenex) antibody overnight and 10µl magnetic beads for 1 hr. Following reversal of the cross-linking and protein digestion with proteinase K, immunoprecipitated DNA was purified with the PCR purification kit (Qiagen). Q-RT-PCR analysis was performed on the input DNA (diluted 1:10) and the ChIP as described above. The primer sequences used for detecting the RNA polII recruitment are as follows:

IFN-α-F 5’AAAGCCTTTGAGTGCAGGTG3’ IFN-α-R 5’TCGGCCTCTAGGTTTTCTGA3’; IL-6-F 5’ATTGGGAGACCAGCTCATTG3’ IL-6-R 5’TTCCTGGCGCATAGTAATCC3’; TNFAIP3-F 5’GACCAGGACTTGGGACTTTG3’ TNFAIP3-R 5’AAAACCAACGCCAGGTAGAC3’.

All oligo sequences were designed using Primer3web (http://primer3.wi.mit.edu/), and were targeted within the 500 nucleotides region downstream of the transcription start site of each gene.

ChIP data is represented as %input minus IgG1 background.

Statistical Analysis

Two Tailed T-test was performed using Prism 4 Software (GraphPad, San Diego, CA). P values of <0.05 were considered significant.

Preface to Chapter 3

The work for this chapter was done in collaboration Søren Beck Jensen, who did the initial work with the generation of the IFI204 knockout mouse and Zhao Zhao Jiang who helped with the breeding and characterization of the IFI204 and cGAS knockout mice.

- Soren Jensen generated the IFI204 knockout mouse
- Zhao Zhao Jiang bred the IFI204 knockout mouse
- Mikayla Thompson and Zhao Zhao Jiang performed the experiments in chapter 3 collectively
- Mikayla Thompson wrote chapter 3

Chapter 3: IFI204 plays a partial role in the type I IFN response to DNA and RNA ligands *in vivo*

Abstract

The Interferon Gamma Inducible protein (IFI)204 has recently been linked to the detection of cytosolic DNA during infection with Herpes Simplex Virus. IFI204 binds dsDNA via HIN200 domains and engages the adaptor molecule Stimulator of Interferon genes (STING) leading to TANK binding kinase-1 (TBK1) dependent phosphorylation of interferon regulatory factor 3 (IRF3) and transcription of type I interferons (IFN) and related genes(130). The role of IFI204 has been studied *in vitro*, however the role of the receptor *in vivo* has yet to be determined. Here we developed a mouse deficient in IFI204 to explore the role of IFI204 in *in vivo* immune responses. We investigated the ability of IFI204 deficient cells to induce type I interferons and other cytokines in response to a panel of DNA and RNA ligands *in vitro*. IFI204 deficient BMDMs display a partial decrease in type I interferon induction in response to both DNA and RNA ligands and viruses. We also observed that this reduction in IFN was transient, type I interferon induction returns to wild type levels at 12 hours post infection. In contrast to these results, expression of the NF-κB regulated cytokines such as IL-6 and IL-1β were unaffected in IFI204 knockdown cells. These results suggest that IFI204 plays a partial role in the induction of type I interferons in response to both DNA and RNA ligands. Additionally, IFI204 may work in tandem with other receptors to amplify the type I interferon response. We also studied the involvement of IFI204

in an *in vivo* model of HSV-1 infection. IFI204 knockout mice produce less IFN-β, IL-6, and IL-1β in the brain and serum, 72 hours post intraperitoneal infection with HSV-1. Furthermore, IFI204 -/- mice are more susceptible to HSV-1 infection as compared to WT mice. These preliminary findings indicate that IFI204 participates in the response to HSV-1 *in vivo* by inducing the production of cytokines that are necessary for the control of viral infection. Further studies are needed to define the *in vivo* cell type(s) responsible.

Introduction

Herpes Simplex Virus -1 is a highly prevalent and contagious pathogen that can range in symptoms from recurrent infections of the oropharynx and genitalia to lethal encephalitis of the brain. The innate immune response is crucial for early detection and clearance of the pathogen. This response is largely governed by PRRs including TLR2, TLR 3, TLR 7, TLR9, and NLRP3(32,59,60,176,177). Activation of these PRRs leads to a cascade of cytokines and chemokines, such as IL-6 and IL-1β, that recruits leukocytes to the site of infection. IL-1β deficient mice have increased susceptibility to HSV-1 due to high viral load and rapid spread of disease(179).

The type I interferons are arguably the most important of these effectors, activating an antiviral state in the host that is essential for viral clearance. Perhaps the most striking evidence for this is the increased susceptibility to HSV-1 infection in mice deficient in key players of type I IFN production or response pathway. Mice deficient in IFNAR, STING, and TBK1 are unable to mount the appropriate immune response, which leads to lethality upon HSV-1 infection (12,89,180,181,214,215). On the other hand, HSV-1 has evolved its own mechanisms to evade the host immune response. The immediate early protein ICP0 ubiquitinates IRF3 and IRF7, and targets them for degradation, thus blocks the production of type I interferons (216-218). The mutant HSV-1 7134 strain, which is deficient in ICP0, induces a more robust immune response. For this reason, in our *in vitro* studies we use HSV-1 7134 strain to investigate the receptors recognizing the viral ligands as well as the critical factors upstream of

IRF3 and IRF7 for type I IFN signaling. We use the WT KOS strain or WT equivalent HSV-1 7134R revertant strain in our *in vivo* studies, as ICP0 is necessary for viral replication.

Many cytosolic nucleic acid sensors have been implicated in the response to HSV-1 including RIG-I and MDA5, which detect viral RNA intermediates, and DAI, DHX9, DHX36, DDX41, IFI16, and cGAS, which detect viral DNA in the cytoplasm (178). Recent studies have defined IFI16 as an important mediator of HSV-1 responses. Knockdown of IFI16 by siRNA or shRNA in THP-1 cells leads to decreased IFN-β production in response to HSV-1 *in vitro*. Furthermore, IFI16 mediates a type I IFN response through STING and TBK-1 dependent phosphorylation of IRF3(130). The role of IFI16 has been extensively studied with knockdown techniques *in vitro*, however its role *in vivo* has yet to be defined.

IFI204 is the only member of the PYHIN family in murine cells that has the same domain structure as IFI16 and is proposed as the mouse orhtologue to the gene. IFI204 contains two HIN200 domains and a pyrin domain and is capable of binding DNA. Similar to IFI16, when IFI204 is knocked down by siRNA in murine RAW 264.7 macrophages, bone marrow derived macrophages, or MEFs, type I interferon production is inhibited in response to dsVACV 70mer, HSV60mer, and HSV-1 virus. Contrastingly, in response to Sendai Virus, an RNA virus that produces a robust type I IFN response through the RNA sensor RIG-I, IFN-β production was unaltered in IFI204 knockdown cells(130). Furthermore, knockdown of IFI204 in corneal epithelium leads to resistance of HSV-1 infection *in vitro* (202).

In the present study, we generated a mouse deficient in IFI204 to explore the role of IFI204 in *in vivo* immune responses. We investigated the ability of IFI204 deficient cells to induce type I interferons and other cytokines in response to a panel of DNA and RNA ligands *in vitro*. Additionally, we studied the involvement of IFI204 in an *in vivo* model of HSV-1 infection. These responses were compared to those of cGAS deficient mice, which were also generated. Like IFI16, cGAS has been shown to play a role in the immune response to HSV-1 *in vitro*. Analysis revealed that IFI204 plays a partial role in type I interferon induction in response to both DNA and RNA ligands in BMDMs as compared to cGAS deficient cells which display a complete abrogation of type I IFN production in response to DNA ligands in all cell types. We also show that IFI204 deficient mice produce less IFN-β, IL-1β, and IL-6 during HSV-1 infection *in vivo* as compared to WT and cGAS deficient mice.

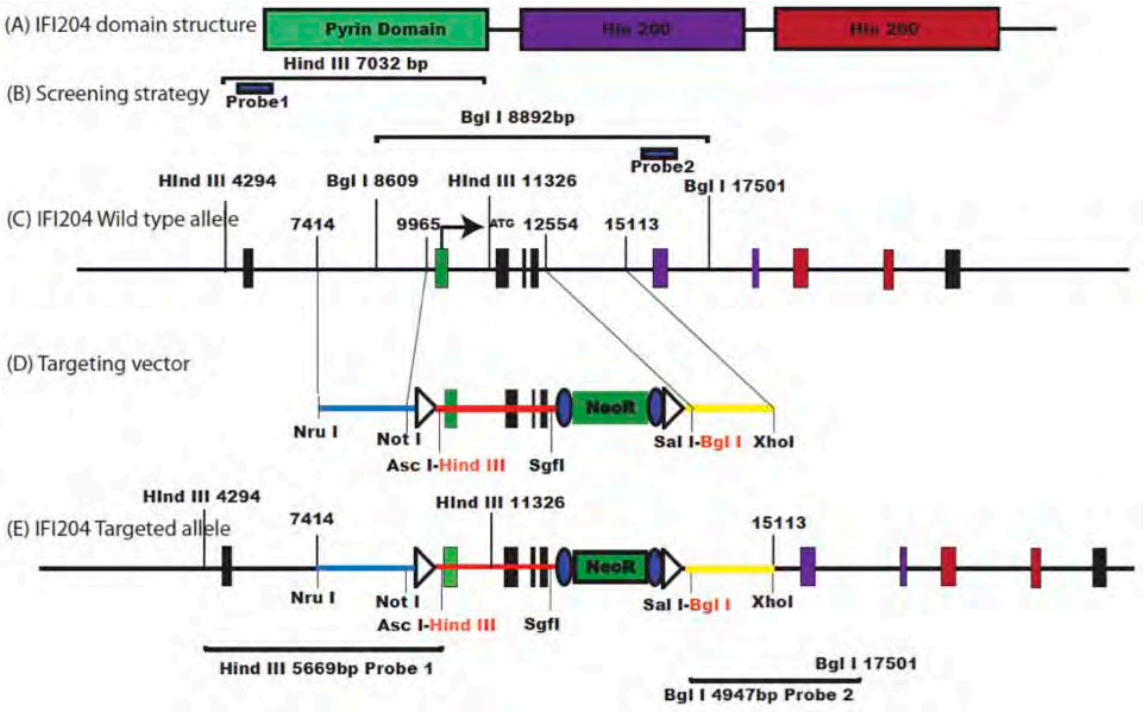
Results

IFI204 plays a partial role in the type I IFN response to DNA ligands.

Recently, IFI16 and its proposed mouse orthologue IFI204 has been identified as a novel cytosolic nucleic acid sensor(219). Much work has been done to study the role of IFI16/IFI204 *in vitro* with knockdown or overexpression of IFI16/IFI204 in cell lines, however to date the role of IFI16/IFI204 *in vivo* is unknown. We have successfully generated an IFI204 gene targeted mouse. A targeting plasmid designed to delete the Pyrin domain (exons 2, 3, 4, and 5) of IFI204 was generated and verified by EcoRI- and HindIII digestions and

sequencing (Figure 3.1). BL6 Embryonic stem cells (ES) were electroporated and 500 ES colonies were expanded and screened for the targeted IFI204 allele by Southern blot analysis. The screening procedure outlined in Figure 3.1 was used. Successfully targeted clones were identified and expanded for verification by southern blot analysis. Correctly targeted ES cells were injected into the blastocyst of albino BL6 mice and chimeric mice were generated. After crossing chimeric mice to albino C57BL6 mice, agouti mice were generated, suggesting that the targeted gene was transmitted to the germline. The mice heterozygous for the IFI204 targeted allele were selected by PCR analysis, which were then bred to ZCRE mice. ZCRE is expressed in the germline and will delete the targeted cassette in order to generate a complete IFI204 knockout allele (Figure 3.1b). The IFI204 knockout mice were born in appropriate Mendelian ratios and appear to be developmentally normal.

A.



B.

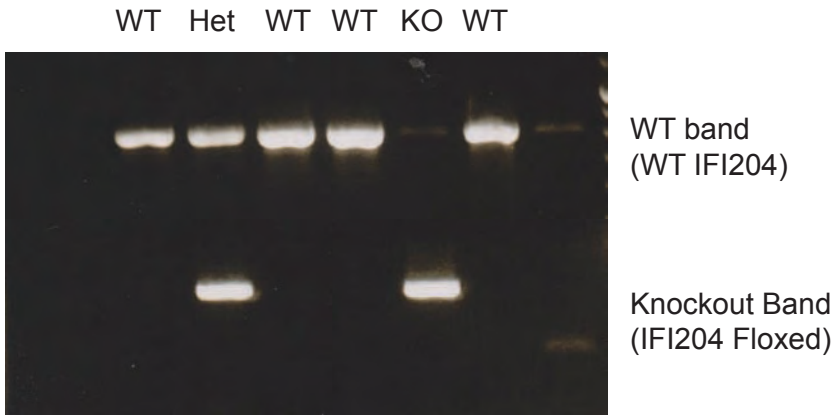


Figure 3.1. Targeting strategy for the generation of the IFI204-/- mouse. A. (a) domain structure for IFI204 (also called p204). (b). Southern blot screening strategy outline. (c). IFI204 IFI204. (d,e,). Targeted IFI204 allele and screening strategy. B. PCR analysis of IFI204 knockout mice. Lanes 1,3,4,6 are WT, lane 2 is IFI204 +/-, and lane 5 is a complete IFI204 -/- mouse.

In order to determine if IFI204 plays a role in type I IFN production, we generated primary cells from IFI204 deficient mice and ZCRE mice as a control. As a control we used cGAS deficient mice, which are known to be limited in their ability to produce type I IFNs in response to HSV-1 and other DNA ligands, Thioglycolate elicited peritoneal macrophages were collected from IFI204 -/-, ZCRE, WT, and cGAS-/- mice and stimulated with a panel of synthetic RNA and DNA ligands as well as RNA viruses and DNA viruses as indicated. After 18 hours of stimulation, supernatants were collected and cytokines were measured by ELISA. We did not notice any significant decrease in IFN-β production in response to poly(dA:dT) or ISD in the IFI204 deficient peritoneal macrophages as compared to ZCRE cells (Figure 3.2a), in contrast to siRNA studies, which implicated IFI204 as a cytosolic DNA sensor marked by decreased type I interferon induction. However, consistent with published reports, IFN-β production in cGAS deficient mice was completely abrogated in response to these ligands as well as to HSV-1 and mCMV, a second DNA virus. Interestingly, HSV-1 and mCMV driven IFN-β production was partially dependent on IFI204 in peritoneal macrophages. We did not notice any significant decrease in IFN-β production in response to cGAMP for either IFI204 or cGAS deficient mice. This result was expected, as cGAMP bypasses the need for a sensor and directly binds STING to induce type I interferons (Figure 3.2a).

