INVESTIGATING THE ROLE OF NEWLY DESCRIBED CYTOSOLIC PATTERN RECOGNITION RECEPTORS IN PROTECTIVE HOST RESPONSES TO NEUROTROPIC VIRUSES

by

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ABSTRACT

EMMA KATELIN CRILL. Investigating the role of newly described cytosolic pattern recognition receptors in protective host responses to neurotropic viruses. (Under the direction of DR. IAN MARRIOTT)

The importance of the effector functions performed by glial cells, the resident cells of the CNS possessing immune functions, in abrogating potentially fatal neurotropic viral infections have only recently begun to gain appreciation. These effector functions would not be possible if it weren't for the presence of innate pattern recognition receptors (PRRs) that serve as sensors for conserved pathogenic components and initiate activation of innate defense mechanisms in response to a non-self invader. The current study investigates the role of retinoic acid inducible gene (RIG)-I and DNA-dependent activator of interferon regulatory factors (DAI) in the recognition and intervention against RNA and DNA viral infections in the glial cells, astrocytes and microglia. Our laboratory has previously shown that astrocytes and microglia express RIG-I and DAI and demonstrated that stimulation of these receptors with synthetic or virally-derived RNA or DNA ligands, respectively, leads to the expression and secretion of proinflammatory mediators. More recently, it has been hypothesized that the utilization of RNA polymerase III confers on RIG-I the ability to indirectly recognize and become activated by DNA pathogens. In the present study, we demonstrate that RNA polymerase III inhibition significantly attenuates protective host responses elicited following challenge with DNA, but not RNA, viruses and ligands. Inhibition of RNA polymerase III activity led to enhanced DNA, but not RNA, viral replication associated with reduced production of the potent antiviral molecule, interferon-β (IFN-β), suggesting that RNA polymerase III licenses RIG-I to mount protective immune responses against DNA pathogens.

DEDICATION

This thesis is dedicated to my forever role-model and hero, my mom.

I could not have done it without you.

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CHAPTER 1: INTRODUCTION

1.1 Viral Meningitis and Encephalitis in the Central Nervous System

20,000 cases of encephalitis are reported each year in the United States (CDC). Since viruses are the primary causative agent of encephalitis, this number is likely to be underreported given the sheer volume of viruses capable of inducing central nervous system (CNS) inflammation. The incidence of encephalitis in westernized nations is estimated to be 6.34 per 100,000 for all age groups (Jmor et al., 2008). Worldwide, the DNA virus, herpes simplex virus (HSV), is recognized as the primary causative agent of virally-induced encephalitis (Jmor et al., 2008). HSV type I (HSV-1) accounts for upwards of 90% of herpes simplex encephalitis (HSE) cases (Steiner and Benninger, 2013). Prompt medical intervention for HSE patients is often difficult since neurodegeneration progresses rapidly and sometimes occurs in the absence of symptoms associated with a prior HSV-1 infection (Steiner and Benninger, 2013). Without treatment, HSE carries a 70-97% morality rate (Steiner and Benninger, 2013). Of those survivors, most are left with permanent cognitive impairment and motor deficiencies (Ghoshal et al., 2007).

More common than encephalitis is meningitis, which accounts for an average of 40,000 cases annually in the United States (CDC). RNA viruses belonging to the enterovirus genus are responsible for 85-95% of viral meningitis cases in adults (Putz et al., 2013). Less common, but far more dangerous, is HSV-induced meningitis which is

identified in about 3% of all meningitis cases and is typically associated with progression to meningoencephalitis (Putz et al., 2013). Acyclovir, a nucleoside analog that is selectively phosphorylated by viral thymidine kinase, is commonly employed for the treatment of meningitis originating from an HSV infection (Tavis et al., 2014). Although acyclovir administration, which inhibits the actions of virally-encoded DNA polymerase, is usually sufficient for precluding HSV manifestations in the CNS, the ability to diagnose it before irreversible neurological deficits are sustained is difficult (Putz et al., 2013).

Viral infections in the CNS are typically characterized by the activation of resident immune cells and the ultimate generation of innate and adaptive immunological responses. The recognition of a viral challenge prompts the production of antiviral and proinflammatory mediators by microglia and astrocytes. Excessive and inappropriate production of these mediators, which are important for inhibiting viral dissemination, can result in the progression to meningitis and/or encephalitis coupled with severe neurological sequelae. Diagnosis of virally-induced CNS inflammation relies on identifying the region of the brain afflicted. Meningitis is characterized by inflammation of the meninges, the membranes surrounding the brain, while inflammation of the brain parenchyma is diagnosed as encephalitis.

Marked inflammation rapidly follows the perception of a viral infection in the CNS thereby making meningitis and encephalitis particularly difficult to diagnose before severe neurological damage is sustained (Furr and Marriott 2012). Patients initially present with high fever and an altered mental state associated with confusion, disorientation and photophobia (Jmor et al., 2008). Without intervention, patients

experience continued deterioration in neurological function leading to coma, seizures and even death (Jmor et al., 2008).

1.2 Protective versus Damaging Host Responses in the Central Nervous System

The perception of an active viral infection in the CNS results in the secretion of a variety of chemical mediators by both immune and non-immune cells. The typical cytokine profile observed during neurotropic viral infection is characterized by the expression of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) which contribute to the promotion of inflammation, as well as type I interferons which are critical for activating antiviral effector functions. Maintaining a balance between bystander damage, a common side effect of proinflammatory mediator functions, and viral clearance via type I interferons is vital for attenuating the progression of an acute viral infection to meningitis and/or encephalitis.

The contribution of type I interferons towards host survival during viral infections was highlighted by the identification of patients deficient in responses mediated by these potent inflammatory molecules. Of the three patients identified, two died from viral infections (Levy et al., 2011). Much like what is observed in humans, mice incapable of generating interferon responses due to deficiencies in the interferon alpha/beta receptor 1 (IFNAR-1) subunit of the type I interferon receptor, are increasingly susceptible to neurotropic viral infections (Sorgeloos et al., 2013). Additionally, type I interferon responses are critical for restricting replication of the primary causative agent of viral encephalitis, HSV-1, suggesting a dually protective role against viral infections and fatal neuroinflammation (Jmor et al., 2008; Roasto and Lieb, 2014).

The type I interferon family is comprised of interferon-α (IFN-α) and interferon-β (IFN-β). The expression of IFN-β, whose transcription is dependent upon the activation of interferon regulatory factor 3 (IRF3), is a prerequisite for IFN-α expression (Levy et al., 2011; Rustagi and Gale, 2014). Binding of newly translated IFN-β to its receptor initiates janus kinases/signal transducers and activators of transcription (JAK/STAT) signaling, which ultimately leads to the transcription of interferon stimulated genes (ISGs) (Sato et al., 2000). The majority of ISGs, including IFN-α, are associated with interference of viral entry into host cells, hindering viral genome translation and obstructing other processes occurring during the early phases of the viral lifecycle (Schoggins and Rice, 2011).

In addition to promoting ISG expression, IFN-β can directly act on cells and promote entrance into an antiviral state by inducing 2',5' (2-5A) oligoadenylate synthetases, protein kinase R (PKR) and Mx proteins (Zhou et al., 1999). The conversion of double-stranded RNA (dsRNA) into 2-5A oligoadenylates by active 2-5A synthetases activates RNaseL, resulting in the degradation of host and viral mRNAs (Zhou et al., 1999). Unlike the RNaseL mechanism which inhibits early stages in the viral lifecycle, PKR-dependent phosphorylation of translation initiation factor eIF2α aids to inhibit viral genome translation (Zhou et al., 1999). Like PKR, Mx proteins are critical for abrogating late viral lifecycle events via their GTPase function which effectively hinders the production of RNA virions (Zhou et al., 1999). The above IFN-β-induced mechanisms act synergistically to prevent viral transcription, translation and replication.

In spite of sharing the same receptor, IFN-α and IFN-β play disparate roles in the CNS during viral challenge. The immunoregulatory functions of IFN-β protect the host against viral dissemination and bystander damage (Dafny and Yang, 2005). IFN-β impedes inflammation by reducing the permeability of the blood-brain barrier, skewing T cell effector functions towards those best suited for viral infections and downregulating the expression of adhesion molecules which are required for the infiltration of inflammatory cells into the CNS (Paul et al., 2007). Unlike its protective relative, IFN-α is notoriously associated with neurotoxicity by acting on CNS resident cells to induce the expression of proinflammatory IL-1β, IL-6 and TNF-α (Fritz-French and Tyor, 2012).

IL-1β, IL-6 and TNF-α alter adhesion molecule expression to permit the recruitment of monocytes into the CNS (Ghoshal et al., 2007). Upon the successful passage of monocytes through the blood-brain barrier, IL-6 activates monocytes and skews them toward the inflammatory M1 macrophage phenotype (Ashhurst et al., 2014). Inflammation then proceeds in a feed-forward mechanism via the secretion of IL-1β, IL-6 and TNF-α by newly activated monocytes which promote the infiltration and subsequent activation of additional monocytes. In addition to contributing to inflammatory cell recruitment, TNF-α promotes the secretion of nitric oxide (NO) by stimulating the expression of inducible nitric oxide synthase (iNOS) (Ghoshal et al., 2007). NO is a precursor of highly reactive intermediates that are capable of inducing extensive neuronal death and tissue damage (Liu et al., 2002).

Although both IFN-α and IFN-β are recognized as potent antiviral molecules, their secretion in the CNS during an active viral infection is tightly regulated so as to

limit the undesirable side effects of IFN-α (Paul et al., 2007). The effector functions of IFN-α, which contribute to necrotic death of infected neurons as well as the compromise of the blood-brain barrier by infiltrating monocytes, can consequently result in viral dissemination and severe inflammation of the brain parenchyma and/or the meninges. Neurotropic viral infections are typically characterized by reduced IFN-α production and tissue destruction at the expense of permitting some viral replication (Paul et al., 2007). As is the case with most immunological mediators, the optimal level of type I interferon secretion is that which promotes the establishment of an antiviral state without sacrificing the integrity of the resident CNS cell population.

1.3 Glial Cell Participation in Protective and Damaging Host Responses to Neurotropic Viral Infection

The activation of microglia and astrocytes is a prerequisite for the initiation of innate immunological responses in the CNS (Aloisi, 1999). Microglia constitute the major myeloid cell population in the brain and are readily activated in response to pathogenic challenges. Their responsiveness coupled with their abundance contributes to the recognition of microglia as the predominating immune effector cell type in the CNS (Kreutzberg, 1996). Astrocytes are primarily responsible for providing neuronal support and maintaining neurological homeostasis, but like microglia, astrocytes can participate in innate immunological functions when activated (Aloisi, 1999).