We also derived bone marrow derived macrophages (Figure 3.2 c and d) and dendritic cells (Figure 3.2 b) from IFI204 -/-, ZCRE, WT, and cGAS-/- mice. We stimulated BMDCs with a panel of stimuli as indicated for 18 hours and

monitored IFN-β production by ELISA. Consistent with results from peritoneal macrophages, BMDCs from IFI204 -/- mice were not altered in their ability to produce IFN-β in response to DNA ligands, poly(dA:dT) and ISD, whereas responses from cells derived from cGAS -/- mice were severely attenuated as compared to WT mice(Figure 3.2 b). Unlike peritoneal macrophages, IFN-β production was not governed by IFI204 in BMDCs in response to HSV-1 or mCMV. Furthermore, we saw no dependency on IFI204 or cGAS in response to Sendai Virus or cGAMP, which is consistent with pervious studies.

We next stimulated BMDMs from IFI204 and cGAS deficient mice with a panel of RNA and DNA stimuli, including poly(dA:dT), ISD, SV, HSV-1, and cGAMP for 6 hours and monitored their ability to induce IFN-β mRNA production by q-RT-PCR (Figure 3.1c and d). When we monitored fold change induction of IFN-β in the IFI204 -/- cells, we noted a 50 percent decrease in IFN-β induction in response to cGAMP, poly(dA:dT), ISD, and Sendai Virus (Figure 3.2d). These results suggest that IFN-β response to both DNA and RNA ligands is partially dependent on IFI204. This is consistent with results from IFI16 knockdown cells shown in chapter 2 of this thesis. Thus, IFI204 may play a broad role in regulating the response to both DNA and RNA ligands both as a sensor and transcriptional regulator. It also suggests that IFI204 may play a role in early responses to infection, since we saw no decrease in IFN-β production in BMDCs after 12 hours. Consistent with previous results, cGAS deficient BMDMs display a decrease in IFN-β production to HSV-1, poly(dA:dT), and mCMV (Figure 3.2c) Interestingly, cGAS deficient cells were also unable to produce IFN-β in response

to *Plasmodium falciparum* genomic DNA and AT5 *Plasmodium* DNA, a 20mer derived from the *Plasmodium falciparum* genome, as well as CpGA. This observation has not been previously described and may suggest a role for cGAS in the detection of *Plasmodium* infection. Furthermore, CpG DNA may be detected by not only TLR9, but also detected by cGAS and driving the synthesis of cGAMP to further induce type I interferon responses. This suggests that cGAS has the ability to detect a broader range of DNA ligands than previously described. Together these results show that while cGAS plays a clear role in the recognition of DNA ligands and the induction of type I interferons, IFI204 may play only a partial or redundant, yet broader role in sensing DNA and RNA ligands.

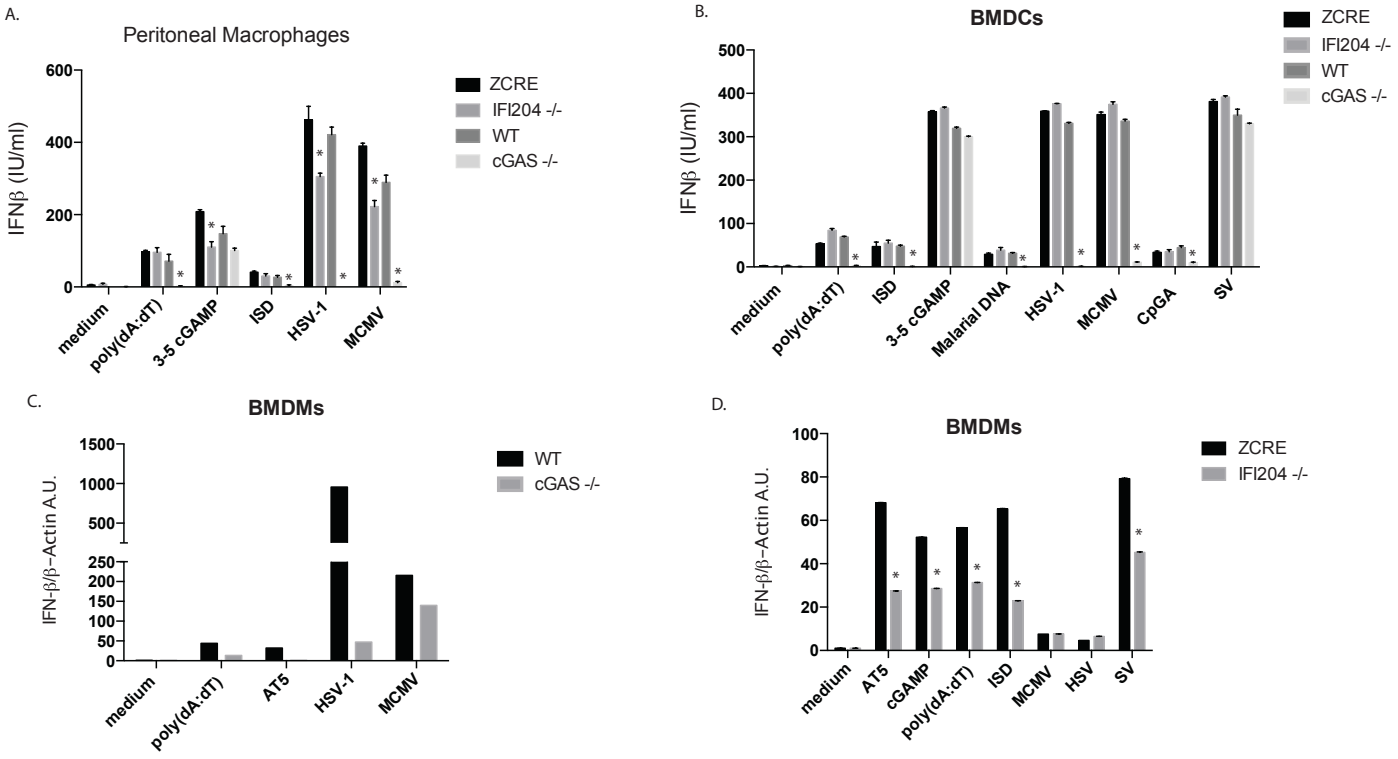


Figure 3.2. IFI204 plays a partial role in the type I IFN response to DNA ligands. A. Peritoneal Macrophages, B. BMDCs or C,D. BMDMs were stimulated with poly(dA:dT), 3-5 cGAMP, ISD, HSV-1, mCMV, Malarial DNA, or SV for 6 hours and monitored for IFNβ expression by q-RT-PCR or ELISA. Results are represented as fold change over media. C. Data is representative of one experiment. (* $p<0.05$ assessed by Two-Tailed T-Test compared to WT or ZCRE mice, data are represented as mean + S.E. Data represents 3 biological replicates). Bars without * are not significant.

In order to further study the role of IFI204 in type I interferon responses, we monitored the induction of a panel of Type I IFNs, cytokines, chemokines, and PRRs before and after DNA stimulation by multiplex gene expression analysis using nCounter (Nanostring) technology in primary BMDMs. We stimulated BMDMs derived from ZCRE and IFI204 deficient mice with poly(dA:dT), ISD, cGAMP, and Sendai Virus for 6 hours and isolated RNA for nanostring analysis. We did not detect any differences in many of the genes that were upregulated in response to these ligands when we compared IFI204 deficient BMDMs to control cells (Figure 3.3 and Table 3.1). We did, however, notice a specific decrease in IFN-β and IFN-α levels in response to both DNA and RNA ligands in IFI204 KO cells. Furthermore, IFI204 deficient cells also displayed lower levels of IRF7 and Viperin after stimulation. This result is consistent with suggesting a partial role for IFI204 in Type I interferon induction. It is also consistent with our results from IFI16 knockdown cells discussed in chapter two of this thesis, in that IFI204 plays a broader role in the induction of type I interferons and ISGs in response to both DNA and RNA ligands. Specifically, there is a more prominent decrease in IFN-α levels supported by the fact that IRF7 is decreased as well.

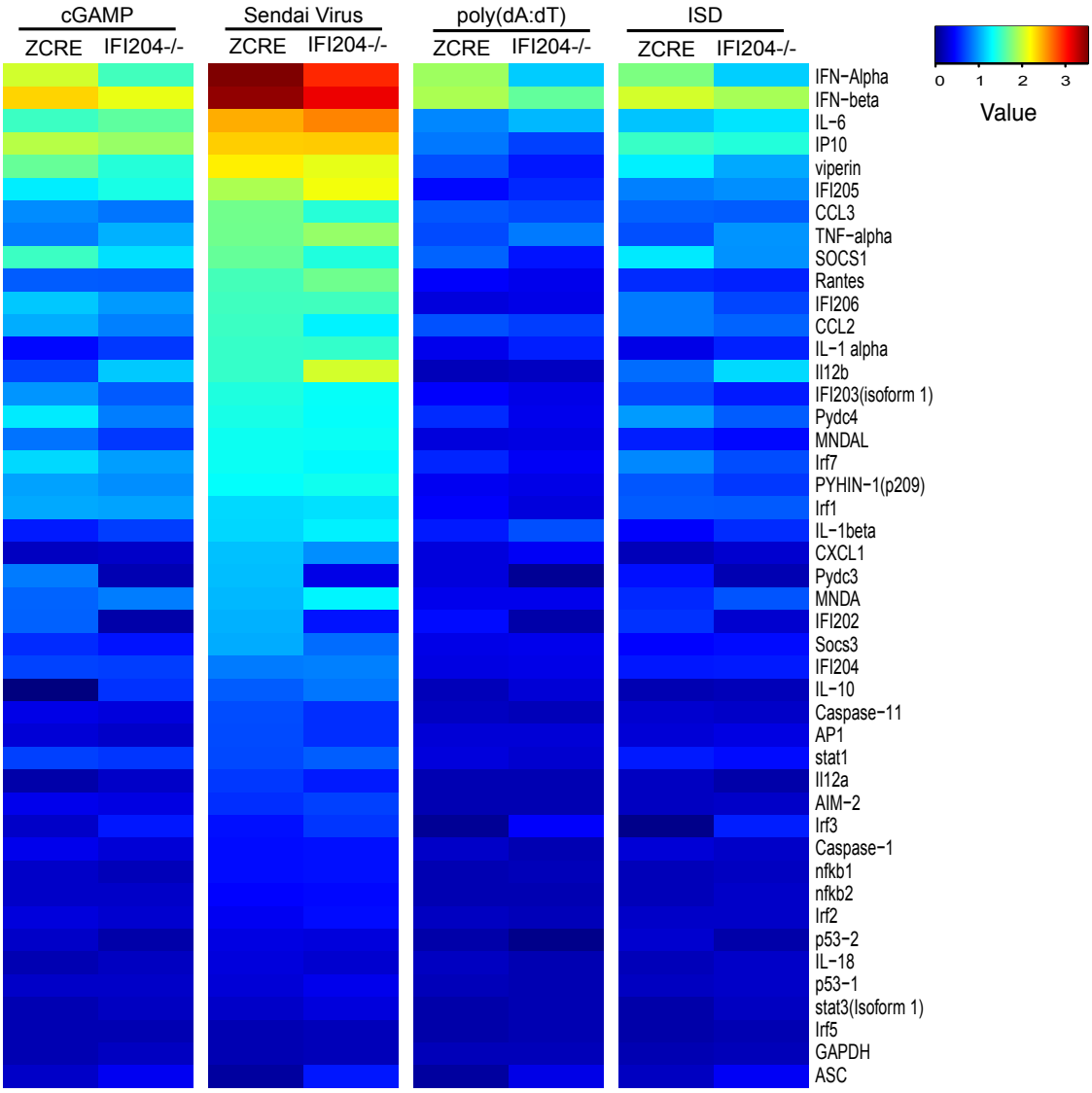


Figure 3.3. Nanostring Analysis of IFI204-/- cells. ZCRE and IFI204 deficient BMDM's were stimulated with poly (dA:dT), Sendai Virus,, ISD, and cGAMP for 6 hours. RNA was collected for nanostring analysis. Results displayed as fold change over medium.

Table 3.1. Nanostring Analysis of IFI204 -/- BMDMs, Fold Change

	cGAMP		Sendai Virus		poly(dA:dT)		ISD	
	ZCRE	IFI204-/-	ZCRE	IFI204-/-	ZCRE	IFI204-/-	ZCRE	IFI204-/-
AIM-2	1.8	1.6	3.4	4.0	0.9	0.9	1.1	1.2
AP1	1.4	1.2	4.4	3.4	1.4	1.4	1.4	1.6
ASC	1.2	1.9	0.7	2.7	0.7	1.7	1.1	2.0
CCL2	10.4	7.1	33.0	18.5	4.7	3.9	6.7	5.5
CCL3	7.9	6.4	50.9	27.7	4.9	4.3	5.4	5.2
CXCL1	1.1	1.2	12.3	8.0	1.5	2.0	1.0	1.3
Caspase-1	1.8	1.4	2.4	2.5	1.2	0.9	1.4	1.2
Caspase-11	1.7	1.5	4.5	3.4	1.1	1.0	1.3	1.2
GAPDH	0.9	1.1	0.9	1.0	1.0	1.0	0.9	1.0
IFI202	5.4	0.8	10.7	2.6	2.4	0.8	3.5	1.3
IFI203	8.5	5.1	26.1	21.3	2.1	1.7	4.3	2.8
IFI205	17.9	24.9	81.7	143.5	2.3	3.2	7.1	8.1
IFI206	13.0	8.9	34.2	34.4	1.5	1.7	6.7	4.2
IFN-Alpha	110.2	34.7	3318.0	884.3	73.3	13.4	55.8	13.7
IFN-beta	220.8	133.7	2865.0	1378.0	81.5	45.3	111.7	78.6
IL-1 alpha	2.3	3.7	31.6	30.8	1.8	2.9	1.7	3.0
IL-10	0.4	3.5	5.2	6.5	1.0	1.4	0.9	1.0
IL-18	0.9	1.1	1.5	1.3	1.1	0.9	1.0	1.2
IL-1beta	2.8	3.9	14.7	18.3	2.8	4.6	2.1	3.3
IL-6	32.9	43.9	301.9	417.9	7.5	11.4	12.6	16.6
IP10	90.0	69.9	232.4	238.6	6.6	4.0	32.2	27.0
Il12a	0.8	1.2	3.7	2.8	0.9	0.9	1.1	0.8
Il12b	4.1	13.1	31.4	111.6	1.0	1.1	6.0	15.1
Irf1	10.0	9.5	15.0	16.0	2.1	1.5	5.2	5.1
Irf2	1.5	1.3	1.9	2.4	1.1	1.0	1.2	1.2
Irf3	1.2	2.7	2.5	3.6	0.6	2.1	0.5	2.9
Irf5	0.9	0.9	0.9	1.0	0.8	0.9	0.8	0.9
Irf7	14.9	9.1	22.2	19.5	3.1	2.0	7.6	4.5
MNDA	5.5	6.9	11.4	18.9	1.8	1.8	3.2	4.8
MNDAL	6.3	3.7	22.5	22.0	1.5	1.6	2.9	2.3
PYHIN-1	9.4	8.0	20.7	23.2	1.9	1.7	4.9	3.7
Pydc3	6.8	0.9	12.0	1.7	1.5	0.6	2.5	0.9
Pydc4	17.4	6.9	24.7	20.9	3.3	1.8	9.0	5.2
Rantes	5.2	5.1	35.5	50.1	2.1	1.8	3.3	3.0
SOCS1	33.0	15.9	46.4	26.4	5.5	2.6	17.2	8.3
Socs3	3.3	2.6	10.3	6.0	1.7	1.8	2.2	2.4
TNF-alpha	6.9	10.7	50.8	68.3	4.4	6.7	4.6	8.4
nfkb1	1.2	1.0	2.4	2.5	0.9	1.0	1.0	1.1
nfkb2	1.2	1.2	2.2	2.3	0.9	0.9	1.0	1.2
p53-1	1.2	1.2	1.4	1.8	1.0	0.9	1.1	1.2

p53-2	1.2	0.8	1.6	1.5	0.8	0.5	1.3	0.8
stat1	4.0	3.6	4.3	5.3	1.5	1.3	2.8	2.4
stat3	0.9	1.1	1.2	1.5	0.8	0.9	0.8	1.1
viperin	46.6	27.5	178.2	131.1	4.6	2.7	18.2	10.0

Table 3.1 Nanostring Analysis of IFI204-/- cells, Fold Change. ZCRE and IFI204 deficient BMDM's were stimulated with poly (dA:dT), Sendai Virus,, ISD, and cGAMP for 6 hours. RNA was collected for nanostring analysis. Results displayed as fold change over medium.

IFI204 does not play a role in the NF-κB response to DNA and RNA ligands, nor is it necessary for pyroptotic cell death.

Previous in vitro studies did not detect a role for IFI204 in upregulating NF-κB associated genes***.*** Furthermore, work in chapter 2 of this thesis discusses that in IFI16 knockdown cells, levels of NF-κB driven cytokines, IL-6 and IL-1 β are unchanged or often slightly increased in response to DNA ligands as compared to EV control cells. To determine if IFI204 plays a role in the NF-κB responses driven by RNA and DNA ligands, we stimulated peritoneal macrophages and BMDCs from IFI204 -/-, ZCRE, WT, and cGAS-/- mice with a panel of ligands and monitored IL-6 production by ELISA. We found that IL-6 production was unchanged or slightly elevated in IFI204 deficient cells after stimulation. We also stimulated ZCRE and IFI204-/- BMDMs with poly(dA:dT), ISD, Sendai Virus, and cGAMP for 6 hours and collected RNA for nanostring analysis. Consistent with the above results, we found that levels of IL-6, TNF-α, and IL-1β were unchanged or slightly increased in response to all ligands. Furthermore, in the IFI204-/- cells, we noticed a slight decrease in expression of SOCS-1, which has been shown to negatively regulate NF-κB driven cytokine production (Figure 3.3 and Table 3.1).

We also noticed a partial defect in IL-6 production in cGAS deficient cells in response to poly(dA:dT), ISD, and malarial DNA. This response was even more dramatic in the cGAS deficient BMDCs (Figure 3.4 a and b). These results suggests that cGAS has a broader impact in immune activation to DNA infection

than the IFI204. It also provides further evidence that cGAS may be an important player in the immune response to malarial DNA. Furthermore, we saw severe attenuation of IL-6 in response to DNA virus mCMV in the cGAS -/- cells as compared to WT cells. This is surprising, as the response to the other DNA virus, HSV-1, is unaltered in cGAS knockouts compared to WT cells. For these studies, we used the ICP0 mutant HSV-1 7134 virus. ICP0 is a viral produced immunosuppressive gene that has been deleted in order to induce a greater immune response in cells. Alteration in this strain as compared to the WT mCMV may be responsible for the differences in the way the viruses are detected by cGAS. Further study is needed in order to determine the role of cGAS in mCMV and *plasmodium* responses.

Previous work has shown that IFI16 participates in the formation of an inflammasome in the nucleus in response to KSHV. We next wanted to explore the ability of IFI204 to induce inflammasome responses to various ligands. Peritoneal macrophages from IFI204 -/-, ZCRE, WT, and cGAS-/- mice were primed with LPS for 2 hours and then stimulated with DNA and inflammasome dependent ligands, ATP and Nigericin. We did not notice any significant changes in IL-1β production for either the IFI204 or cGAS deficient cells (Figure 3.4 c and d), suggesting that neither gene plays a role in inflammasome activation by the poly(dA:dT), HSV-1, ATP, or Nigericin.

Studies have also defined a role for IFI16 in pyroptotic cells death during HIV infection. We next monitored IFI204 BMDMs and BMDCs for their ability to undergo pyroptotic cell death using the cell glo assay. Cells were primed with

LPS for 2 hours and then stimulated with DNA ligands. We did not detect any difference in cell death in the IFI204 deficient cells as compared to control ZCRE cells. (Figure 3.4e and f). These results suggest that IFI204 does not play a role in NF-κB activation, inflammasome induced IL-1β production, or cell death in response to DNA ligands.

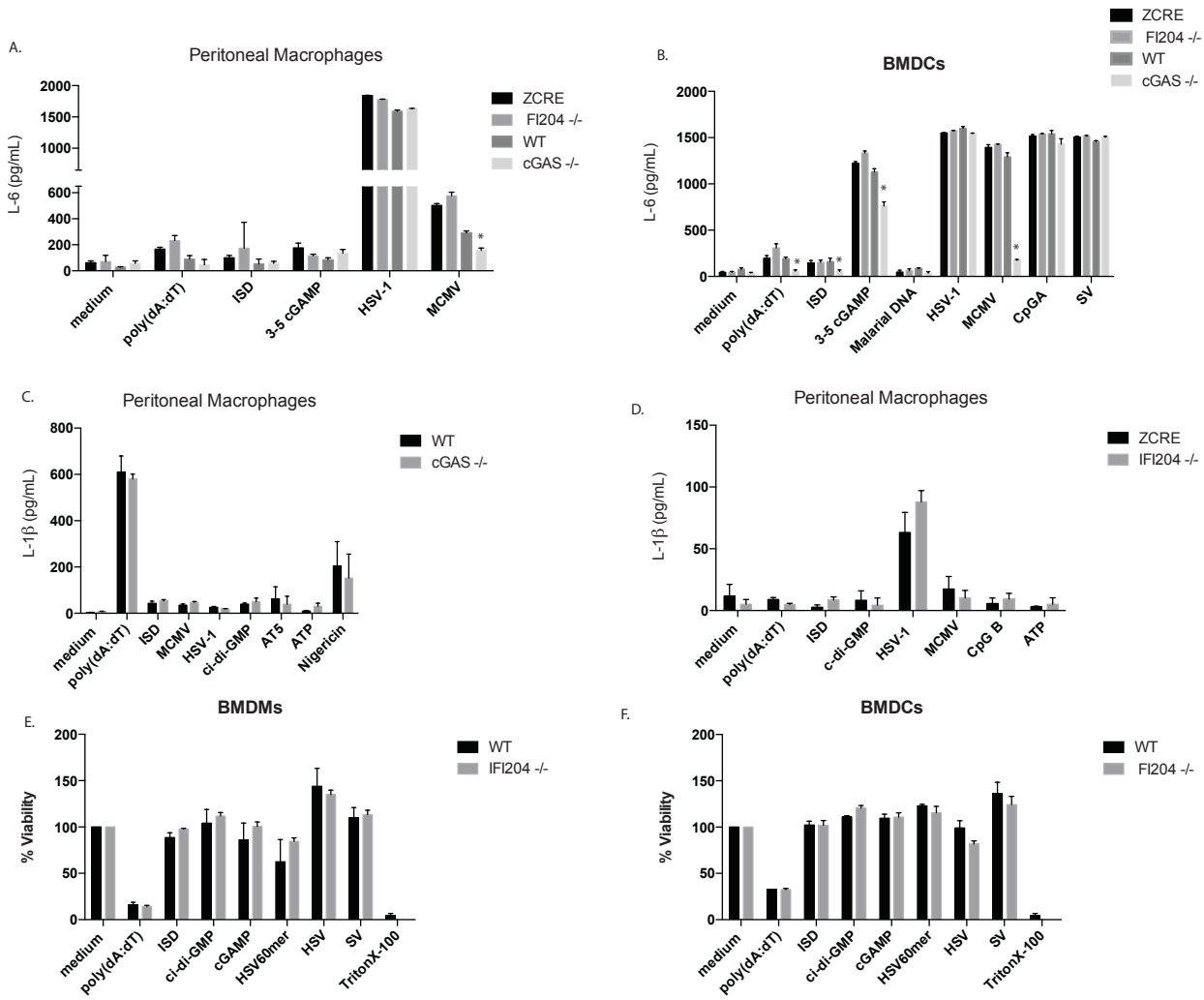


Figure 3.4 IFI204 does not play a role in the NF-κB response to DNA and RNA ligands, nor is it necessary for pyroptotic cells death. A. Pertitoneal Macrophages and B. BMDCs were stimulated with poly(dA:dT), 3-5 cGAMP, ISD, HSV-1, mCMV, Malarial DNA, or SV for 6 hours and monitored for IF-6 production by ELISA. C. cGAS or D. IFI204 deficient cells were primed with LPS for 2 hours and stimulated with poly(dA:dT), 3-5 cGAMP, ISD, HSV-1, mCMV, Malarial DNA, or SV for 12 hours and monitored for IL-1β production ELISA. E. BMDMs or F. BMDCs from IFI204 deficient mice were primed with LPS for 2 hours and stimulated with poly(dA:dT), 3-5 cGAMP, ISD, HSV-1, mCMV, Malarial DNA, or SV for 12 hours and monitored for cell death by Cell Glo Assay. (* $p<0.05$ assessed by Two-Tailed T-Test compared to WT or ZCRE mice, data are represented as mean + S.E. Data represents 3 biological replicates). Bars without * are not significant.

IFI204 mediates the innate immune response to HSV-1 in vivo

To date, the role of IFI204 *in vivo* has not been investigated. Thus, with the generation of the IFI204 knockout mouse, we explored the role IFI204 in the response to HSV-1 infection *in vivo*. We infected IFI204 -/-, WT, cGAS-/-, and TBK1-/- mice with $5.0X10^8$ pfu HSV 7134R per mouse via intraperitoneal injection. None of the mice showed obvious sign of susceptibility upon virus infection during 72 hours. We collected serum at 24 hour and 72 hour time points, and harvested brain, liver and spleen. We extracted RNA from half of the brain tissue, liver and spleen, and collected brain homogenates from the other half of the brain tissue. We did not detect a difference in IFN-β mRNA expression in the brains of the mice by q-RT-PCR, however TBK-1 mice displayed higher IL-6 and IP-10 levels suggesting that an active infection was taking place (Figure 3.5a-c). We did detect a higher HSV genome copy number in the brain of cGAS -/- mice, which was consistent with previous studies (Figure 3.5h). Furthermore, the viral load was below the limit of detection of the plaque assay performed. Studies have shown that cGAS -/- mice are more susceptible to HSV-1 infection, marked by decreased survival, decreased IFNα/β serum cytokine production, and increased viral load(151). We also saw higher HSV genome copy number in the liver and spleen of cGAS-/- mice (data not shown). While IFN-β and IL-6 mRNA levels were not changed, we were able to detect differences in cytokine production from brain homogenates by ELISA. WT and cGAS-/- mice induced higher levels of IL-β, IFN-β, and IL-6 than the IFI204 -/- mice (Figure 3.5d, e, and f). IFN-β levels were significantly lower in the IFI204 -/- mice as compared to WT

mice, while levels of IL-1β and IL-6 were approaching significance. In subsequent studies, a higher dose of virus and younger mice may be necessary in order for the virus to travel to the brain in high amounts.

We also wanted to determine if IFI204 mediates the immune response to HSV-1 systemically. We analyzed blood serum collected at 24 and 72 hours post infection for production of IFN-β, IL-6, and IL-1β by ELISA. Consistent with results from brain homogenates, IFI204 -/- mice produced lower amounts of IL-6, IL-1β, and IFN-β in the serum 72 hours post infection as compared to WT mice (Figure 3.5i, j, and k). IL-1β levels were significantly lower in the IFI204 -/- mice as compared to WT mice, while levels of IFN-β and IL-6 were approaching significance. These results provide further evidence that IF204 mediates that innate immune response to HSV-1 *in vivo*.