The secretion of hallmark inflammatory cytokines, IL-1β, IL-6 and TNF-α, by glial cells has been documented in response to an array of viral challenges including, but not limited to, RNA viruses like Japanese encephalitis virus (JEV) and DNA viruses like HSV-1 (Ghoshal et al., 2007). In addition to inflammatory cytokine production,

IFN-α can indirectly stimulate the production of NO by microglia and astrocytes by promoting the expression of the NO secretory molecule, TNF-α (Liu et al., 2002).

Microglia and astrocytes are known to be potent sources of type I inteferons, IFN-α and IFN-β, in response to challenge with both DNA and RNA viruses, as well as, synthetic nucleic acids (Paul et al., 2007). In contrast to the neuroinflammatory mediators discussed above, IFN-β limits bystander damage and further viral infiltration. Although virally-induced neuroinflammation carries a high mortality rate, the number of viral meningitis and encephalitis cases reported annually is significantly lower than would be expected given the sheer volume of viruses capable of causing this type of pathogenesis. Since viral infections in the CNS are generally not associated with extensive inflammation, the production of IFN-β by microglia and astrocytes must be sufficient to counteract the damaging side effects of IL-1β, IL-6, TNF-α and NO. Vergara and colleagues (2010) have demonstrated the ability of IFN-β to inhibit NO secretion by activated glial cells thus attenuating neighboring cell and tissue death. In addition to neutralizing IFN-α-induced products, the utilization of a shared receptor by type I interferons serves as a compensatory mechanism that allows IFN-β to minimalize IFN-α signaling. The duration that IFN-β remains bound to IFNAR is considerably longer than that observed with IFN-α (Reder and Feng, 2014). Prolonged binding of IFN-β not only induces the expression of an expanded set of IFN-β stimulated genes, but also physically blocks IFN-α from binding thus abolishing the activation of IFN-α-induced ISG expression (Reder and Feng, 2014).

1.4 Innate Recognition of Virus by Host

The diverse nature of viruses poses a challenge to the innate immune system which relies on highly conserved pathogen associated molecular patterns (PAMPs) for the recognition of foreign invaders (Stetson and Medzhitov, 2006). Germline-encoded pattern recognition receptors (PRRs) capable of detecting viral nucleic acids will confer an advantage to host cells that possess them because unlike other structural components which vary significantly from virus-to-virus, all viruses possess a genome comprised of RNA or DNA. The ability to perceive the DNA virus, HSV-1, before it can establish a robust infection is particularly important since these infections are associated with severe neurological sequelae and a staggering mortality rate. The envelopment of HSV-1 and vesicular stomatitis virus (VSV), a neurotropic RNA viral pathogen capable of inducing encephalitis, virions effectively hides their viral genomes when outside of the host cell (Salameh et al., 2012; Hastie et al., 2013). This type of genome sequestration restricts the sites for innate immune recognition to those where viral transcription, translation and replication occur. Upon fusion with a host cell, the contents from both the HSV-1 and VSV envelope are released into the cytoplasm (Salameh et al., 2012; Hastie et al., 2013). Unlike VSV which replicates in the cytoplasm using its own RNA-dependent RNA-polymerase, the nucleic acids and proteins from HSV-1 are transported into the nucleus for viral replication via host encoded enzymes (Salameh et al., 2012; Hastie et al., 2013). Intracellular PRRs, specifically those located in the cytosol, will be best suited to initiate antiviral defenses against both HSV-1 and VSV.

Toll-like receptors (TLRs) are type I transmembrane domains that utilize a Toll-interleukin 1 receptor (TIR) domain to activate signal transduction pathways (Bussey et

al., 2014). The initiated signaling cascade is secondary to the recognition of specific PAMPs, which is mediated by leucine rich repeats (LRRs) (Takeuchi et al., 2007). Membrane bound TLR2 and TLR4 have been recognized to detect structural components of herpesvirus family members, specifically those derived from murine gammaherpesvirus-68 (MHV-68) (Bussey et al., 2014). Knockdown of TLR2 in murine embryonic fibroblasts (MEFs) significantly attenuates the production of proinflammatory mediators in response to MHV-68 infection (Bussey et al., 2014). In spite of the recognition that TLR2 and 4 participate to some degree in antiviral innate immune signaling, their location is disadvantageous because it forces them to rely on the detection of continuously evolving virion surface components. In addition, most causative agents of viral meningitis and encephalitis produce virions that are coated with an envelope comprised of host cell proteins and thus will not be recognized as foreign by membrane bound TLRs (Salameh et al., 2012). Of the remaining TLRs, the location of TLR3,7 and 9 on cytoplasmic vesicles coupled with their specificity for dsRNA, single-stranded RNA (ssRNA) and DNA with unmethylated CpG motifs, respectively, makes them ideal candidates for neurotropic viral recognition (Takeuchi et al., 2007). Viral infection has been demonstrated to be a potent stimulus for upregulating the expression of these receptors in glia and a number of other cell types (Bowman et al., 2003; Carpentier et al., 2005; Jack et al., 2005; McKimmie and Fazakerley, 2005; McKimmie et al., 2005; Bsibsi et al., 2006). TLR 9 -/- mice infected with HSV-1 demonstrated attenuated IFN-α production in the absence of increased viral pathogenesis suggesting the role of another PRR that is potentially better suited for recognizing and dealing with these infections in an IFN-α-independent manner (Krug et

al., 2004). Furthermore, the orientation of the LRRs responsible for PAMP recognition on TLR3, 7 and 9 towards the extracellular space or the endosomal lumen inhibits their ability to detect PAMPs in the cytoplasm (Takeuchi et al., 2007). An inability to detect virally-derived nucleic acids in the cytosol restricts the innate immune system's recognition of viral pathogens to less conserved and highly variable structural components leaving host cells more susceptible to neurotropic viral infection.

Although DNA and RNA viruses such as HSV-1, MHV-68 and VSV exhibit lifecycle disparities, nucleic acids from each of these viruses is released into the cytoplasm during the initial stages of infection. The ability of cytoplasm bound receptors to recognize conserved viral nucleic acids coupled with their location in a common viral genome release site provides a mechanism to compensate for the lack of perception of cytosolic nucleic acids by TLRs. The localization of two newly discovered innate receptors, retinoic acid inducible gene (RIG)-I and DNA-dependent activator of interferon regulatory factors (DAI), confers the ability to survey the cytoplasm for RNA and DNA motifs, respectively, in order to assess its sterility (Furr and Marriott, 2012). The ideal intracellular positioning and specificities of RIG-I and DAI suggest a pivotal role for them in innate immune recognition and downstream signaling during viral infections.

RIG-I is comprised of a C-terminal DExD/H box RNA helicase domain which specifically detects 5'triphosophorylated single-stranded or double-stranded RNA and an N-terminal caspase activation and recruitment domain (CARD) that mediates downstream signaling events (Kato et al., 2005). Binding of the PAMP to the C-terminal helicase domain induces a conformational change that separates the RIG-I

repressor domain from the N-terminal CARD (Rustagi and Gale, 2014). TRIM25 and other chaperone proteins interact with the newly exposed N-terminal CARD and facilitate the transport of RIG-I to the mitochondrially associated endoplasmic reticulum (Rustagi and Gale, 2014). Interactions between the N-terminal CARD of RIG-I with the CARD of the adaptor molecule, mitochondrial antiviral signaling (MAVS), which is also known as interferon-beta promoter stimulator 1 (IPS-1), leads to the assembly of a signaling complex which is responsible for the activation of nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and interferon regulatory factors (IRF) 3 and 7 (IRF3 and IRF7) (Levy et al., 2011).

DAI, whose genes were first identified in tumor stromal tissue, possesses two DNA binding domains with an affinity towards double-stranded DNA in its canonical helical formation (B-DNA) (Takaoka et al., 2007). When cytosolic DNA is recognized, a complex forms between the D3 region of the DNA binding domain, B-DNA, Z_α and Z_β which leads to a conformational change that fosters the recruitment of TANK-binding kinase 1 (TBK1) and IRF3 (Wang et al., 2008). TBK1-dependent serine and threonine residue phosphorylation promotes further downstream signaling via activation of additional TBK1 and IRF3 molecules (Wang et al., 2008).

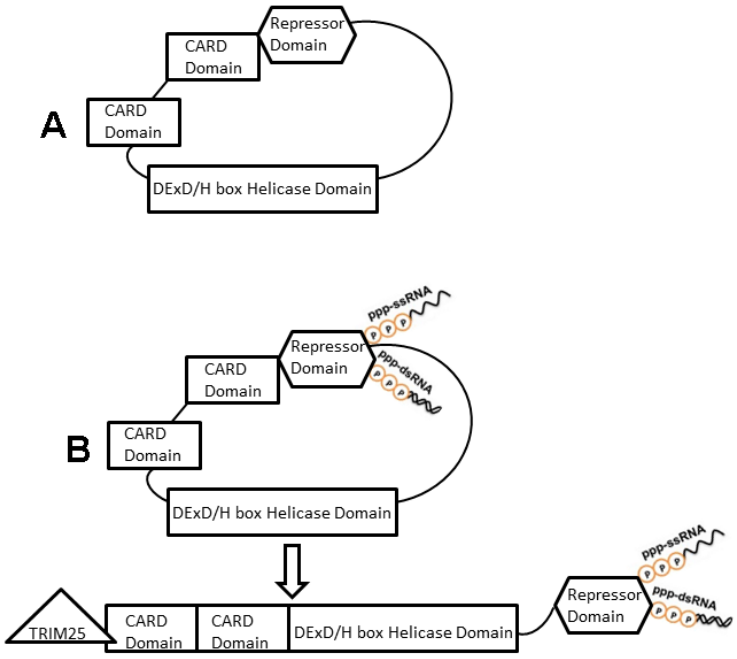

Figure 1: Native and active conformations of RIG-I. In the absence of a RIG-I ligand, the N-terminal CARD domain is kept inactive and sequestered from chaperone molecules by a repressor domain (A). Binding of 5'triphosphorylated single-stranded or double-stranded RNA to the repressor domain induces a conformational change that results in the separation of the repressor domain from the CARD domain (B). Separation of the repressor domain creates binding sites for TRIM25 and other chaperone molecules on the CARD domain.