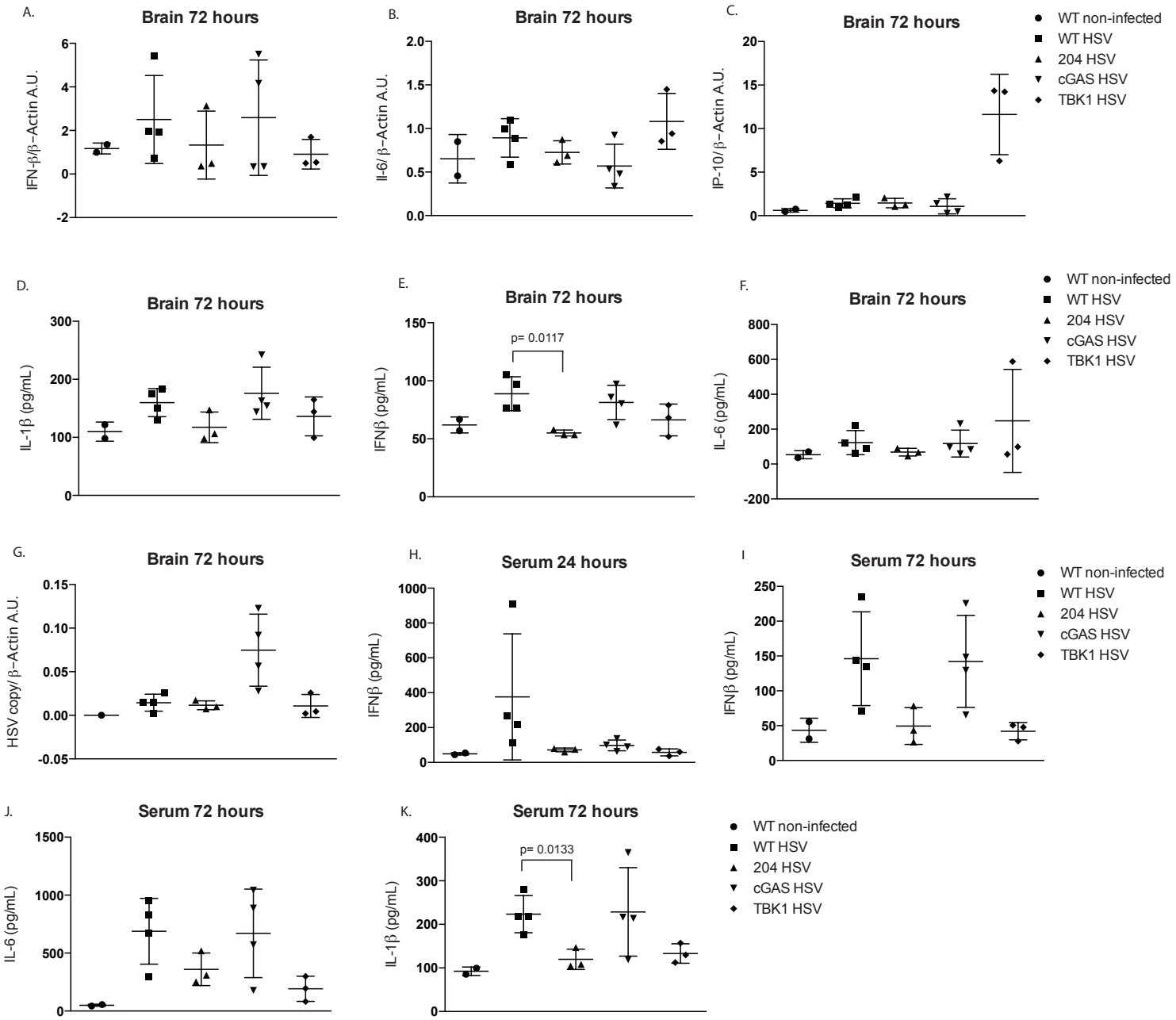


Figure 3.5. IFI204 mediates the innate immune response to HSV-1 *in vivo*. Brain Homogenates were collected 72 hours post infection and monitored for A. IFNβ, B. IL-6, C. IP-10, or G. HSV-1 copy number by q-RTPCR. Brain homogenates were monitored for D. IL-1β, E. IFNβ, or F. IL-6 production by ELISA. H. Serum was collected at 24 hours post infection and monitored for IFNβ by ELISA and at 72 hours post infection and monitored for I. IFNβ, J. IL-6 and K. IL-1β production by ELISA. (* $p<0.05$ assessed by Two-Tailed T-Test compared WT infected mice, data are represented as mean + S.E.) Data without p values are not significant.

Lastly, we wanted to determine if the IFI204 -/- mice are more susceptible to HSV-1 infection *in vivo*. We infected 15 day old WT, STING -/-, and IFI204 -/- mice via intraperitoneal injection with 1.0X10^7 pfu HSV-1 7134R. 100% of STING -/- mice, which have been shown to be more susceptible to HSV-1 infection(89), died at day 4 post infection. >65% of IFI204-/- mice died at day 5 post infection as compared to WT mice, which did not succumb to infection at all (Figure 3.6). 3 of the 9 WT mice developed hind leg paralysis on day 5 and recovered from the paralysis on day 7 post infection, which is an indication that an active infection was taking place in these mice. Of the remaining IFI204-/- mice, 2 developed hind leg paralysis on day 5 post infection and the paralysis remained until the mice were sacrificed on day 14 post infection. Further study is necessary to determine which cells types are responsible for lower cytokine production observed in the *in vivo* experiments.

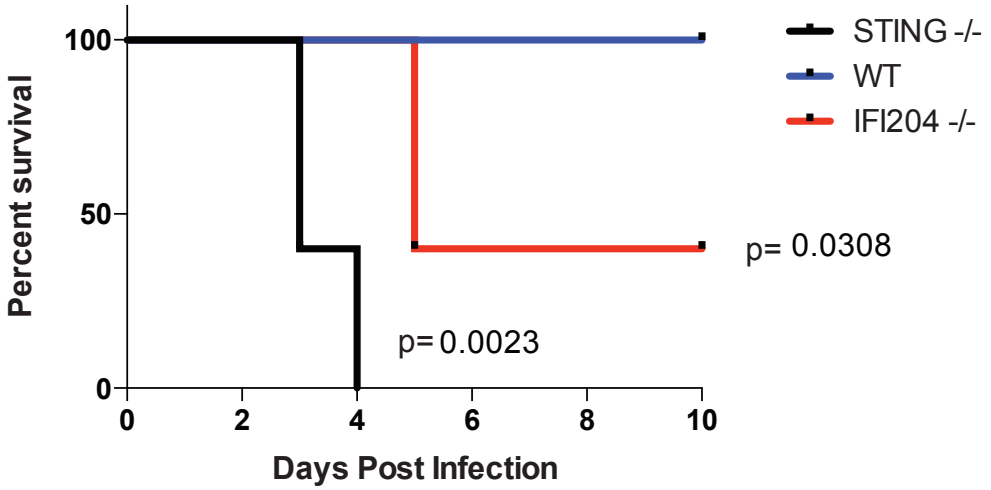


Figure 3.6. HSV-1 Survival Study. 15 day old STING -/-, IFI204-/- and WT -/- mice were infected with 1.0X10^7 pfu HSV-1 7134R via intraperitoneal injection and monitored for survival. (* $p<0.05$ assessed by Mantel-Cox test compared WT infected mice).

Discussion

The type I interferons play a critical role in HSV-1 infection by activating an antiviral state that leads to clearance of the virus. Mice deficient in key players of type I IFN production, such as IFNAR, STING, TBK1, as well as IRF3 and display increased susceptibility to HSV-1 infection (12,89,180,181,214,215). IFI16 and its mouse orthologue IFI204 have been extensively studied for their role in HSV-1 recognition *in vitro*, however to date the role of IFI16 or IFI204 *in vivo* has yet to be determined. In this study, we developed an IFI204 knockout mouse and studied its role in immune responses to a panel of ligands *in vitro* and its role in HSV-1 infection *in vivo*.

We began by stimulating peritoneal macrophages, BMDMs, and BMDCs with a panel of DNA and RNA stimuli and viruses and monitoring the cells for cytokine production. IFI204 deficient cells were unaltered in their ability to produce type I interferons to many of the stimuli, including poly(dA:dT) and ISD in peritoneal macrophages, in contrast to cGAS deficient cells. We did, however, observe a partial phenotype in response to both HSV-1 and mCMV in the IFI204 deficient peritoneal macrophages. Furthermore, in BMDCs, IFI204 was not required for IFN-β production by ELISA. These results are in contrast to previous siRNA studies that show that IFN-β production is strongly dependent on IFI204 in response to these ligands in RAW cells. It is unclear if siRNA targeting of IFI204 was specific and it is possible that siRNA yielded some off target effects that enhanced the observed phenotype. Many of the PYHIN proteins are closely related to one another and siRNA may have some overlapping effects. IFI204

may work in tandem with other PYHIN proteins or other cytosolic sensors to induce these responses and a double knockout mouse may be necessary in order to see full effects.

In contrast to these results, when IFI204 deficient BMDMs were stimulated with both DNA and RNA ligands for 6 hours and RNA was analyzed for IFN-β production by q-RT-PCR, we noticed a 50 percent decrease in IFN-β induction in response to all ligands. The results are consistent with IFI16 knockdown studies found in chapter two of this thesis, suggesting that IFI204 plays a broader role in the production of type I interferons to both DNA and RNA ligands. Furthermore, nanostring analysis confirms that levels of viperin and IRF7 are also decreased. The lower levels of viperin and IRF7 may provide an explanation for why IFN-α levels are more greatly attenuated. Further work is necessary in order to determine whether IFI204 also plays a role in the regulation of transcription of type I interferons. It would be interesting to determine if levels of RIG-I are decreased in the IFI204 knockdown cells, since there is a partial defect in SV induced production of IFN-β in BMDMs. In contrast to IFI16 studies, basal levels of ISGs are not attenuated. It is important to note that the PYHIN proteins are closely related genes and other PYHIN proteins may contribute to type I interferon production at a basal level, although IFI204 is important for type I interferon induction after stimulation. Furthermore, IFI204 may be playing both a redundant and time dependent role along with other cytosolic sensors. This may explain why we saw no dependence on IFI204 after 12 hours, but a partial decreased in IFN-β production after 6 hours was observed.

We also monitored levels of NF-κB driven cytokines in IFI204 and cGAS deficient cells. In all cell types, IL-6, IL-1β, and TNF-α were unchanged or slightly increased in the IFI204 deficient cells after stimulation. This suggests that although IFI204 plays a role in type I interferon production, the NF-κB axis of activation is unaffected. Slight increases in cytokine production may be due to compensation mechanisms of the cell to ensure pathogen clearance. Interestingly, we found that IFN-β production in response to CpG and malarial DNA is dependent on cGAS. These results suggest a role for cGAS in malaria infection and a broader role for cGAS in the recognition of DNA ligands.

Previous studies indicate that IFI16 is capable of assembling into an inflammasome complex with ASC in response to KSHV. We investigated the ability of IFI204 to induce IL-1β after LPS priming in response to a panel of stimuli. We did not see a defect in IL-1β production in either cGAS or IFI204 deficient cells. There is much debate as to whether or not IFI16 forms an actual inflammasome since studies show only indirect evidence of inflammasome assembly via ASC colocalization with IFI16. Contradictory studies find that IFI16 expression suppresses caspase-1 activation by the NLRP3 and AIM2 inflammasomes. The role of IFI16 and type I interferon production is well defined, thus the production of IL-1β may be due to type I interferon induction and not bona fide inflammasome assembly. Furthermore, studies have shown that type I interferons inhibit IL-1β production by STAT-1 inhibition of NLRP3 (220). With less type I interferon production in the IF204 deficient cells, you would expect that this pathway would not be engaged, and IL-1β production would be normal.

Recent studies have linked IFI16 as the receptor responsible for inflammasome activation and pyroptotic death of bystander CD4 T cells during HIV infection. Thus, we also monitored IFI204 deficient cells their ability to induce pyroptosis in response to various stimuli after LPS priming. We did not detect any differences in cell death in the IFI204 deficient BMDMs or BMDCs. Although this is in contrast to HIV studies mentioned above, IFI16 dependent pyroptosis may be ligand and cell type specific and dispensable for HSV-1 responses in macrophages and dendritic cells.

We next wanted to determine if IFI204 plays a role in HSV-1 infection *in vivo*. Previous studies have shown that type I interferon induction is important for HSV-1 viral clearance. Mice deficient in cGAS and TBK1 display increased susceptibility to HSV-1. Although we did not detect higher viral load in IFI204 deficient mice, we did detect lower levels of IFN-β, IL-6, and IL-1β in the brain and serum post infection. These results suggest that HSV-1 is recognized by IFI204 to induce these responses. Furthermore, IFI204-/- mice display increased susceptibility to HSV-1 as compared to WT mice. Further study is necessary to determine how IFI204 mediates the response to HSV-1 *in vivo*.

To date, there has been much discussion as to whether or not IFI16 is a bona fide nucleic acid sensor for DNA viruses. This study is the first to describe a role for IFI204 *in vivo* during a viral infection. Further work is necessary in order to determine how IFI204 interacts with other cytosolic sensors and the role it plays during HSV-1 infection.

Materials and Methods

Reagents and Antibodies

LPS, 3-5 cGAMP, and poly(dA:dT) were obtained from Sigma-Aldrich (St. Louis, MO). HSV 60mer, and ISD oligonucleotides were synthesized as described in (130), Cyclic-di-GMP was from Biolog (Hayward, CA). HSV-1 (7134) and HSV-1 7134R was a gift from D. Knipe (Harvard Medical School, MA). Sendai virus (SeV, Cantrell strain) was purchased from Charles River Laboratories (Wilmington, MA). mCMV (Smith strain) was a gift from R. Welsh (UMASS Medical School, MA)Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Genejuice was from Novagen (Madison, WI).

Cell Culture, Stimulation and ELISA

Peritoneal Macrophages were harvested 3 days after i.p. injection of 1 ml of thioglycolate. As described in (32). Bone marrow from the femur of mice was harvested and BMDMs and BMDCs were derived as described in (157). For stimulations, poly(dA:dT) (1 µg/ml), ISD (3 µM) HSV 60mer (3 µM) ci-di-AMP (3 µM), 5'ppp RNA (600ng/mL), cGAMP 3'-5' (3 µM) were transfected into the cells with lipofectamine in accordance with the manufacturer's instructions. Cells were infected with mCMV or HSV-1 7134 viruses at multiplicities of infection (MOI) of 10 for *in vitro*. Cells were infected with Sendai virus at 200 IU/ml. Cells were challenged with stimulants or microbes for 6 hrs (for RNA analysis) or 12 hours (for protein analysis by ELISA). Cytokine and IFN levels in culture supernatants

were assayed for IL-1β and IL-6 (BD Biosciences, Franklin Lakes, NJ) and IFN-β by sandwich ELISA.

In Vivo Studies

6 week old mice were infected with 5.0X10^8 pfu via intrapertioneal injection with HSV-1 7134R and brain, liver, spleen, and serum were collected from the mice 72 hours post infection. 15 day old mice were infected with 1.0X10^7 pfu HSV-1 7134R via intraperitoneal injection and monitored for survival over 14 days.

Mice

ZCRE mice were from Jackson Laboratories (Bar Harbor, ME). cGAS embryos were obtained from International Mouse strain resource. All experiments were conducted with mice maintained under specific pathogen-free conditions in the animal facilities at the UMASS Medical School and were carried out in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee.

Nanostring and RT-QPCR experiments

Cells were treated for 6 hours and RNA was purified with a quick RNA mini prep kit (Zymo Research, Irvine, CA). RNA transcript counting was performed on total RNA hybridized to a custom gene expression CodeSet and analyzed on an nCounter Digital Analyzer. Counts were normalized to internal spike-in and

endogenous controls per Nanostring Technologies' specifications. A pseudo count was added to all values such that the smallest value in the dataset was equal to 1.

cDNA was synthesized, and quantitative RT-PCR analysis was performed as described (212,213). Gene expression is shown as a ratio of gene copy number per 100 copies of HPRT ± SD.

Cell Viability Assay

Cell Glo stain was obtained from Promega. Cells were stimulated as described above and cell viability was assayed according to the manufacture's protocol. Cells were washed in PBS and incubated in cell glo stain for 30 min at room temperature. Cells were monitored for FITC fluorescence.

Chapter 4: Discussion and Perspectives

The innate immune system is the body's first line of defense against pathogens. This response is governed by a variety of PRRs that recognize PAMPS and DAMPS and activate downstream signaling molecules. Effectors such as type I interferons (IFNs) and pro-inflammatory cytokines and chemokines aid in mediating this response. Nucleic acids that are released into the cytosol during bacterial and viral infection are recognized by a range of intracellular RNA and DNA sensors that activate the type I interferon response.