1.5 RIG-I and DAI Expression and Functionality in Non-glial Cell Types

Differential RIG-I expression was suspected to be the source of disparities between the functional roles of two distinct human dendritic cell subsets, $CD1a^+$ and $CD1a^-$. $CD1a^+$ dendritic cells contribute to inflammatory responses by secreting an array of cytokines that attract immune cells to compromised sites to perform their effector functions (Szatmari et al., 2004). Conversely, the $CD1a^-$ subset employs phagocytosis to eliminate apoptosed cells and debris post-infection in order to promote homeostatic return and hinder any additional recruitment of proinflammatory immune cells (Szatmari et al., 2004). Transfecting and infecting both dendritic cell subtypes with poly

I:C and influenza A virus, respectively, revealed that expression of RIG-I mRNA and protein was significantly higher in $CD1a^+$ than $CD1a^-$ cells (Szabo et al., 2012). As expected based on the RIG-I expression profiles obtained from these cells, signaling through RIG-I leading to the downstream production of IFN-β was also augmented in $CD1a^+$ dendritic cells upon stimulation with RNA virus and synthetic ligands (Szabo et al., 2012). This data suggests that RIG-I functionality and expression is cell type specific and directly coincides with the effector functions of a given cell. Thus, RIG-I expression should be most pronounced in cytokine competent cells capable of limiting viral dissemination, like $CD1a^+$ dendritic cells, in order to permit early detection and exploitation of antiviral effector functions.

In addition to preferential expression by certain dendritic cell subsets, RIG-I expression following viral or ligand challenge has also been identified in two human epithelial cell lines, HEK293 and A549, and primary cortical neurons (Sirén et al., 2006; Nazmi et al., 2011). HEK293 cells were transfected with either a RIG-I or TLR3 expression plasmid and a fluorescent reporter under the control of the IFN-β promoter in order to confirm that the expressed RIG-I was veritably functional and solely responsible for the observed IFN-β production during RNA viral challenge (Sirén et al., 2006). Luciferase data obtained following infection with influenza A virus, a known stimulus for RIG-I activation, revealed enhanced activation of the IFN-β promoter, which was amplified in a multiplicity of infection (MOI)-dependent fashion when cells were transfected with the RIG-I expression plasmid (Sirén et al., 2006). Enhanced luciferase activity was absent following transfection with the TLR3 expression plasmid,

indicating that intact signaling through RIG-I is responsible for the secretion of antiviral cytokines by HEK293 cells in response to influenza A infection (Sirén et al., 2006).

The presence of a functional RIG-I receptor is not only advantageous in response to common viruses, like influenza A, but also in abrogating other infections like JEV and VSV, which are known to cause encephalitis. In the absence of intact RIG-I signaling, MEFs infected with GFP VSV emitted a significantly greater fluorescence signal than those obtained from wild-type MEFs suggesting that RIG-I functionality is absolutely critical for attenuating RNA viral replication and dissemination (Kato et al., 2005). Similarly, abolition of RIG-I signaling in mouse neuroblastoma Neuro2a (N2a) cells permitted enhanced JEV replication as evidenced by increased production of virally-derived NS 5 protein (Nazmi et al., 2011). The contribution of RIG-I knockdown to the enhancement of viral pathogenesis can be attributed to the attenuation of IFN-β expression following RNA viral challenge.

Fluorescence resonance energy transfer (FRET) analysis of HeLa cells, a human epithelial cell line, stimulated with fluorescently tagged synthetic DAI ligands demonstrated that signaling through HeLa-expressed DAI was not only functional, but also specific to dsDNA ligands (Takaoka et al., 2007). As expected, the binding of B-DNA to DAI was the only interaction that led to a FRET signal thus authenticating DAI's ability to specifically bind its ligand in HeLa cells (Takaoka et al., 2007). Augmentation of DAI expression was observed in MEFs, L929 fibroblasts and HeLa cells following transfection with B-DNA (Takaoka et al., 2007). Active signaling through DAI, which is itself an ISG, acts in a feed-forward manner to amplify DAI expression (Rustagi and Gale, 2014). The observed upregulation of DAI expression in

these cells following stimulation with a known DAI ligand indicates entrance into the characteristic PRR positive feedback loop which can only be set in motion if signaling through DAI is functional.

The expression of a functional DAI receptor is particularly advantageous in attenuating infections with the major causative agents of viral meningitis and encephalitis. The DNA composition of the HSV-1 genome is central to DAI's ability to detect challenge with HSV-1 and other herpesvirus family members because the D3 region of its binding domain is specific for DNA. Silencing RNA (siRNA)-mediated knockdown of DAI in RAW264.7, a murine macrophage cell line, and L929 cells resulted in attenuated IFN-β expression following HSV-1 challenge (Takaoka et al., 2007). The knockdown of DAI expression in HepG2, a human hepatocarcinoma cell line, contributed to enhanced HSV-1 viral replication, which was demonstrated by plaque assay (Pham et al., 2013). The production of HSV-1 immediate early and early proteins, which are notoriously good at counteracting innate antiviral host defenses was also significantly amplified in HepG2 cells whose DAI signaling was abrogated (Pham et al., 2013). It is no surprise that the expression of a functional DAI receptor has been reported in so many cell types since intact signaling through this receptor appears to be critical for producing significant quantities of IFN-β, which functions to attenuate viral dissemination and limit inappropriate inflammatory responses to DNA viral infections.

1.6 RIG-I and DAI Expression and Functionality in Astrocytes and Microglia

The administration of various doses of the synthetic RIG-I ligand, 5'triphosphorylated single-stranded and double-stranded RNA, into primary murine astrocytes has been recognized as a potent stimulus for augmenting RIG-I protein

expression (de Rivero Vaccari et al., 2012). In addition to RIG-I expression in primary murine astrocytes, the expression of RIG-I in human astrocyte-like cells has also been reported. Western blot analysis has revealed that infection with the neurotropic RNA virus, Chikungunya virus (CHIKV), induced RIG-I mRNA and protein expression in the cytosol of U-87 MG cells, a human astrocytic cell line derived from a glioblastoma (Abraham et al., 2013). Expression of RIG-I in U-87 MG cells increased with increasing CHIKV MOI (Abraham et al., 2013).

The functionality of the RIG-I receptor expressed by astrocytes was authenticated by the completion of two necessary downstream signaling events. The exploitation of IRF3's capacity for transcriptional activation of IFN-β cannot occur without phosphorylation, which precedes its translocation to the nucleus (Rustagi and Gale, 2004). The levels of phosphorylated IRF3 were significantly enhanced in response to transfection with a ligand specifically and exclusively detected by RIG-I (de Rivero Vaccari et al., 2012). If signaling through RIG-I is intact, an increase in phosphorylated IRF3 should also lead to an increase in the number of IFN-β transcripts. As expected, stimulation with RIG-I ligands induced rapid secretion of type I interferons by astrocytes (Paul et al., 2007).

The expression of RIG-I has also been observed in murine microglia. Furr and colleagues (2008) detected expression and production of RIG-I mRNA and protein, respectively, by primary murine microglia that occurred in the absence of stimulation by virus or ligands. RIG-I protein expression was significantly elevated above constitutive levels in a MOI-dependent fashion following infection of primary murine microglia with Sendai virus (SeV), a RNA virus (Furr et al., 2008). Infection and

transfection with JEV and poly I:C, respectively, led to a significant upregulation in RIG-I production in the murine microglia cell line BV-2 (Jiang et al., 2014). Functionality of the RIG-I receptor in microglia was initially demonstrated in response to synthetic RIG-I ligands. Similar to the trend observed in astrocytes, poly I:C transfection has been shown to induce robust IFN-β production by microglia in a RIG-I-dependent fashion (Paul et al., 2007). Obstruction of RIG-I signaling in BV-2 cells permitted significantly enhanced JEV propagation (Jiang et al., 2014). The inability to limit RNA viral dissemination in the absence of RIG-I is attributed to inadequate production of TNF-α, IL-6 and other proinflammatory mediators as well as failure to establish an antiviral state due to deficient IFN-β production.

The expression of DAI was markedly enhanced in astrocytes and microglia following stimulation with poly(dA:dT) (Furr et al., 2011). Silencing RNA (siRNA)-mediated knockdown of DAI signaling in microglia significantly hindered the production of potent inflammatory molecules in response to HSV-1 challenge (Furr et al., 2011). Similarly, astrocytes infected with HSV-1 produced less TNF-α and IL-6 when signaling through DAI was abrogated (Furr et al., 2011).

Expression of RIG-I and DAI has been identified in the two predominating CNS immune cell populations, astrocytes and microglia. Obstruction of RIG-I and DAI signaling pathways by siRNA in murine astrocytes and microglia significantly reduced the expression and production of proinflammatory mediators following viral infection or ligand transfection. Insufficient production of proinflammatory mediators and type I interferons contributes to enhanced viral replication thus making the host more susceptible to neurotropic viral dissemination.

1.7 Indirect Detection of DAI ligands by RIG-I via RNA Polymerase III

The documentation of RIG-I activation during Hepatitis C virus (HCV), VSV and a number of other RNA viral infections is not surprising considering its specificity for RNA motifs (Samanta et al., 2006). However, recently it has been demonstrated that infection with a DNA virus, Epstein-Barr virus (EBV), can precipitate RIG-I activation in B lymphocytes (Samanta et al., 2006). The phenomenon of RIG-I recognition of atypical PAMP, like those of EBV origin, has been reported in a number of other cell types. Western blot analysis has revealed that RIG-I protein expression is augmented in N20.1 cells, a murine oligodendritic-like cell line, following infection with MHV-68 (Li et al., 2010). The observed upregulation in RIG-I expression in N20.1 cells also appears to be functionally significant for antiviral defenses because siRNA-mediated knockdown of RIG-I significantly attenuates the production of type I interferons in response to MHV-68 infection (Li et al., 2010). Unlike the slew of RNA viruses classically associated with RIG-I activation, the genomes of EBV and MHV-68 are comprised of DNA, which was previously only deemed recognizable by specific DNA sensors such as DAI.

A number of studies employing siRNA to disrupt DAI signaling have verified that the observed enhancement of RIG-I expression following DNA viral challenge is functionally relevant and contributes to a host's artillery of antiviral defense mechanisms. DAI knockdown in HepG2 cells did not completely abolish IRF3 phosphorylation which permitted downstream IFN-β production and expression in response to transfection with B-DNA (Pham et al., 2013). Similarly, Takaoka and colleagues (2007) demonstrated that obstruction of signaling through DAI by siRNA in

L929 fibroblasts retained the ability to produce type I interferons in response to HSV-1 infection.

The ability of synthetic or virally-derived ligands of specific DNA sensors, like DAI, to augment the expression and activation of RIG-I suggests the existence of an alternative mechanism that permits the indirect detection of DNA motifs by RIG-I. RNA polymerase III has been proposed as a putative mediator in atypical RIG-I signaling due to its ability to transcribe a number of small RNAs including but not limited to 5s ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) (Ablasser et al., 2009). Unlike RNA polymerase I and II, the majority of RNA polymerase III activity is sequestered to the cytoplasm where RIG-I resides (Jaehning and Roeder, 1977). The identified functions of RNA polymerase III coupled with the location of their targets led to the hypothesis that RNA polymerase III converts cytoplasmic DNA into a RNA ligand that can be served up and recognized by RIG-I.

This hypothesis is supported by Ablasser and colleagues (2009) who demonstrated that RNA polymerase III catalyzes the transcription of EBV encoded RNAs from AT-rich dsDNA sequences. The conversion of DNA viral genome components into RNA sequences licenses the induction of RIG-I signaling in response to a DNA virus like EBV (Ablasser et al., 2009). In vitro inhibition of RNA polymerase III via ML-60218, a pharmacological inhibitor of RNA polymerase III activity, exemplified this enzyme's pivotal role in permitting atypical DNA viral recognition by RIG-I. Inhibition of RNA polymerase III in RAW264.7 cells, a murine macrophage-like cell line, resulted in a significant downregulation in IFN-β production in response to infection with two different DNA viruses, HSV-1 and adenovirus (Chiu et al., 2009).

RAW264.7 cells were also infected with a RNA virus, SeV, to confirm that the inhibitory functions of ML-60218 were specific for RNA polymerase III-dependent transcription of ligands detectable by RIG-I and not the function of the RIG-I receptor itself (Chiu et al., 2009). Pretreatment with ML-60218 did not affect the competency of RAW264.7 cells to induce IFN-β production in response to Sendai viral infection, which indicates that ML-60218 does not abrogate direct recognition of RIG-I ligands and that the requirement for RNA polymerase III by RIG-I is restricted solely to the perception of DNA viral challenges (Chiu et al., 2009).

1.8 Broadening the Classic Role of RIG-I in Viral Infections

Viral detection by the host can be particularly challenging because viruses like VSV, a known inducer of fatal murine encephalitis, and many others encode their own polymerases for genome replication (Hastie et al., 2013). Virally derived polymerases are notoriously error-prone and contribute to the evasion of host detection by inducing mutations in a number of structural elements. Unlike the proteins comprising the outer surface of virions, which can be mutated and still permit viral survival, mutations in the viral genome would be detrimental to the virus. The viral genome possessed by HSV-1, VSV and other agents of encephalitis will be released into the cytosol at some point during the viral lifecycle, regardless of where genome replication takes place (Salameh et al., 2012; Hastie et al., 2013). The ability to detect conserved viral nucleic acids in the cytoplasm, a prevalent viral genome release site, amplifies the contribution of PRRs towards host antiviral defense mechanisms.

In spite of the fact that redundancy has been suggested to be a common theme in innate immune signaling, the induction of robust type I interferon production in

response to DNA viral infection was formerly considered exclusive to DAI. Since the primary causative agent of viral encephalitis is a DNA virus, HSV-1, expanding the ability to perceive conserved DNA viral components by receptors other than DAI would be exceptionally valuable to host cells (Jmor et al., 2008). Increasing the repertoire of PRRs capable of detecting DNA ligands will intensify antiviral responses and potentially abrogate severe neurological manifestations, like HIV-induced encephalitis. The sequestration of RNA polymerase III activity to the cytoplasm as well as its ability to convert classic DAI ligands into a form detectable by RIG-I suggests that it plays a pivotal role in expanding the functions of RIG-I beyond RNA viral recognition.

Signaling through RIG-I is central for production of the protective type I interferon subset, IFN-β. The secretion of IFN-β not only induces the expression of a number of ISGs with potent antiviral functions, but also promotes the recruitment of natural killer (NK) cells, which are particularly good at inducing apoptosis in virally-infected cells with minimal bystander damage (Carrero 2013). Administration of IFN-β to RIG-I -/- MEFs and lung fibroblasts effectively reverses their enhanced susceptibility to neurotropic VSV infection (Kato et al., 2005). Employment of ML-60218 in RAW264.7 cells led to a marked reduction in IFN-β secretion in response to HSV-1 infection (Chiu et al., 2009). RNA polymerase III inhibition precludes RIG-I activation in response to DNA viral challenge as a direct consequence of the failure to convert DNA sequences into a RNA intermediate recognizable by RIG-I. Abrogating RIG-I signaling via direct or indirect means consequently attenuates production of the potent antiviral cytokine, IFN-β, which promotes extensive viral dissemination.

1.9 A Putative Role for RIG-I in Non-viral Infections

In addition to the direct and indirect recognition of RNA and DNA motifs, respectively, RIG-I is also suspected to play a role in promoting protective host responses to bacterial and protozoan infections. Intracellular DNA from *Mycobacterium tuberculosis*, *Legionella pneumophila* and the protozoan responsible for malaria, *Plasmodium falciparum*, have been recognized as potent stimuli for PRR activation and signal enhancement (Manzanillo et al., 2012; Chiu et al., 2009; Sharma et al., 2011). Since DAI is specifically activated by DNA PAMPs it is capable of directly detecting bacterially- and potentially protozoan-derived nucleic acids. RIG-I on the other hand can only be directly activated by RNA motifs and thus relies on RNA polymerase III for recognition of these pathogens. RNA polymerase III inhibition promoted the growth of *Legionella pneumophila*, which was a direct consequence of obstructing indirect RIG-I activation and the subsequent production of antipathogenic mediators, like IFN-β (Chiu et al., 2009). The utilization of RNA polymerase III by RIG-I not only promotes crosstalk between RIG-I and DAI, but also contributes to the fulfillment of a more general role by conferring innate immunity to pathogens other than just RNA viruses.

1.10 Hypothesis of the Present Study

In the present study we have tested the hypothesis that RIG-I has a broader importance than previously thought due to its ability to recognize not only RNA viral motifs, but also DNA viral motifs and bacterial pathogens through the employment of RNA polymerase III. In a number of former studies, RIG-I has been demonstrated to indirectly detect viral DNA by utilizing the enzymatic functions of RNA polymerase III. Since the expression of a functional RIG-I receptor by microglia and astrocytes has

already been established, we sought to determine if RNA polymerase III plays the same role in microglia and astrocytes as it does in fibroblasts, macrophages and other cell types.

Employment of siRNA and a pharmacological RNA polymerase III inhibitor to directly or indirectly, respectively, interfere with RIG-I signaling permits enhanced viral replication in prominent immune cells like macrophages and dendritic cells as well as non-immune cells like fibroblasts. The pronounced viral dissemination observed in an array of cell types with obstructed RIG-I signaling is hypothesized to be a consequence of insufficient IFN-β production. The downstream activities of IFN-β function to impede virtually all stages of the viral lifecycle and are critical for limiting viral dissemination.

Given the importance of IFN-β in establishing an antiviral state within host cells, we sought to test the hypothesis that inhibition of RNA polymerase III contributed to enhanced viral replication as a direct consequence of insufficient IFN-β production by RIG-I. To emphasize that functional RNA polymerase III is required for RIG-I-mediated production of IFN-β in response to DNA motifs, we stimulated cells with DNA viruses and ligands in the presence and absence of RNA polymerase III inhibition. With these findings, we sought to demonstrate that RNA polymerase III contributes to the enhanced production of IFN-β as a result of permitting indirect RIG-I signaling. Conversely, we sought to demonstrate that inhibition of RNA polymerase III led to enhanced viral replication that can be directly correlated to inadequate production of IFN-β.

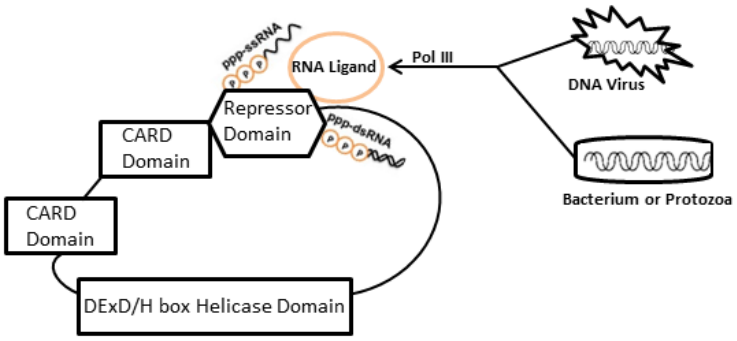

Figure 2: Proposed roles of RIG-I. RIG-I, which was previously only implicated in RNA viral infections due to its specificity for 5'triphosphorylated single-stranded and double-stranded RNA ligands, may have a broader role than originally thought through the employment of RNA polymerase III. RNA polymerase III is hypothesized to convert DNA ligands of viral, bacterial or protozoan origin into a RNA ligand recognizable by RIG-I. The indirect detection of DNA pathogens by RIG-I leads to the activation of RIG-I signaling pathways and ultimately to RIG-I-dependent production of proinflammatory mediators and type I interferons in response to pathogens not traditionally detected by RIG-I.

CHAPTER 2: MATERIALS AND METHODS

2.1 Isolation and Culture of Primary Murine Astrocytes

Mixed glial cultures were obtained from the brains of murine neonates 1-3 days after birth. Skulls from C57BL/6J neonates were surgically detached from the body with sterile scissors. Surgical forceps were used to dissect the brain, which was then finely minced and forced through a sieve with 0.25% Trypsin-1 mM EDTA (Gibco, Grand Island, NY). Trypsinized brain homogenates were combined with RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and gentamicin and spun at 500 *g* for 3 minutes. Supernatant was discarded and mixed glial cultures were resuspended in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and gentamicin in a T75 tissue culture treated flask (Corning Life Sciences, Acton, MA). Approximately 10-12 days after isolation, RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and gentamicin was removed. Cultures were washed with serum-free RPMI1640 supplemented with gentamicin in order to remove residual FBS and then incubated with 0.25% Trypsin-1 mM EDTA (Gibco, Grand Island, NY) diluted 1:4 in serum-free RPMI 1640 supplemented with gentamicin (Saura et al., 2003). Murine astrocytes, which completely detached approximately 20 minutes after mild trypsinization, were collected and resupended in supplemented RPMI 1640. Astrocytes were spun at 33 RPM for 3 minutes to remove residual trypsin. Precipitated cells were transferred to

T75 flasks and resuspended in supplemented RPMI 1640. Through histochemical staining for the microglia surface marker CD11b, the intact layer of cells that remained adhered to the bottom of the original flask following the removal of the astrocyte layer was demonstrated to be >98% microglia (Saura et al., 2003). The large population of pure microglia absent from the astrocytic layer indicates successful separation of the astrocytes from the microglia. Astrocytes were determined to be >96% pure based on morphological characteristics.