It is well known that type I interferons play a critical role in the clearance of viruses. The production of IFNαβ activates an antiviral state which initiates the transcription of many interferon stimulated genes and production of other cytokines and chemokines that enhance the inflammatory response and viral clearance. To date over 400 ISGs have been identified and found to play a role in viral pathogenesis. Among these ISGs are the well known PKR, Mx Proteins, and OAS. PKR is a serine-threonine kinase that binds to dsRNA intermediates that are formed during viral replication. Once bound, PKR phosphorylates the eukaryotic initiation factor 2a (eIF2a), which in turn inhibits viral gene translation. PKR has been shown to play a role in HSV-1, Vaccinia Virus, and EMCV(190,221). The Mx Proteins are GTPases that are expressed in the cytosol after type I interferon induction. Here, they bind to viral capsid proteins and prevent import to the nucleus, thus inhibiting vial replication. Mx Proteins have been implicated in Influenza virus and VSV infection(190,221,222). Lastly OAS

has been shown to bind dsRNA and convert ATP into 2'-5' linked AMP-oligomers (2-5A), which in turn activates RNAseL. RNAseL then cleaves viral which inhibits viral replication. This cleaved RNA also activates RIG-I which amplifies the type I interferon response. OAS has been shown to play a role in SV and EMCV infection(190,221,223).

The importance of the type I interferon pathway is evident as viruses have evolved ways to evade the type I interferon response. Herpes Simplex Virus encodes an immediate early protein ICP0 which inhibits IRF3 and IRF7 and subsequent type I interferon responses(218). The crucial role type I interferons play in innate immunity has made them a target of many therapeutic approaches. Type I interferons are well known to inhibit tumor growth(224) and have been used in the clinic to treat leukemia, melanoma, and Kaposi's sarcoma(225). Furthermore, Type I interferons have also been implicated in a variety of diseases such as inflammatory bowel disease, psoriasis, and Systemic lupus erythematous (SLE).

Since type I interferons have such effects on immunomodulation, it is important to understand how they are regulated. Studies have shown that SOCS1 can negatively regulate type I interferon production by inhibition of the JAK/STAT pathway. Patients with SLE have defects in SOCS1 regulation, thus exhibiting a hyper production of type I Interferons(226). The key to understanding other ways in which type I interferons are regulated is first determining how they are induced.

To date, many cytosolic DNA sensors have been described, however it is unclear how these receptors work in concert to mediate pathogen clearance. In chapter two of this thesis, we describe a broader role for the intracellular DNA sensor, IFI16, in regulating these responses. IFI16 is the human member of the PYHIN family of proteins. Initial reports of IFI16 described it to be a negative regulator of cell cycle regulation. However recent siRNA studies have shown that IFI16 binds cytosolic DNA via its HIN200 domains and activates type I interferon response through STING, TBK1, and IRF3. In contrast to these findings, we determined that when IFI16 is knocked down in human THP1 cells by shRNA, type I IFN production is abrogated in response to both DNA and RNA ligands. We have now shown that IFI16 plays a role in regulating transcription of IFNα. Given the critical function of type I interferons in disease, our studies have unveiled an important additional way for type I interferons to be activated. By regulating IFNα gene expression, IFI16 bypasses the viral evasions mechanisms that shut down type I IFN responses through IRF3.

Our work also provides insight as to how IFI16 may be targeted as a potential therapeutic for disease. SLE is an autoimmune disease that is characterized by overproduction of type I Interferons. In fact, the hallmark of disease is elevated levels of IFNα in the serum of patients(227-230). Clinical trials in which neutralizing antibodies to IFNα were administered to patients greatly reduced symptoms of disease(231). IFI16 has also been linked to SLE. Several groups have shown the production of anti-IFI16 antibodies in patients with SLE(127,129,232). Furthermore, patients with the IFI16 SNP rs866484,

which causes an amino acid change in the protein, have higher incidences of SLE(233). Although the production of type I interferons is beneficial for viral clearance, the over production of type I interferons in autoimmune diseases demonstrates the need for a tight balance of these responses. Taken together with our studies, the presence of IFI16 autoantibodies in SLE and the fact that IFI16 plays a role in the regulation of IFNα expression suggest that IFI16 may be a potential therapeutic target for autoimmunity. Furthermore, the onset of autoimmune diseases has been linked to persistent viral infection. This observation provides a correlation between the induction of IFI16 after viral infection and its importance in SLE immunopathology.

These results also provide insight into how IFI16 may work with other cytosolic sensors. Recent studies have suggested that although many cytosolic DNA sensors have been discovered, cGAS is the only bona fide DNA sensor. Since much of the work has been limited to siRNA studies, more stable knockdown or complete knockout models of these receptors may prove that the proposed molecules are not actual receptors at all. Our results suggest that IFI16 is not only an innate sensor for DNA, but works also in tandem with other receptors to amplify the type I interferon response to viruses. IFI16 is interferon inducible, as such its levels are increased after viral infection and initial induction of type I interferons. cGAS levels remain constant in cells. In fact, cGAS is expressed broadly across all tissue types, whereas IFI16 has a very specific and limited pattern of expression. We have shown that cGAS levels are normal in IFI16 knockdown cells. It is possible that cGAS directly recognizes DNA to initiate

the type I interferon response and upregulate IFI16, which then itself regulates IFN production to further the antiviral state. This idea would lead us to believe that viral evasion mechanisms would exist to directly target IFI16. In fact, it has been shown that HSV-1 ICP0 protein targets IFI16 for degradation in the nucleus of HFF cells(133). Perhaps this persistent and long term antiviral state contributes to the activation of LAT transcripts, pushing HSV-1 into latency.

In addition to the fact that DNA sensors may act in a timing dependent manner, a cell type dependence may explain the redundancy of receptors as well. As mentioned above, it is necessary for complete knockout of proposed DNA sensors in order to fully elucidate their roles in type I interferon response. In chapter 3 of this thesis we generated an IFI204 knockout mouse to determine the role of the proposed DNA sensor *in vivo*. In contrast to siRNA studies, we demonstrate that IFI204 plays only a partial role in the response to viral DNA ligands in BMDMs. This is in contrast to cGAS, which responds to a broader range of DNA ligands in multiple cell types. These results are consistent with the idea that cGAS is a bona fide DNA sensor for all intracellular DNA signaling, whereas the IFI204 response is only ligand and cell type specific. Further work is needed in order to determine if timing is necessary for IFI204 signaling and whether it works with cGAS in a sequential manner. We also demonstrated that IFNαβ levels in IFI204 knockout mice were decreased in BMDMs in response to both DNA and RNA ligands, which is consistent with IFI16 data presented in Chapter two. More work is needed in order to determine if IFI204 is in fact the mouse orthologue to IFI16 and functions to regulate type I interferon production.

There has been much question as to whether or not IFI204 is the true orthologue of IFI16. IFI204 and IFI16 were hypothesized to be orthologues mainly due to their similarities in domain structure, as each contains two HIN200 domains and one Pyrin domain. In addition, studies by Unterholzner et al., found that the two function similarly, producing less IFN-β in response to DNA ligands when IFI204 or IFI16 are knocked down with siRNA (124). However, phylogenetic analysis of both the Pyrin domain and HIN200 domains revealed that IFI16 and IFI204 are not evolutionary orthologues (234). Both IFI16 and IFI204 are more closely related to other members of the ALR family with respect to their HIN200 and Pyrin domains than they are to one another. Furthermore, conflicting results show that when IFI204 is knocked down in BMDMs and MEFs with a siRNA that was designed to target IFI204 specifically and not any of the other PYHIN family members, IFN-β production is not impaired in response to a panel of DNA ligands (234). Despite the similarity in domain structure, IFI204 and IFI16 are not evolutionary orthologues. It remains to be determined whether or not the two are functional orthologues, however. Data from this thesis suggests that the two function similarly by playing a broader role in the detection of both DNA and RNA ligands. However, IFN-β production is only partially dependent on IFI204, suggesting that IFI204 is a redundant DNA sensor and may require other PYHIN proteins for its full function.

The role of type I interferons in HSV-1 infection is well known. Mice deficient in IFNAR, IRF3, and TBK1 are more susceptible to HSV-1 viral infection. However the role of DNA sensors that activate this pathway is unclear.

Here we show that, IFI204 plays a role in pathogenesis to HSV-1 infection *in vivo.* Mice deficient in IFI204 are limited in their ability to produce IFNβ, IL-6 and IL-1β after HSV-1 infection as compared to WT infected mice. Our work also provides evidence that IFI204 works in conjunction with cGAS to mediate HSV-1 clearance. Previous studies have shown that cGAS-/- mice produce less IFNβ up to 12 hours post HSV-1 infection as compared to WT mice. However, after 12 hours, levels of IFNβ return to normal. In our work, we found that levels of IFNβ in the brain and serum of cGAS -/- mice are normal as compared to WT mice 72 hours post infection, which is consistent with previous studies. However, IFI204-/- mice produce less IFNβ in both the brain and serum 72 hours post infection. These results suggest that cGAS is important for the initial early responses to HSV-1 and IFI204 plays a role later during infection. Furthermore, IFI204 -/- mice display increased susceptibility to HSV-1 as compared to WT mice. This result, coupled with the lower levels of IFN-β production seen in the IFI204 -/- mice after HSV-1 infection, suggest the mice deficient in IFI204 would be hindered in their ability to modulate viral load. Further work is needed to determine if HSV-1 replication is affected in the IFI204 -/- mice after infection, and what cell types are responsible for the decreased cytokine production. These results provide a better understanding of how type I interferons are regulated in response to viral infection *in vivo*.

In Appendix B we highlight the importance of STING in type I interferon responses. MOLF mice are limited in their ability to produce type I interferons in response to DNA ligands. Using forward genetics we show that this defect is due

to the fact that MOLF mice have a mutation in STING. This emphasizes the importance of STING as it has evolved to play a central role in the antiviral response. Studies have shown that the C terminal domain of STING is important for binding of ligands and its activity. DMXAA has been shown to bind to mouse STING, but not human STING due to a SNP mutation in the C terminus. This provides an explanation for why DMXAA failed to target tumors in clinical trials. However in our studies, we found the mutation in the N terminus of STING to be responsible for lack of activity. It is possible that the N terminus of STING interacts with DNA sensors to regulate type I interferon responses. These results contribute to the knowledge of how STING is activated and can provide insight into how therapeutics such as DMXAA can be altered to bind STING more effectively in humans.

Lastly, this thesis highlights the importance of equilibrium of innate immune responses. The production of type I interferons is important for the clearance of viruses and activation of the antiviral state. However, it is clear that over production of type I interferons can be detrimental to the host as is seen with autoimmune diseases such as SLE. In chapter two of this thesis, we show that although IFI16 knockdown cells produce decreased amounts of type I interferons, these cells produce similar or higher amounts of NF-κB driven cytokines, IL-6 and IL-1β. It is possible that the host releases these cytokines in excess to act as compensation for the lack of type I interferons necessary to clear infection. Type I interferon and ISG induction play a crucial role in containing pathogen load. When this line of defense is disrupted, pathogen

replication can then induce the production of NF-κB driven cytokines, shifting to a pro-inflammatory driven response. Furthermore, it has been shown that these cytokines regulate one another to maintain tight balance of immune responses. Type I interferons have been shown to inhibit pro-IL-1β production in a STAT-1 dependent manner(220). Furthermore, patients receiving IFN-β treatment have weakened immune responses due to a decrease in IL-1β production(220). Knowledge of how these cytokines work together to control the immune response will aid in creating therapeutics that are better targeted. Perhaps IFI16 can be targeted for inflammatory diseases as well.

During HSV infection, the production of cytokines and chemokines is important for viral clearance, however cytokine storm can lead to severe inflammation and death if the response is not tightly regulated. In Appendix A we discuss the role of the CD200R1 in HSV-1 infection. While it is well known that TLR2 plays a role in the HSV-1 survival in vivo, how TLR2 is regulated was unknown. CD200R -/- mice are more susceptible to HSV-1 infection. They also lack the ability to upregulate TLR2 and produce cytokines in response to TLR2 ligands. These results suggest a role for CD200R in ensuring a tight balance of TLR2 activation in response to HSV-1. We also found a role for CD200R in regulating IL-1β production. IL-1β has been used as a therapeutic for multiple inflammatory diseases such as rheumatoid arthritis. Blocking IL-1β production has been used in treatment of the disease, however side effects due to inability to mount immune responses have been noted. A greater understanding of how

IL-1β is regulated introduces the possibility that receptors such as CD200R may be targeted to produce more specific and effective therapeutics.

In conclusion, this thesis provides a model for the activation of cytosolic nucleic acid sensors by HSV-1 and other ligands, and the regulation of type I interferons by IFI16. IFI16 transcriptionally regulates the basal expression of IFN-α and ISGs, including RIG-I. Type I interferons then signal through the IFNAR and STAT pathway to further induce type I Interferons in a feedback loop. This tonic type I interferon signaling exists to maintain ISGs at appropriate basal levels. Disruption of this balance can lead to increased susceptibility to disease or cancer in the case of too little type I IFN being produced, or the development of autoimmune disease if too much type I IFN is produced.

Upon infection with DNA ligands, such as HSV-1, cGAS induces the production of type I Interferons and ISGs, such as IFI16, through activation of STING and phosphorylation of IRF3. IFI16, which is predominantly nuclear, plays a role in regulating the transcription of type I interferons and other ISGs, further amplifying the antiviral response. Viral evasion mechanisms, such as the HSV-1 ICP0 gene, block this response by inhibiting IRF3, or IFI16.

Lastly, there are long term effects of type I interferon signaling. After viral infection, IFI16 regulates IFN-α and subsequent ISG expression. Receptors such as RIG-I, which recognize Sendai Virus are upregulated as a result of IFI16 activation. Thus in the absence of IFI16, type I interferon signaling in response to Sendai Virus is abrogated. However, HSV-1 can also be recognized by TLR2 to induce NF-κB dependent cytokines, IL-6 and IL-1β. In the absence of IFI16, the

host shifts to a pro-inflammatory response to compensate for lack of type I interferon antiviral activity. Figure 4.1 shows a model for IFI16 regulation of type I interferons.

Collectively, this thesis defines a broader role for IFI16 in the regulation of type I interferons. Furthermore, we provide insight into how cytosolic nucleic acid sensors work together to regulate the type I interferon response to HSV-1 and other ligands. We highlight the importance of the balance of the immune response as is relates to the development of disease. Thus, this knowledge can be used to create safe and effective therapeutics.