2.2 Source and Propagation of Cell Lines

EOC 13.31 (ATCC® CRL-2468), an immortalized murine microglia cell line, was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% FBS, gentamicin, and 20% LADMAC conditioned media derived from LADMAC (ATCC number CRL-2420), a murine macrophage-like cell line that secretes colony stimulating factor-1 (CSF-1). EOC 13.31 cells are classified as microglia due to the presence of CD11b and other microglia-specific cell surface markers (Walker et al., 1995).

2.3 Source and Propagation of Viruses

The following viruses were used in this study: VSV-ΔM51-GFP, VSV (Indiana Strain), Herpes Simplex Virus-1 (HSV-1) (MacIntyre strain; ATCC, VR-539), and Murine gammaherpesvirus-68 (MHV-68). VSV-ΔM51-GFP is characterized by the insertion of the green fluorescent protein (GFP) ORF at position 5 of the viral genome and a methionine deletion in the M protein (Wollmann G, 2010). Wild-type and mutant VSV viral titers were quantified using a standard plaque assay of serial dilutions in baby hamster kidney (BHK-21) cells (ATCC, CCL-10). Plaque assays to determine HSV and

MHV-68 titers were executed in African green monkey kidney cells (Vero) (ATCC, CCL-81). Viral titers are expressed as CFU/ml.

2.4 RNA Polymerase III Inhibition

InSolution RNA polymerase III inhibitor (N-(1-(3-(5-Chloro-3-methylbenzo[b]thiophen-2-yl-1-methyl-1H-pyrazol-5-yl))-2-chlorobenzenesulfonamide, 2-Chloro-N-(3-(5-chloro-3-methylbenzo[b]thiophen-2-yl)-1-methyl-1H-pyrazol-5-yl)benzenesulfonamide) was purchased from Calbiochem (San Diego, California). A 25 uM solution of the RNA polymerase III inhibitor was combined with supplemented culture media and added to seeded cells once 60% or greater confluency was achieved. Cells were incubated at 37°C in 5% CO_2 for 24 hours.

2.5 Viral Infections

Following RNA polymerase III inhibition, viral stocks were diluted in serum-free culture media supplemented with gentamicin until the desired multiplicity of infection (MOI) was achieved. Cells were washed with serum-free culture media supplemented with gentamicin prior to infection in order to remove residual FBS. At 1 hour post-infection (h.p.i.), media containing non-adsorbed viral particles was removed and replaced with a 25 uM solution of the RNA polymerase III inhibitor in serum-free culture media or serum-free media alone. Culture supernatants, total cellular RNA, and protein were collected 3-24 h.p.i.

2.6 Transfection Reagents

The synthetic B-DNA analog, poly(deoxyadenylic-deoxythymidylic) acid sodium salt (Poly(dA:dT)), 5’ triphosphate double stranded RNA (5’ ppp-dsRNA), and LyoVec transfection reagent were purchased from Invivogen (San Diego, CA). All

reagents were received lyophilized and were rehydrated according to the manufacturer's protocol.

2.7 Ligand Transfection

Following RNA polymerase III inhibition, B-DNA and 5'ppp-dsRNA (PPP) were complexed with LyoVec to achieve a 3 ug/ml or 4 ug/ml concentration, respectively. LyoVec/ligand complexes were added directly to culture media in a 1:20 ratio. Cells were incubated at 37°C in 5% CO_2. Culture supernatants and total RNA were collected at 9 or 24 hours post transfection.

2.8 RNA Isolation

Cellular RNA was isolated from primary murine astrocytes and EOC 13.31 cells according to a modified version of the protocol provided by the TRIzol reagent (Ambion) manufacturer. The protocol modifications are described below. After the addition of isopropanol in the RNA precipitation phase, samples were placed in -80°C for a minimum of 30 minutes. Washed samples were centrifuged for 15 minutes at 13,500 *g*. Upon resuspension of the pellet in 30 ul HyPure molecular biology grade water (Thermo Scientific), RNA concentration and purity was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). All samples were further diluted to achieve a standard concentration of 1.0-2.0 ug/ul.

2.9 Reverse Transcription

A master mix comprised of equal volumes, 12.5% each of 10 mM dNTP (GenScript), random hexamer (Integrated DNA Technologies), 0.1 M DTT (Invivogen) and M-MLV reverse transcriptase (Promega) and 50% M-MLV reverse transcriptase 5X Reaction Buffer (Promega) was combined with 12 ul of diluted RNA. The RNA-

master mix complex was incubated in a 37°C water bath for 1.5 hours. Reverse transcription was terminated by incubation at 95°C for 3 minutes and the subsequent addition of 95% ethanol containing 2% 10X sodium acetate. Samples were kept in -80°C for a minimum of 30 minutes before the final centrifugation and resuspension.

2.10 Semi-quantitative PCR

Semi-quantitative PCR was used to demonstrate the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), MHV-encoded ORF65 and IFN-β by astrocytes and EOC 13.31cells prior to quantifying variations in their expression via Real-Time PCR. Following cDNA resuspension in 50 ul HyPure molecular biology grade water, 5 ul of template DNA was combined with 0.5 ul GoTaq DNA polymerase (Promega), 1 ul each of 10 mM dNTP (GenScript) and the forward and reverse primers (Integrated DNA Technologies), and 10 ul each of 25 mM $MgCl_2$ (Promega) and 5X GoTaq Flexi buffer (Promega).

PCR primers used to amplify cDNA derived from cellular mRNA were as follows:

GAPDH Forward: 5'-CCA TCA CCA TCT TCC AGG AGC GAG-3'

GAPDH Reverse: 5'-CAC AGT CTT CTG GGT GGC AGT GAT-3'

IFN-β Forward: 5'-GGT GGA ATG AGA CTA TTG TTG-3'

IFN-β Reverse: 5'-AGG ACA TCT CCC ACG TC-3'

MHV ORF65 Forward: 5'-ATG CTC CAG AAG AGG AAG GGA CAC-3'

MHV ORF65 Reverse: 5'-TTG GCA AAG ACC CAG AAG AAG CC-3'

All primers were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD) based on their location in different exons of

the genomic sequences and their lack of significant homology to sequences present in GenBank (National Center for Biotechnology Information, Bethesda, MD). The identity of the polymerase chain reaction (PCR) amplified fragments were further verified by size comparison with DNA standards (Promega).

Initially, samples underwent three cycles of a 94°C 45 second denaturation phase, 60°C 1.5 minute annealing phase, and a 72°C 2.5 minute extension phase. The remaining 27 (GAPDH) and 30 (IFN-β) cycles were comprised of a 94°C 35 second denaturation, 60°C 1.15 second annealing, and a 72°C 1.5 minute extension. Gel electrophoresis was used to visualize PCR products.

2.11 Real-Time PCR

A master mix was prepared by combining 10 ul 2X QuantiTect SYBR Green PCR master mix (Qiagen) with variable amounts of the forward primer, reverse primer and RNase-free water (Qiagen) in order to achieve a final primer concentration of 400 nM. 2 ul of template cDNA was then added to a 96-well plate containing the master mix. The plate was loaded into a 7500 Fast Real-Time PCR machine (Applied Biosystems) which was programmed with an initial 15 minute denaturation phase at 95°C followed by 40 cycles alternating between a 15 second segment at 95°C and a 1 minute segment at 60°C. Variations in mRNA expression were quantified using the comparative C_T method, $\frac{2^{\text{Cont.G3 Ct}-\text{Sample G3 Ct}}}{2^{\text{Cont.gene ofinterest Ct}-\text{Sample gene of interest Ct}}}$, which calculates the expression of the gene of interest relative to the expression of the GAPDH housekeeping gene.

2.12 Quantification of IFN-β Secretion

A commercially available mouse IFN-β ELISA kit (BioLegend) was used for the detection and quantification of IFN-β secretion from primary murine astrocytes and EOC 13.31. Pre-coated plates were used according to the manufacturer's protocol.

2.13 Western blot analyses

Western blot analyses were performed as described previously by our laboratory (Bowman et al., 2003; Rasley et al., 2006; Sterka and Marriott, 2006; Sterka et al., 2006) to detect the presence of endogenous total and phosphorylated IRF-3, various VSV virion proteins and VP16, an HSV-1 tegument protein, in primary murine astrocytes and EOC 13.31 cells following viral challenge. The primary antibodies used for detection of total and phosphorylated IRF3 were a purified rabbit monoclonal antibody directed against IRF-3 (Cell Signaling Technology) and a purified rabbit monoclonal antibody specific for IRF-3 that has been phosphorylated at Ser396 (Cell Signaling Technology). Western blot analyses for total and phosphorylated IRF-3 were carried out according to the protocol provided by Cell Signaling Technology and a goat anti-rabbit IgG was used as the secondary antibody (SouthernBiotech). The primary antibodies used to detect viral proteins were a purified mouse monoclonal antibody directed against HSV-1 and HSV-2 VP16 (abcam) and 1:10,000 rabbit polyclonal anti-VSV antibodies directed against VSV virion proteins in 5% BSA and 1% of 2% sodium azide. 1X TBST with 5% w/v nonfat dry milk was used as the primary antibody dilution buffer, blocking buffer and secondary antibody dilution buffer. A goat anti-mouse and goat anti-rabbit IgG (SouthernBiotech) were used as the secondary antibody for protein

detection in samples probed for HSV-1 and VSV proteins, respectively. To assess total protein loading in each well, immunoblots were reprobed with a goat anti-mouse β-actin antibody (Santa Cruz Biotechnology). Total IRF-3 was normalized to β-actin expression levels and phosphorylated IRF3 was expressed relative to the levels of total IRF-3. ImageJ software (National Institutes of Health) was used for densitometric analysis.

2.14 Fluorescence Measurement

VSV-ΔM51-GFP GFP fluorescence levels were measured in EOC 13.31 using a CytoFluor multi-well plate reader (PerSeptive Biosystems) at 0, 8 and 24 h.p.i. with VSV-ΔM51-GFP. The machine was programmed with the following parameters: excitation filter of 450/50 nm, emission filter of 530/25 nm and gain=50 (Moerdyk-Schauwecker et al., 2013).

2.15 Statistical Analysis

All statistical analyses were performed using GraphPad Prism, version 5.03 for Windows (GraphPad Prism, GraphPad Software Inc. La Jolla, CA). One- or two-way analyses of variance (ANOVA) with Tukey's post-hoc test were used to analyze all results. Results are presented as the mean +/- SEM. An n of 3-4 was used for all experiments.

CHAPTER 3: RESULTS

3.1 RIG-I Signaling is Involved in Protective Antiviral Responses in EOC 13.31 Cells and Primary Cultures of Murine Astrocytes and Mixed Glial Cells.