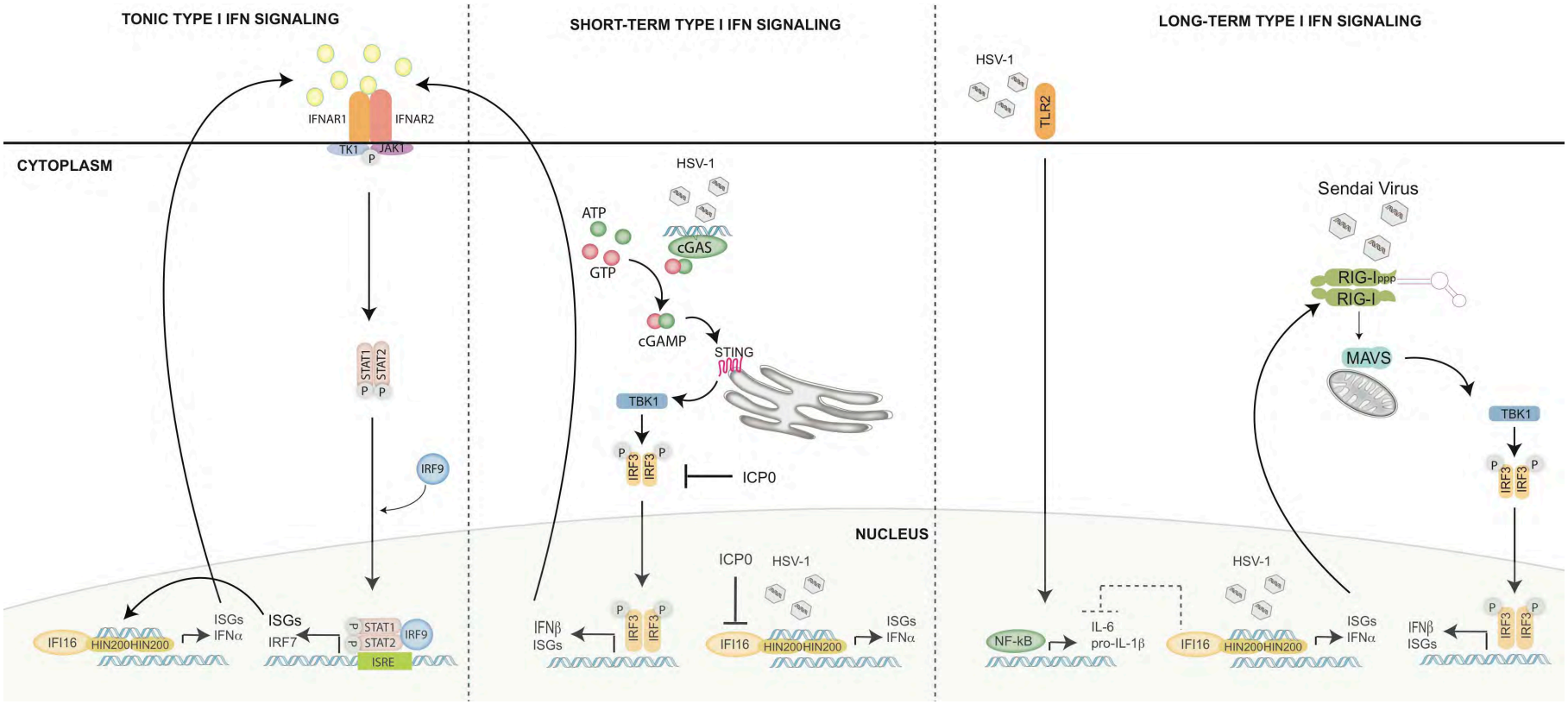


Figure 4.1 IFI16 regulation of type I IFNs. IFI16 regulates basal transcription of IFNα and ISGs. During HSV-1 infection, DNA that is leaked into the cytosol during infection signals through cGAS to induce type I IFNs and ISG expression. Thus, IFI16 is upregulated in the nucleus, where it also recognizes HSV-1 DNA to induce IFNα and ISG expression and amplify the type I IFN response. HSV-1 ICP0 can inhibit IRF3 and IFI16 to block type I IFN production. IFI16 activation maintains expression of ISGs such as RIG-I, which recognizes Sendai Virus. In the absence of IFI16, the host switches to a pro-inflammatory response in which TLR2 is activated to produce NF-κB dependent cytokines, IL-6 and pro-IL-1β. Figure adapted from Mikayla R. Thompson, John J. Kaminski, Evelyn A. Kurt-Jones, Katherine A. Fitzgerald. 2011. *Pattern recognition receptors and the innate immune response to viral infection*. Viruses. 6:920-40.

Appendix A: CD200R1 Supports HSV-1 Viral Replication and Licenses Pro-Inflammatory Signaling Functions of TLR2

Preface to Appendix A

This Appendix has appeared in the following publications/manuscripts:

Soberman, Roy J., MacKay, Christopher R., Vaine, Christine A., Ryan, Glennice Bowen., Cerny, Anna M.,**Thompson, Mikayla R,.** Nikolic, Boris., Primo, Valeria., Christmas, Peter., Sheiffele, Paul., Aronov, Lisa., Knipe, David M., Kurt-Jones, Evelyn A. (2012) "CD200R1 Supports HSV-1 Viral Replication and Licenses Pro-Inflammatory Signaling Functions of TLR2." *PLoS ONE* 7(10).

- Mikayla Thompson performed the experiments in 5.1b and c
- Mikayla Thompson wrote the introduction and added to and adapted the results and discussion from the paper listed above.

Introduction

The innate response to pathogens activates cells of the immune system, which aids in viral clearance; however prolonged activation or severe inflammation can be harmful to the host. In order to maintain this delicate balance, negative regulators, such as suppressor of cytokines (SOCS) (235,236), and apoptosis mechanisms (236) exist to restore the system back to its basal state. In fact, defects in negative regulation can lead to the onset of autoimmune disorders (236-239). In the case of herpes simplex virus 1 (HSV-1), the interaction of the virus with Toll-like receptor (TLR) 2 is critical.

Studies have shown that HSV-1 signals through TLR-2 to trigger the production of NF-κB driven cytokines. (32,240-242) This activation leads to the recruitment of leukocytes to the site of infection to aid in viral clearance. While these responses can be beneficial, excessive TLR2 activation can cause a cytokine storm, which causes harmful inflammation, tissue damage, and potential lethality (240,242,243). TLR2-/- mice are less susceptible to HSV-1 infection, marked by decreased production of CCL5 (Rantes), CCL2 (MCP-1) and IL-6 in the brain. Levels of these cytokines have been correlated to the severity of HSV-1 infection and the development of disease (242,244-247). Therefore, survival from HSV-1 encephalitis is determined not only by viral replication but also by the balance between pro-inflammatory and down-regulatory responses. To date, the mechanisms by which TLR2 signaling is down regulated are poorly described.

The CD200 receptor (CD200R1), which is expressed on all myeloid cells (248) as well as some T and B cell subsets (249), has been shown to down

regulate the immune response when it interacts with its ligand, CD200, which can be released by a wide range of cell subtypes (250-253). Myeloid cells that display the receptor receive an “off” signal from a cell that produces the CD200 ligand. This has been observed in models of experimental autoimmune encephalitis (EAE) (254), collagen induced arthritis (CIA) (255), and renal allografts (256). The CD200R signals by recruiting the adaptor molecules Dok1 and Dok2, which recruit RasGAP and mediate inhibition of the MAP kinase pathway (257). However, the specific pro-inflammatory signal transduction pathways in macrophages, dendritic cells, and mast cells that are down regulated by CD200R1 (251-253) have yet to be elucidated. How CD200R1 impacts TLR2 signaling, particularly in macrophages, is not known.

To understand how CD200R1 signaling impacts TLR2 function, we generated CD200R1-/- mice. We determined the ability of macrophages generated from CD200R1-/- mice to produce cytokines and chemokines in response to the TLR2 agonist Pam2CSK4 and to HSV-1. CD200R1-/- macrophages were limited in their ability to produce both IL-6 and CCL5 (Rantes) in response to stimulation by both Pam2CSK4 and HSV-1. However, we did not notice a decrease in cytokine production in response to LPS. CD200R1-/- macrophages also lacked the ability to up-regulate TLR2 expression in response to HSV-1 infection. Furthermore, CD200R1-/- embryonic fibroblasts and macrophages exhibited a defect in HSV-1 replication.

Results

CD200R1 Licenses Pro-inflammatory Signaling by TLR2 in Macrophages

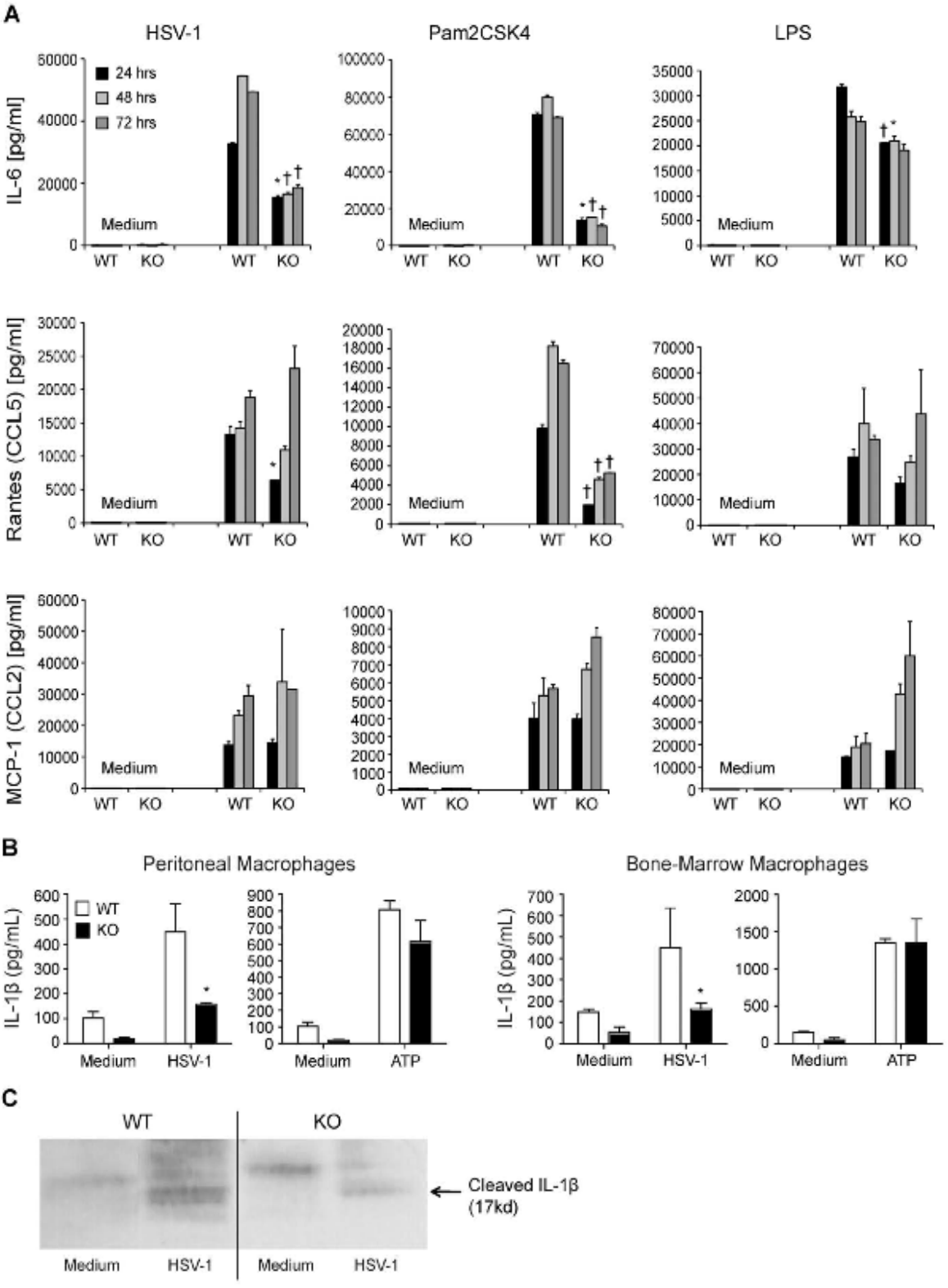
In order to determine how CD200R interacts with TLR2 in response to HSV-1 infection, we generated peritoneal macrophages from CD200R1+/+ and CD200R1-/- mice. Cells were infected with HSV-1 (multiplicity of infection; MOI = 10), or challenged with Pam2CSK4 (100 ng/ml), or LPS (100 ng/ml), and levels of IL-6, CCL5 (Rantes), or CCL2 (MCP-1) were measured at 24, 48, and 72 hours post infection by ELISA. HSV-1 infected CD200R1+/+ macrophages produced 35,000 pg/ml IL-6 24 hours after stimulation, which rose to 60,000 pg/ml by 48 and 72 hours (Figure 4A). In contrast, CD200R1-/- macrophages only generated between 20,000 to 30,000 pg/ml IL-6 in response to HSV-1 stimulation. Pam2CSK4 stimulated IL-6 levels averaged 70,000 to 80,000 pg/ml over the 3 day experiment in CD200R1+/+ cells, CD200R1-/- macrophages generated only 10,000 to 20,000 pg/ ml. Similar to the IL-6 response, the generation of CCL5 (Rantes) by CD200R1-/- macrophages stimulated with HSV-1 was decreased when compared to CD200R1+/+ macrophages. To address whether the decrease in CCL5 (Rantes) was a property of signaling by TLR2, or secondary to low levels of IFN, we determined whether CD200R1-/- peritoneal macrophages exhibit a blunted CCL5 (Rantes) response to stimulation with the TLR4 ligand, LPS. In contrast to the attenuated response to TLR2 stimulation, there was only a slight decrease in the levels of IL-6 at 24 and 48 hours and no difference in the levels of CCL5 (Rantes) generated in response to TLR4

stimulation via LPS in CD200R1+/+ or CD200R1-/- cells at any time point (Figure 5A). In the case of CCL2 (MCP-1) generation, no difference was found between CD200R1-/- and CD200R1+/+ with any stimulant; though LPS induced a marked increase in CCL2 (MCP-1) expression in CD200R1-/- cells.

The formation of mature IL-1β requires two distinct steps (258,259). The first signal induces the transcription and translation of pro-IL-1β. Expression of pro-IL-1β is regulated at the transcriptional level by NF-κB, which, for HSV-1, is activated downstream of TLR2 signaling. The second signal activates assembly of the inflammasome complex and cleavage of pro-IL-1β into its mature secreted form, IL-1β. This can be triggered by a variety of mechanisms, including extracellular ATP acting on the P2X receptor, reactive oxygen species, or potassium efflux triggered by the antibiotic Nigericin (260). The interaction of double stranded DNA and viral replication intermediates with intracellular DNA sensors can also induce inflammasome assembly. (152,153,157,168,169,261)The assembly and function of the inflammasome is therefore dependent on the quantity of viral replication intermediates within cells. We therefore hypothesized that if CD200R1 was required for efficient viral replication, then CD200R1-/- macrophages should show lower levels of inflammasome formation and impaired conversion of pro-IL-1β to mature IL-1β.