We assayed IRF3 phosphorylation, a prerequisite of type I interferon production, and IFN-β mRNA expression and secretion to investigate whether or not signaling through RIG-I promoted antiviral responses in EOC 13.31 cells and primary cultures of murine astrocytes and mixed glial. Infection of EOC 13.31 cells with the RNA virus, VSV, stimulated the phosphorylation of IRF3 (Figure 3). To ensure that the observed phosphorylation was specific to signaling through RIG-I and not another PRR, we transfected EOC 13.31 cells and primary cultures of murine astrocytes and mixed glial with the RIG-I ligand, 5'triphosphorylated single-stranded RNA. RIG-I ligand transfection led to the induction of IFN-β mRNA expression (Figures 6A, 7A and 8A) and this expression was significantly greater than that observed in the unstimulated control. In accordance with the phosphorylated IRF3 data, we saw an upregulation in IFN-β mRNA expression by EOC 13.31 cells (Figure 9) and primary murine astrocytes (Figure 10) following VSV infection.

We also analyzed the secretion of IFN-β in order to verify that the observed message was successfully translated. Similar to the mRNA expression profiles, elevated levels of secreted IFN-β were observed in EOC 13.31 following transfection with

5’triphosphorylated single-stranded RNA (Figure 14A) or infection with VSV (Figure 16). Correspondingly, transfecting primary murine astrocytes with the RIG-I ligand also led to an increase in secreted IFN-β (Figure 15A). These findings suggest that RIG-I assumes a protective role in both primary glial and EOC 13.31 cells through the production of the potent antiviral mediator, IFN-β, when challenged with RNA ligands.

3.2 RNA Polymerase III is Essential for Maximal Anti-viral Glial Responses by Murine Glial Cells to DNA Viruses.

We have assessed the role of RNA polymerase III to begin to determine the mechanisms underlying the involvement of RIG-I in glial responses to DNA viral infection. As shown in Figures 4 and 5, pretreatment of cells with a selective RNA polymerase III inhibitor significantly attenuated IRF3 phosphorylation in EOC 13.31 cells challenged with HSV-1 and MHV-68, respectively. To ensure that this phenomenon was a consequence of abrogating indirect detection of DAI ligands by RIG-I, we transfected EOC 13.31 cells and primary cultures of murine astrocytes and mixed glial with B-DNA in the presence and absence of a RNA polymerase III inhibitor. RNA polymerase III inhibition led to a significant reduction in IFN-β mRNA expression by mixed glia (Figure 4B), EOC 13.31 (Figure 5B) and primary murine astrocytes (Figure 6B) following challenge with B-DNA. Similarly, EOC 13.31 cells infected with HSV-1 (Figure 11) and MHV-68 (Figure 13) experienced a significant reduction in IFN-β mRNA expression in the presence of the RNA polymerase III inhibitor. Like EOC 13.31 cells, the expression of IFN-β by primary murine astrocytes infected with HSV-1 was significantly attenuated when RNA polymerase III was inhibited (Figure 12).

We also quantified the secretion of IFN-β by EOC 13.31 cells and primary murine astrocytes in order to determine the effect that RNA polymerase III inhibition has on protein production. EOC 13.31 (Figure 14B) and primary murine astrocytes (Figure 15B) transfected with B-DNA experienced attenuated secretion of IFN-β in the presence of the RNA polymerase III inhibitor. Similarly, RNA polymerase III led to a reduction in IFN-β secretion by EOC 13.31 cells infected with HSV-1 (Figure 17) and MHV-68 (Figure 19) and primary murine astrocytes infected with HSV-1 (Figure 18). Taken together, these findings suggest that stimulation of primary glia and EOC 13.31 cells with DNA ligands requires RNA polymerase III functionality in order to achieve maximum expression and secretion of IFN-β.

3.3 The Effects of RNA Polymerase III Inhibition are Specific to the Detection of DNA Ligands by RIG-I.

To confirm the specificity of the effect of pharmacological RNA polymerase III inhibitors on viral DNA motif-induced type I interferon responses, we have assessed their effect on IRF3 phosphorylation and IFN-β production induced by VSV and specific RNA ligands for RIG-I. RNA polymerase III inhibition did not attenuate phosphorylation of IRF3 in EOC 13.31 cells stimulated with the RNA virus, VSV (Figure 3). As shown in Figures 6B, 7B and 8B, the intracellular introduction of the RIG-I ligand, 5’triphosphorylated single-stranded RNA, elicits a marked increase in the expression of mRNA encoding IFN-β in cultures of primary mixed glial cells, isolated primary astrocytes or EOC 13.31 cells and unlike B-DNA transfection, is unchanged by the presence of a RNA polymerase III inhibitor. In accordance with the IFN-β mRNA

expression profiles, RNA polymerase III inhibition does not alter the secretion of IFN-β by EOC 13.31 (Figure 14A) and primary murine astrocytes (Figure 15A) transfected with 5'triphosphorylated single-stranded RNA. Additionally, RNA polymerase III inhibition has no effect on IFN-β secretion by EOC 13.31 cells following stimulation with VSV (Figure 16). These results indicate that the side effects of RNA polymerase III inhibition are only applicable to DNA viral challenge.

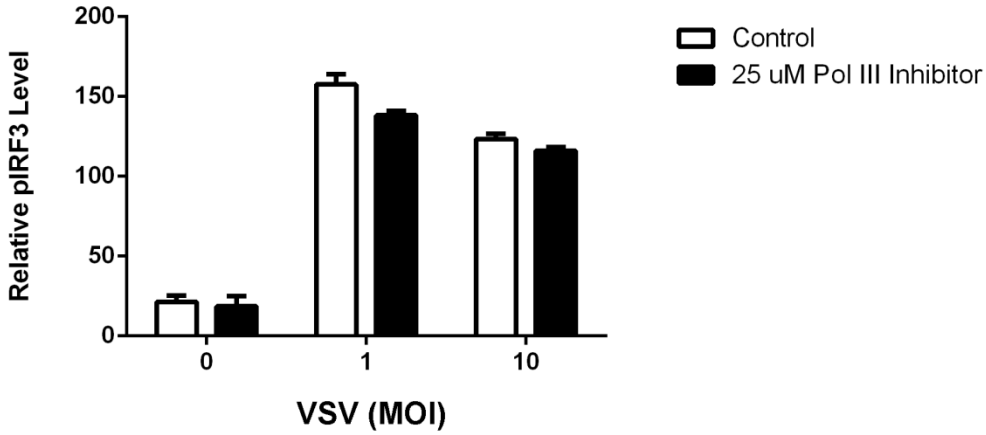

Figure 3: Phosphorylation of IRF3 in EOC 13.31 is enhanced and unaffected by the presence of a RNA polymerase III inhibitor following infection with WT VSV. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with WT VSV at MOI 1 and 10. At 24 h.p.i., whole-cell isolates were collected and pIRF3 protein expression was determined by immunoblot assay. Densitometric analysis data is presented as mean expression ± SEM, normalized to total IRF3 and β-actin.

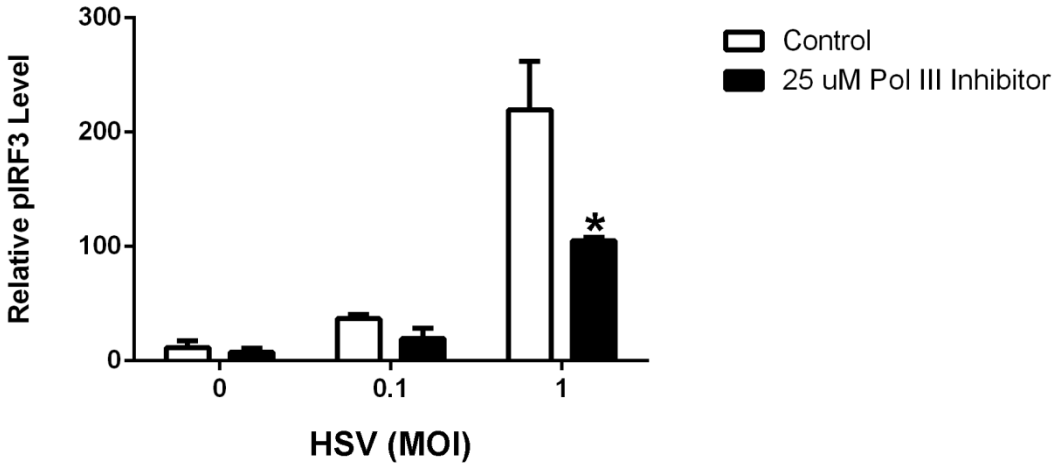

Figure 4: Phosphorylation of IRF3 in EOC 13.31 is attenuated in the presence of a RNA polymerase III inhibitor following infection with HSV-1. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with HSV-1 at MOI 0.1 and 1. At 24 h.p.i., whole-cell isolates were collected and pIRF3 protein expression was determined by immunoblot assay. Densitometric analysis data is presented as mean expression ± SEM, normalized to total IRF3 and β-actin. The asterisk denotes a statistically significant difference from uninhibited cells.

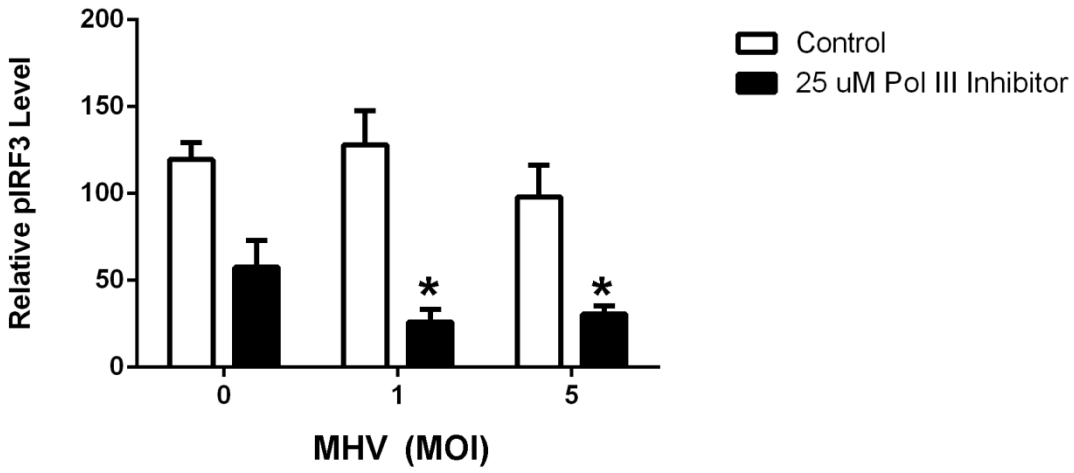

Figure 5: Phosphorylation of IRF3 in EOC 13.31 is attenuated in the presence of a RNA polymerase III inhibitor following infection with MHV-68. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with MHV-68 at MOI 1 and 5. At 24 h.p.i., whole-cell isolates were collected and pIRF3 protein expression was determined by immunoblot assay. Densitometric analysis data is presented as mean expression ± SEM, normalized to total IRF3 and β-actin. The asterisk denotes a statistically significant difference from uninhibited cells.

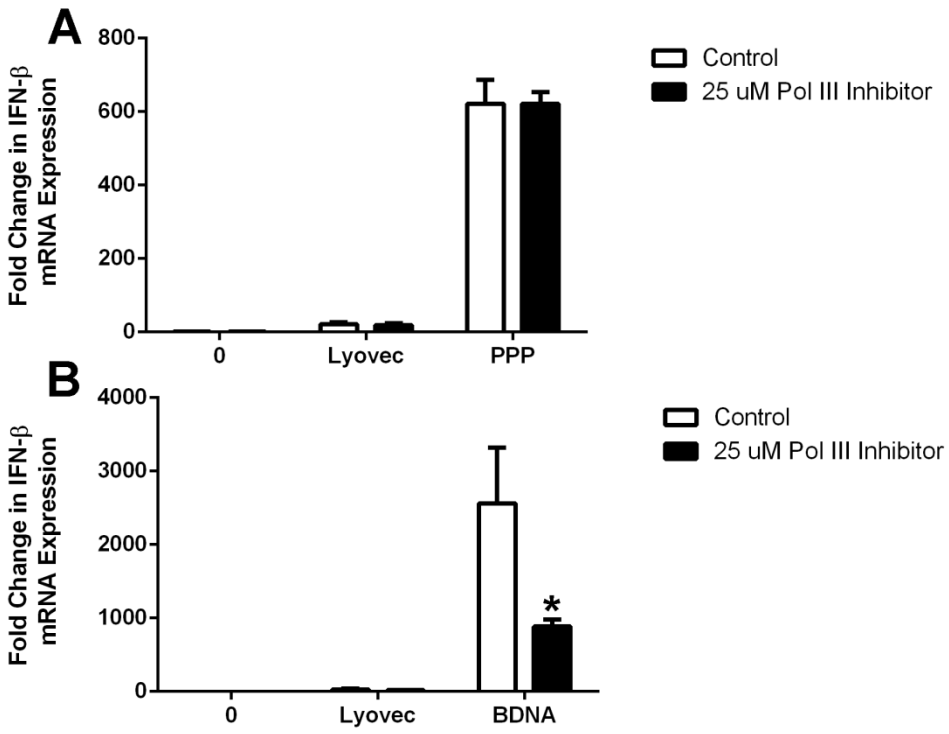

Figure 6: The presence of a RNA polymerase III inhibitor attenuates IFN-β expression following transfection with the DAI ligand, B-DNA, but not the RIG-I ligand, PPP, in mixed glial cells. Primary mixed glial cells were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then untransfected or transfected with 4 ug/ml PPP (Panel A) or 3 ug/ml B-DNA (Panel B). Total RNA was collected at 24 hours post-transfection and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. The asterisk denotes a statistically significantly difference from uninhibited cells.

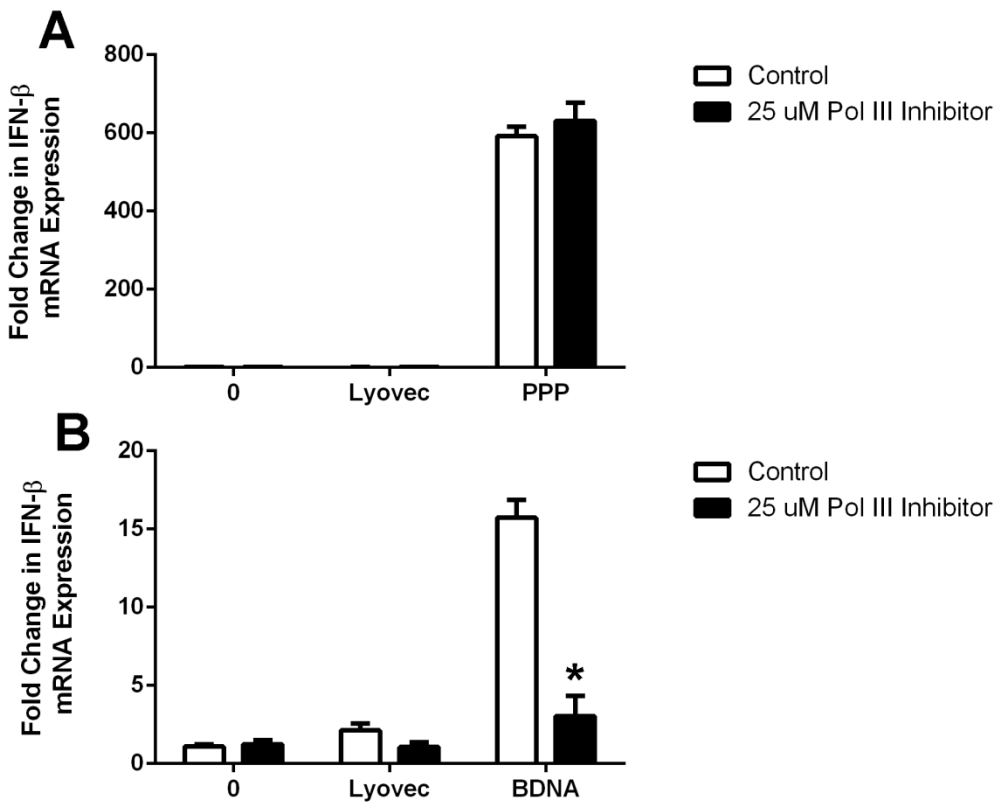

Figure 7: The presence of a RNA polymerase III inhibitor attenuates IFN-β expression following transfection with the DAI ligand, B-DNA, but not the RIG-I ligand, PPP, in EOC 13.31. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then untransfected or transfected with 4 ug/ml PPP (Panel A) or 3 ug/ml B-DNA (Panel B). Total RNA was collected at 24 hours post-transfection and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. The asterisk denotes a statistically significantly difference from uninhibited cells.

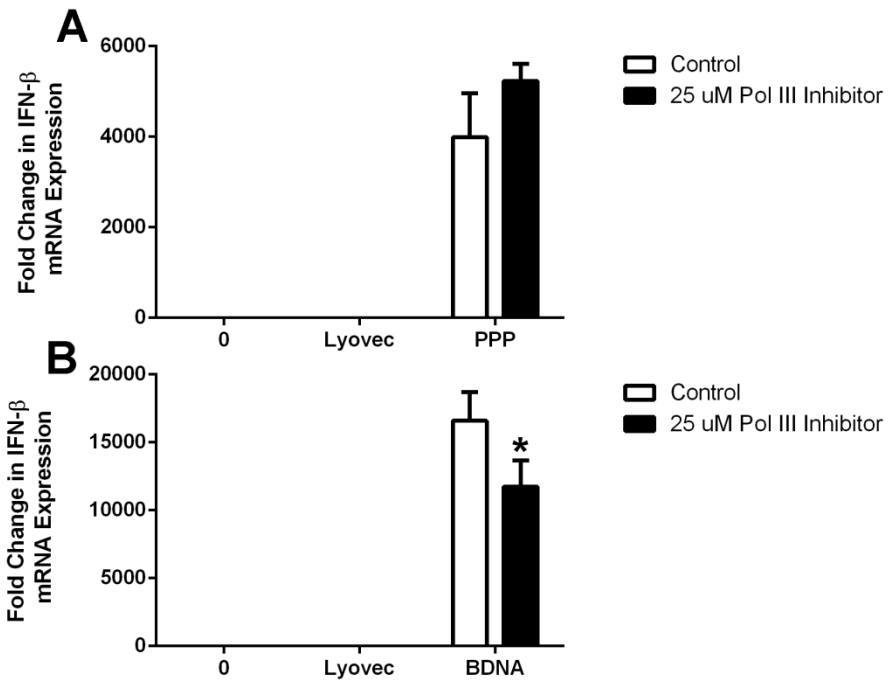

Figure 8: The presence of a RNA polymerase III inhibitor attenuates IFN-β expression following transfection with the DAI ligand, B-DNA, but not the RIG-I ligand, PPP, in astrocytes. Primary murine astrocytes were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then untransfected or transfected with 4 ug/ml PPP (Panel A) or 3 ug/ml B-DNA (Panel B). Total RNA was collected at 24 hours post-transfection and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. The asterisk denotes a statistically significantly difference from uninhibited cells.

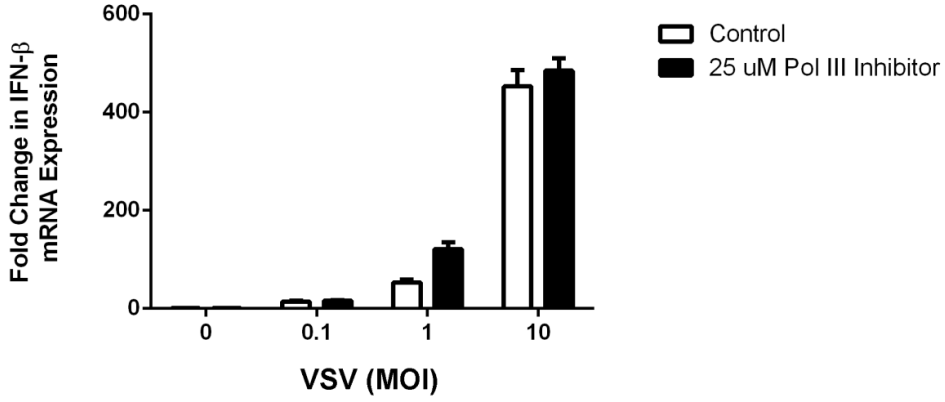

Figure 9: IFN-β mRNA expression in EOC 13.31 is enhanced and unattenuated in the presence of a RNA polymerase III inhibitor following infection with WT VSV. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with WT VSV at MOI 0.1, 1 and 10. At 6 h.p.i., total RNA was collected and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression.