Stimulation of CD200R1-/- peritoneal or bone marrow- derived macrophages with HSV-1 led to the production of approximately 20% of the mature IL-1β levels seen in CD200R1+/+ cells (Figure 5B). In contrast, no difference was found in mature IL-1β levels between CD200R1+/+ or CD200R1-

/- macrophages stimulated with LPS (100 ng/ml) for 3 hours followed by the addition of ATP (1 mM) for 1 hour. These results suggested that that there was no intrinsic defect in inflammasome formation or in the capacity of CD200R1-/- macrophages to generate pro-IL-1β mRNA, while virus-induced inflammasome formation was significantly attenuated in CD200R1-/- macrophages. The difference in the production of mature IL-1β between CD200R1+/+ and CD200R1-/- macrophages following HSV-1 infection were confirmed by Western blot (Figure 5C).


A
HSV-1
Pam2CSK4
LPS
24 hrs
48 hrs
72 hrs
IL-6 [pg/ml]
Rantes (CCL5) [pg/ml]
MCP-1 (CCL2) [pg/ml]
Medium
WT
KO
B
Peritoneal Macrophages
Bone-Marrow Macrophages
IL-1β (pg/mL)
Medium
HSV-1
ATP
C
WT
KO
Cleaved IL-1β
(17kd)
Medium
HSV-1

Discussion

A delicate balance between immune activation and suppression is crucial for the proper detection and clearance of viruses. Improper regulation of these responses can lead to severe inflammation and tissue damage and lethality. In the case of HSV-1, signaling through TLR2 is important for this balance. The CD200R1 has been shown to play a role in down regulating immune responses when it interacts with its ligand CD200, however its role in TLR2 responses are unknown. Here we defined a unique role for CD200R1 in supporting (licensing) pro-inflammatory signaling by TLR2. This role was revealed by studies with peritoneal macrophages in which the generation of IL-6 and CCL5 (Rantes) in CD200R1-/- macrophages was blunted by 80% in response to TLR2 ligands and HSV-1; this was not observed in response to LPS suggesting a specific defect in TLR2-driven responses. Furthermore, CD200R1-/- macrophages lacked the ability to assemble a functional inflammasome. Both the NLRP3 and AIM2 Inflammasomes have been implicated in HSV-1 signaling, however further studies are needed to determine which inflammasome complex is being activated by CD200R1.

It is unclear why IL-6 and CCL5 (Rantes) but not MCP-1 levels were reduced in CD200R1-/-. It may be that there is differential regulation of IL-6 and CCL2 (MCP-1) generation via TLR2 signaling. Although these cytokines are both NF-κB driven, there are distinct transcription factors that drive expression of IL-6 or MCP-1. In previous studies we have noted that HSV-induced IL-6 is strictly dependent on TLR2 expression while some MCP-1 secretion still occurs in

TLR2-/- cells, albeit at reduced levels compared to TLR2+/+ macrophages (262). Currently, the mechanism(s) are not known. It is also possible that CD200R1 regulates IL-6 and CCL2 (MCP-1) differently. It is also unclear why the expression of CCL5 (Rantes) but not CCL2 (MCP-1) is decreased in CD200R1-/- mice. It is possible that CCL5 (Rantes) secretion is secondary to decreased IFN, though ultimately this also is a consequence of the control of TLR2 signaling by CD200R1. An additional defect in TLR2 function, the inability to up-regulate the expression of TLR2 in response to HSV-1 infection, may contribute to the inability to perpetuate and amplify HSV-1 infection, though this may be secondary to the role of CD200R1 in regulating viral replication. These studies provide evidence for a new checkpoint and balance during HSV-1 infection.

Materials and Methods

Ethics Statement

This study was approved and performed in strict accordance with the guidelines set forth by both the University of Massachusetts Medical School Department of Animal Medicine Institutional Animal Care and Use Committee (IACUC) (assurance number A3306-01) and by the Massachusetts General Hospital (MGH) Center for Comparative Medicine, Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee (IACUC) at MGH (assurance number A3596-01). Mice were bred and maintained under specific-pathogen-free conditions at the animal facilities at both the University of Massachusetts Medical School and the Massachusetts General

Hospital Charlestown Facility, and all efforts were made to minimize suffering.

Antibodies

Anti-IL-1β was goat polyclonal anti–mouse IL-1β (AF-401-NA; R&D Systems). The secondary anti-rabbit antibody used in blotting studies was rabbit anti-goat (H+L) IgG HRP conjugate (172–1034; Bio-Rad)

Preparation of Viruses

HSV-1 strains were generated in the laboratory of Dr. David Knipe. Viruses were added to peritoneal macrophages at an M.O.I. of 10:1.

Preparation and Stimulation of Peritoneal Macrophages

Mice were injected with 4% thioglycollate and peritoneal exudate cells were routinely harvested 3–4 days later. To generate macrophages, peritoneal exudate cells were plated at 106 cells per well in 24-well plates in DMEM containing 10% FCS. Lipopolysaccharide (LPS) was obtained from Sigma and phenol extracted. Pam2CSK4 was obtained from EMC Microcollections (Tubingen, Germany).

Statistical Analysis

An unpaired, two-tailed Student's t-test was used to determine statistical significance where indicated. Statistics were performed using GraphPad (Prism v5.0d) software. Values of P,0.05 were considered significant.

Appendix B: Forward genetic analysis of type I IFN responses to DNA ligands reveals a novel polymorphism in the MOLF/Ei STING gene.

Preface to Appendix B

The work of this chapter was done in collaboration with Jennie Chan and collaborators at the Tufts Sackler School of Biomedical Sciences, Alexander Poltorak, Guy Surpris, and Joe Sarhan.

- Mikayla Thompson and Jennie Chan performed experiments in Figures 6.1.
- Mikayla Thompson wrote all of Appendix B with edits from Jennie Chan.
- Alexander Poltorak, Guy Surpris, and Joe Sarhan performed experiments in figures 6.2 and 6.3.

Introduction

The innate immune response is the body's first line of defense against infection. The ability of pattern recognition receptors to recognize various moieties from microbes is crucial for innate immune activation and clearance of pathogens. The role of Toll-like Receptors during host-defense is well known. Recently, intracellular receptors have been implicated in sensing nucleic acids that accumulate in the cytosol during viral and bacterial infections. RNA and DNA are potent activators of pro-inflammatory cytokines, such as IL-1, IL-6 and type I interferons, all of which aid in the clearance of pathogens. A wide range of cytosolic RNA and DNA sensors have been defined, although how these receptors work together has yet to be determined. The ER-bound protein STING is central to these responses as many cytosolic sensors utilize the molecule as an adaptor to induce type I interferons in response to cytosolic DNA.

In unstimulated cells, STING localizes to the ER and perhaps ER-associated mitochondria (91). It contains 4 transmembrane helices and a cytosolic carboxyl terminal domain. Following stimulation with cytosolic DNA or HSV-1, STING translocates to perinuclear foci, via the Golgi, where it initiates downstream effects (89). Activation of STING leads to TANK binding kinase-1 (TBK1) dependent phosphorylation of interferon regulatory factor 3 (IRF3) and transcription of type I IFN genes. Many cytosolic sensors, including IFI16, DDX41, and cGAS act upstream of STING. However, it is possible for cyclic-di-nucleotides to bind STING directly to induce type I interferons. Although the role

of all sensors upstream of STING have yet to be clearly elucidated, it is evident that STING plays an important role in antiviral immunity to DNA viruses.

Because the type I interferon response is so critical to antiviral immunity, it is likely that genes involved in this pathway would be favorable for evolutionary selection. This notion allows us to use evolutionarily distinct mouse strains to reveal novel signaling pathways. These experimental approaches use wild-derived inbred strains of *Mus musculus molossinus MOLF/Ei (MOLF),* which are genetically divergent from the conventional laboratory *Mus musculus musculus C57Bl/6 (B6)* strains by several million years. When bred together, they produce fertile offspring with genetic differences that can be used to identify new alleles that contribute to activate or repress innate immune responses (263). Such approaches have been used to define an anti-inflammatory role for IRAK1BP1(264) and susceptibility to *Salmonella typhimurium*(265).

In the present study, we observed that the wild derived MOLF mice lack the ability to produce type I interferons in response to DNA ligands. Using a forward genetic approach and quantitative trait locus analysis (QTL), we identified a mutation in STING that limits the production of type I interferons in response to DNA ligands.

Results

MOLF/Ei mice have a blunted IFN-β response to DNA ligands

Since wild derived and B6 mice are genetically diverse, yet can be bred to produce fertile offspring, we used these two strains for forward genetic analysis.

We harvested peritoneal macrophages from B6 and MOLF mice and stimulated the cells with poly(dA:dT), HSV-1, MCMV, and Sendai Virus (SV) for 12 hours and then collected supernatant for analysis by ELISA. As compared to B6 mice, the wild derived MOLF mice have severely attenuated IFN-β production in response to all DNA ligands (Figure 6.1a). In contrast to these results, we did not see any difference in IFN-β production in response to Sendai Virus, suggesting that the phenotype is specific to DNA ligands. Furthermore, IL-1β production remained unchanged, suggesting the phenotype is specific to the type I interferon pathway (Figure 6.1b). F1 mice (B6XMOLF cross) yielded a phenotype similar to that of B6 mice, where IFN-β production was not attenuated in response to DNA ligands, suggesting that the gene responsible for the phenotype is dominant for B6 (Figure 6.1a and b). Thus we used a forward genetic screen of 35 N2 mice (F1XB6) in order to map the phenotype back to the genetic locus. Peritoneal macrophages were stimulated with poly(dA:dT) (Figure 6.1c), HSV-1 (Figure 6.1e), Listeria, ci-di-AMP, and SV (data not shown) for 12 hours and then supernatant was analyzed for IFN-β production by ELISA. N2 mice varied in IFN-β production. The cells were also analyzed for IL-6 production (Figure 6.1d and f) as a control to confirm that the cells are functional and can produce other cytokines normally.

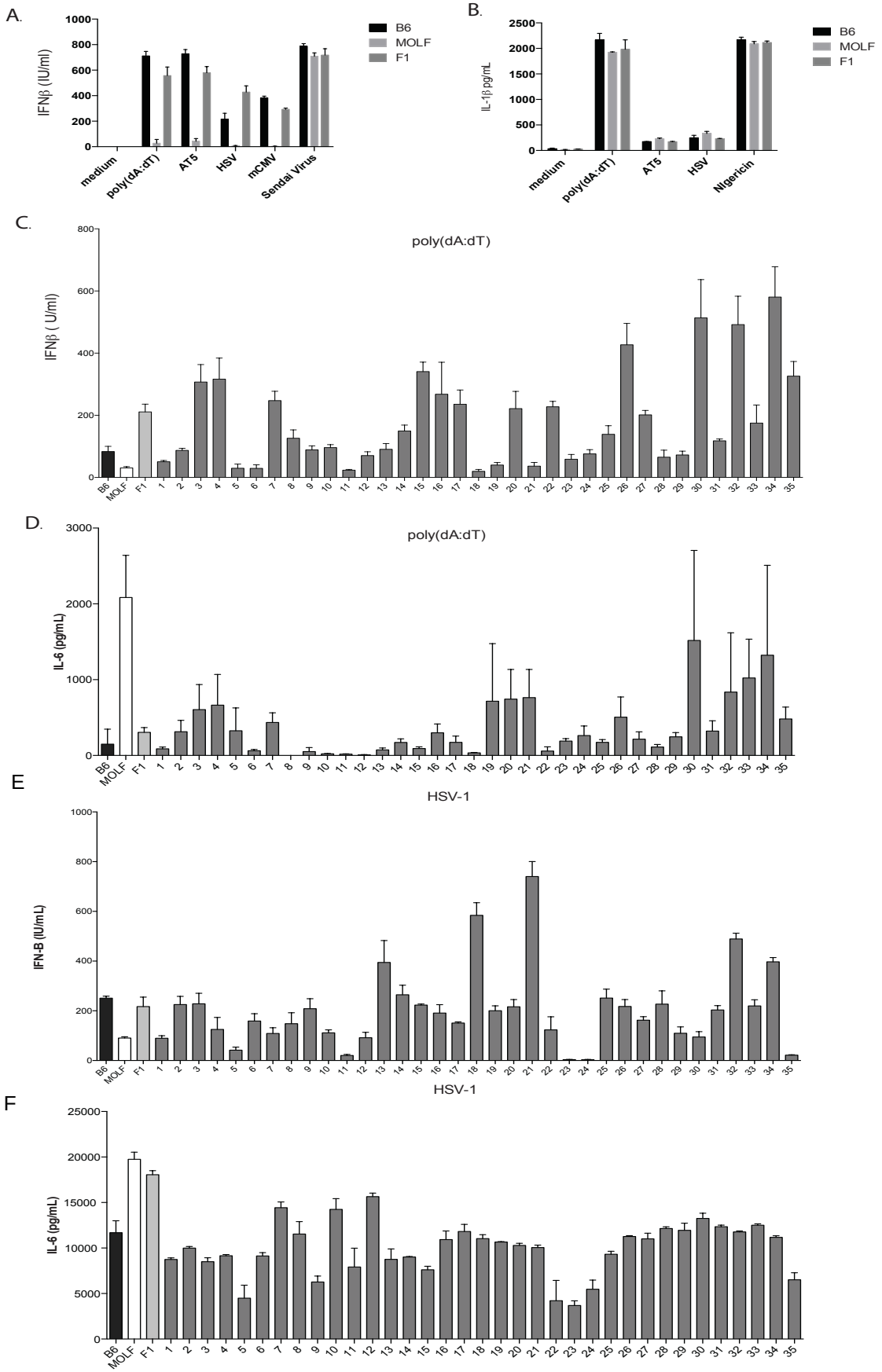


Figure 6.1. MOLF/Ei mice display a blunted IFN-β response to DNA ligands. A. Peritoneal macrophages from B6, MOLF, and F1 mice were stimulated with poly(dA:dT), AT5, HSV, MCMV, and Sendai Virus for 12 hours supernatant was analyzed for IFN-β or B. IL-1β by ELISA. C. B6, MOLF, F1, and N2(1-35) mice were stimulated with poly(dA:dT) (C and D) or HSV-1 (E and F) for 12 hours. Supernatant was analyzed for IFNβ or IL-6 by ELISA.

Decreased IFN-β production in MOLF mice can be mapped to Chromosome 18 and STING.

To map the loci responsible for the observed phenotype, our collaborators at Tufts performed Quantitative trait loci analysis. In response to poly(dA:dT), they found a significant Logarithm of odds score (LOD) at both chromosomes 11 and 18 (Figure 6.2a). A significant LOD score was also found for chromosome 18 in response to ci-di-GMP (data not shown). Therefore, chromosome 18 was pursued further. Fine mapping of the chromosome revealed that D18Mit202 conferred the phenotype. After analysis of genes in the region that were polymorphic between the two mouse strains, we identified STING as a probable candidate in that region. The presence of both D18Mit202 and STING correlated with the parent strains and amount of IFN-β production, suggesting that STING is responsible for the observed phenotype (Figure 6.2b and c).

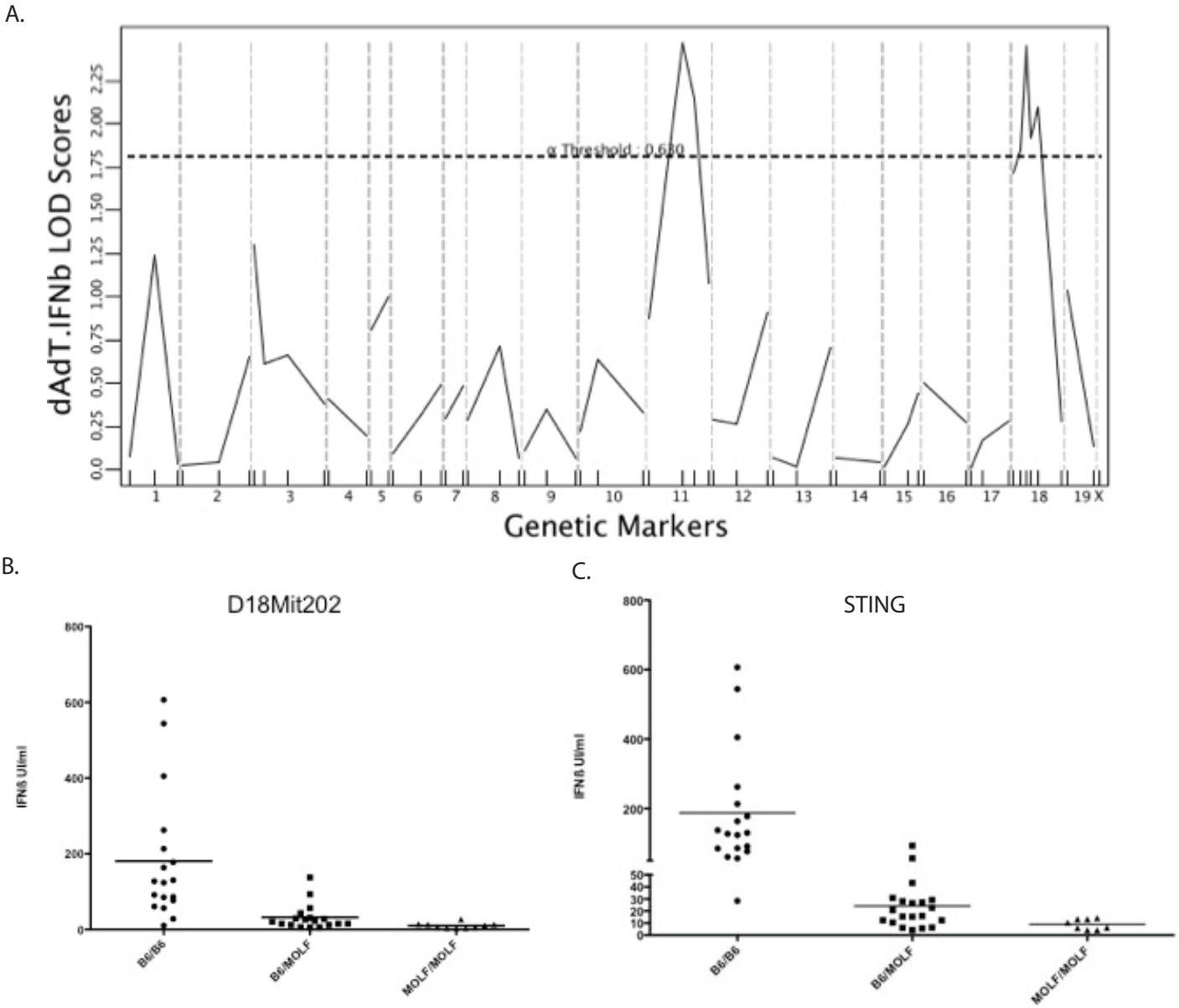


Figure 6.2 Decreased IFN-β production in MOLF/Ei mice can be mapped to Chromosome 18 and STING. A. QTL Analysis of N2 panel in response to poly(dA:dT). Threshold indicates significant LOD scores. B. Effect of D18Mit202 and C. STING inheritance on IFN-β production for individual N2 mice.

MOLF mice have multiple polymorphisms in STING

In order to determine how MOLF STING is functionally different from B6 STING, a sequence analysis was performed. Sequence analysis revealed several polymorphisms in the MOLF STING allele as compared to B6 (Figure 6.3a). To test the functionality of MOLF STING, we cloned the gene and looked for its ability to activate the ISRE promoter by luciferase assay. B6 STING induced the IFN-β ISRE promoter more than 2 fold above MOLF STING (Figure 6.3b). Furthermore, upon stimulation with DMXAA, cyclic-di-AMP, poly(dA:dT) and cyclic-di-GMP, MOLF STING induced less ISRE reporter activity. Lastly, we mutated B6 STING to mimic that of the MOLF STING polymorphisms. We found that in response to DMXAA, cyclic-di-AMP, poly(dA:dT) and cyclic-di-GMP, mutated B6 phenocopied the MOLF hypo-IFNβ responses, marked by less ISRE activation. These results provided further evidence that mutations in MOLF STING hinder the functionality of the gene.

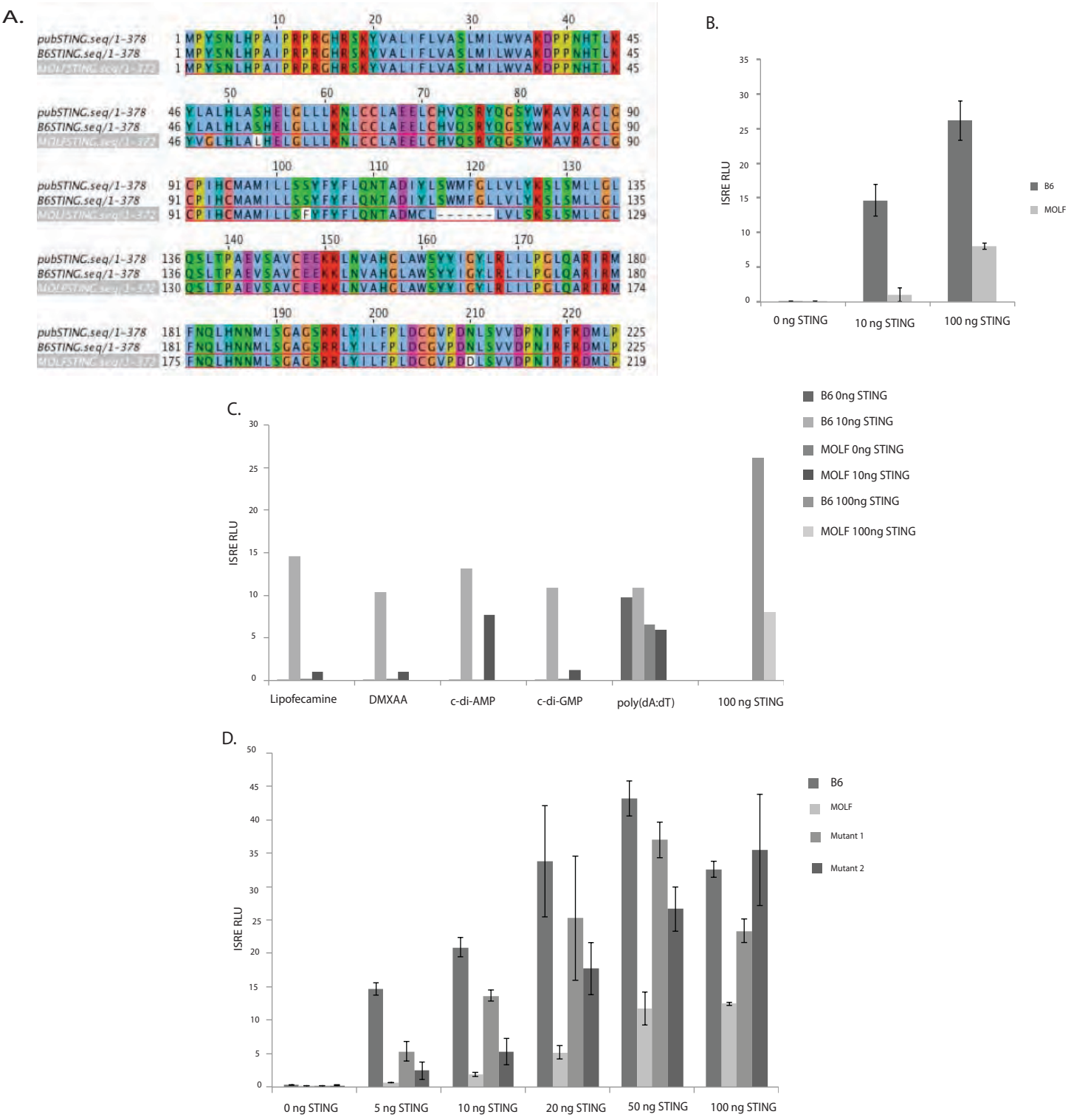


Figure 6.3. MOLF/Ei mice have multiple polymorphisms in STING. A. Sequence analysis of B6 and MOLF STING. B. B6 and MOLF STING were transfected into cells and reporter assay was used to determine activation of ISRE luciferase. Cells are normalized to renilla. C. B6 and MOLF STING were transfected into HEK cells and stimulated with DMXAA, ci-di-AMP, ci-di-GMP, and poly(dA:dT). Cells were monitored for ISRE activation by reporter assay. D. Increasing concentrations of B6, MOLF, and mutant B6 STING were transfected into cells and reporter assay was used to determine activation of ISRE luciferase. Cells are normalized to renilla plasmid.

Discussion

Here we used a forward genetic approach and QTL analysis to determine that a mutation in the wild derived MOLF STING is responsible for decreased functionality of the gene and an inability to produce type I interferons in response to DNA stimuli. This finding highlights the importance of the evolution of STING as a central mediator for these responses in antiviral immunity. We found that MOLF mice are unable to produce type I interferons in response to HSV-1, MCMV, and *Listeria monocytogenes*. Further work is needed in order to determine whether MOLF mice are more susceptible to these pathogens in vivo. Mice deficient in STING are more susceptible to both HSV-1 and Listeria infections (89,266), suggesting that MOLF mice may be susceptible to these pathogens as well.

DMXAA is a tumor-vascular disrupting agent that has been shown to induce type I interferons by activating STING (267,268). Surprisingly mouse STING, but not human STING had the ability to respond to DMXAA, providing explanation for why the drug failed as an anti-tumor medicine in clinical trials(269). The decreased functionality of mouse STING was mapped to the C-terminal domain (CTD). When the C terminal domain of mouse STING was replaced with the C terminal domain of human STING, the ability to respond to DMXAA was restored(269). These studies highlight the importance of the C terminal domain of STING. Previous studies have shown that the CTD not only binds cyclic-di-nucleotides, but also interacts with TBK1 and IRF3 to activate downstream signaling effects. Furthermore, the CTD is important for keeping

STING in its auto-inhibited state, which is then lifted upon binding on cyclic-di-nucleotides.

Interestingly, our studies unveiled a mutation in the N-terminus of MOLF STING. This discovery provides further insight into the functional domains of the gene. In our work we find that although IFN-β responses are blunted in MOLF mice, IL-1β and IL-6 responses are unaltered or increased, respectively. This finding is in contrast to previous results, which show that mutations in the C terminal domain of STING lead to abrogated NF-κB responses as well. Our results suggest that while the C terminus is responsible for the overall activation of STING, the N terminus may have more specific function related to the sensing of DNA ligands. Further work is needed to determine if MOLF STING forms a functional protein and is able to induce NF-κB responses.

Materials and Methods

Mice

C57BL/6J and MOLF/Ei parental strains were obtained from The Jackson Laboratory. All mice were housed in a pathogen-free facility at the Tufts University School of Medicine. All mouse procedures were performed under a protocol approved by the Tufts University/Tufts Medical Center Institutional Animal Care and Use Committee as described in (264).

Genetic Analysis

Genome-wide scanning was performed according to standard procedures, using two to three known polymorphic microsatellite markers per chromosome. Both genotypic and phenotypic data were analyzed using QTX software for genetic mapping of quantitative trait loci as described in (264).

Reagents and Antibodies

LPS and poly(dA:dT) were obtained from Sigma-Aldrich (St. Louis, MO). HSV 60mer, and AT5 oligonucleotides were synthesized as described in (130), Cyclic-di-GMP was from Biolog (Hayward, CA). *L. monocytogenes* (clinical isolate 10403s) was from V. Boyartchuk (NTNY, Trondheim, Norway). HSV-1 (7134) was a gift from D. Knipe (Harvard Medical School, MA). Sendai virus (SeV, Cantrell strain) was purchased from Charles River Laboratories (Wilmington, MA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Genejuice was from Novagen (Madison, WI).

Cell Culture, Stimulation and ELISA

Mice were injected with 4% thioglycollate and peritoneal exudate cells were routinely harvested 3–4 days later. For stimulations, poly(dA:dT) (1 μg/ml) or ci-di-GMP (3 μM) were transfected into the cells with lipofectamine in accordance with the manufacturer's instructions. Cells were infected with mCMV or HSV-1 viruses at multiplicities of infection (MOI) of 10. Cells were infected with Sendai virus at 200 IU/ml. For bacterial infection, cells were challenged with *L.*

monocytogenes at an MOI of 5 for 1 hr. Infected cells were then washed twice and medium containing gentamicin (100 μg/ml) was added to kill extracellular bacteria. Knockdown and control cells were challenged with stimulants or microbes for 12 hours (for protein analysis by ELISA). Cytokine and IFN levels in culture supernatants were assayed for IL-1β and IL-6 (BD Biosciences, Franklin Lakes, NJ) and IFN-β by sandwich ELISA.

Luciferase promoter assays

The indicated regions of the STING were amplified from C57BL/6J or MOLF/Ei genomic DNA and cloned into the pGL4.20 (Promega) firefly luciferase vector. HEK 293T cells were transfected with lipofectamine as per the manufacturer's protocol, stimulated, and lysed using Passive Lysis buffer. Luciferase was read using Dual Luciferase reagent (Promega) in a single tube luminometer as described in (264).

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