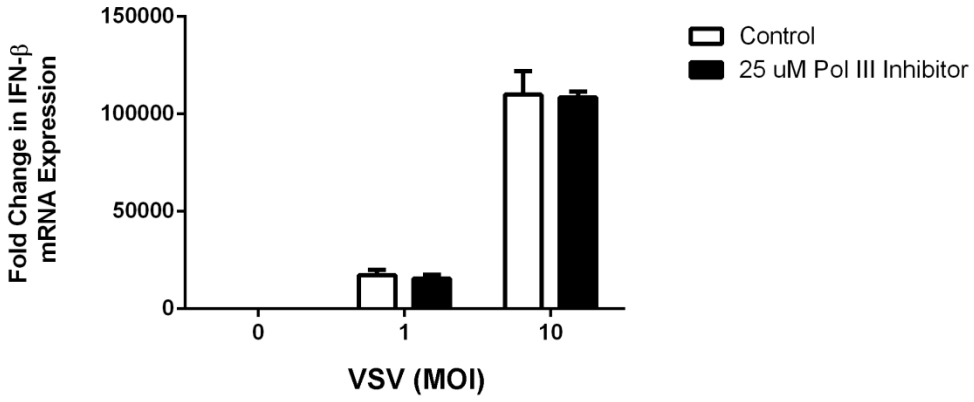

Figure 10: IFN-β mRNA expression in astrocytes is enhanced and unattenuated in the presence of a RNA polymerase III inhibitor following infection with WT VSV. Primary murine astrocytes were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with WT VSV at MOI 1 and 10. At 6 h.p.i., total RNA was collected and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression.

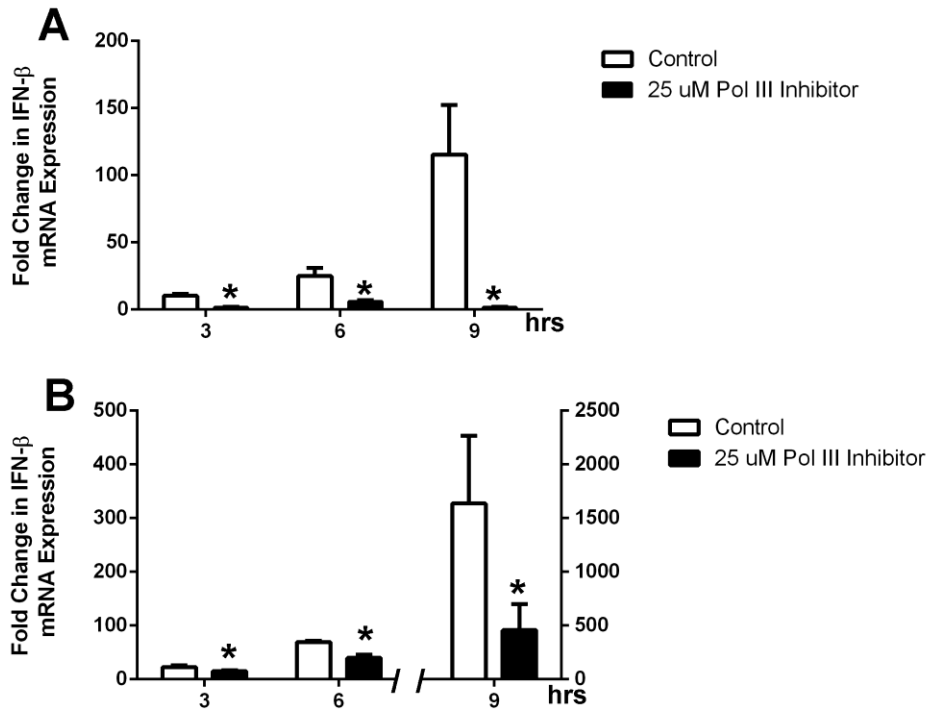

Figure 11: IFN-β mRNA expression in EOC 13.31 is attenuated in the presence of a RNA polymerase III inhibitor following infection with HSV-1. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with HSV-1 at MOI 0.1 (Panel A) or 1 (Panel B). At 3, 6 and 9 h.p.i., total RNA was collected and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. Asterisks indicate a statistically significant difference from uninhibited cells.

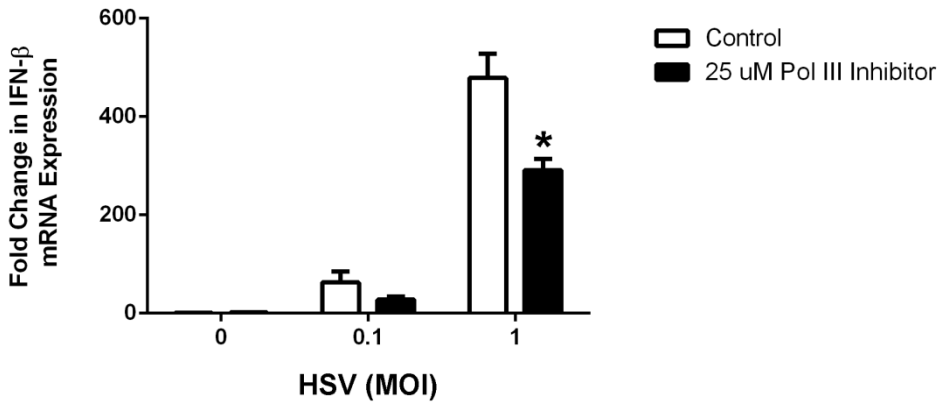

Figure 12: IFN-β mRNA expression in astrocytes is attenuated in the presence of a RNA polymerase III inhibitor following infection with HSV-1. Primary murine astrocytes were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with HSV-1 at MOI 0.1 and 1. At 6 h.p.i., total RNA was collected and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. The asterisk denotes a statistically significant difference from uninhibited cells.

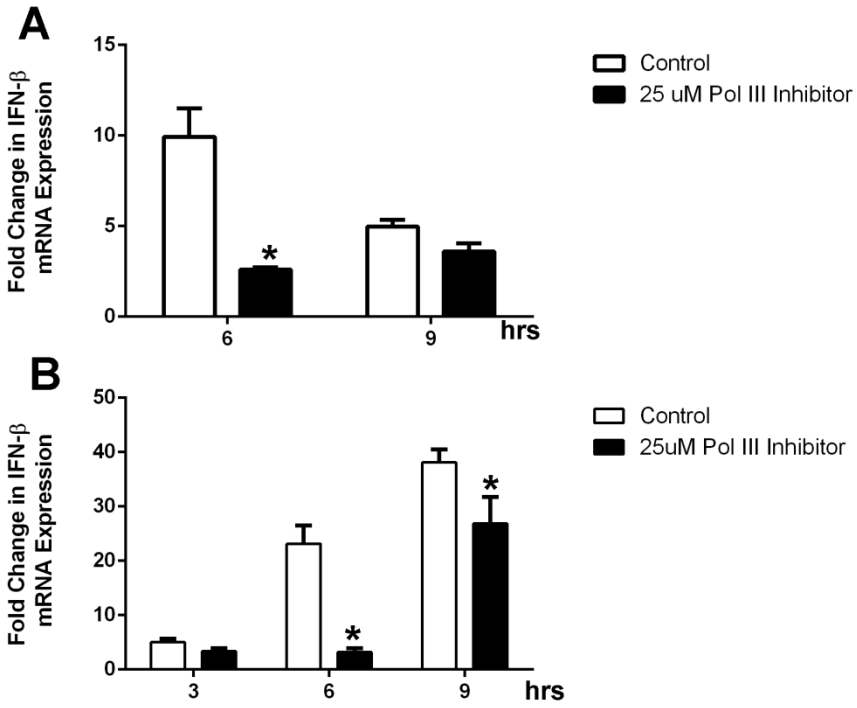

Figure 13: IFN-β mRNA expression in EOC 13.31 is attenuated in the presence of a RNA polymerase III inhibitor following infection with MHV-68. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with MHV-68 at MOI 0.1 (Panel A) or 1 (Panel B). At 3, 6 and 9 h.p.i., total RNA was collected and expression of IFN-β and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. Asterisks indicate a statistically significant difference from uninhibited cells.

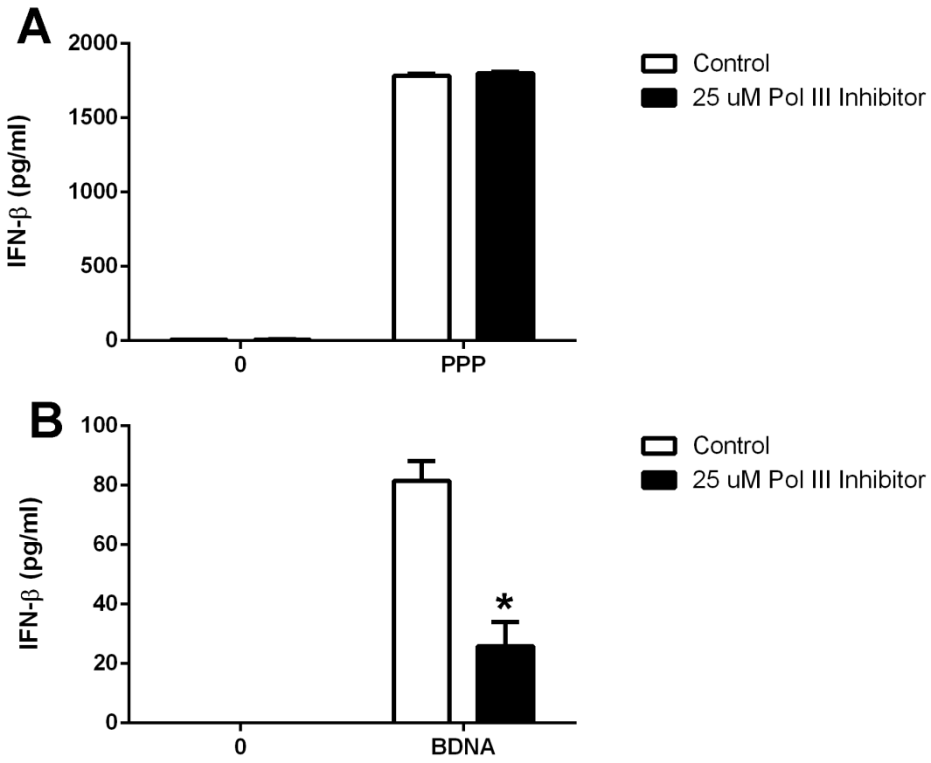

Figure 14: The presence of a RNA polymerase III inhibitor attenuates IFN-β protein secretion following transfection with the DAI ligand, B-DNA, but not the RIG-I ligand, PPP, in EOC 13.31. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then untransfected or transfected with 4 ug/ml PPP (Panel A) or 3 ug/ml B-DNA (Panel B). Culture supernatants were collected at 24 hours post-transfection and IFN-β secretion was determined by specific capture ELISA. Data are presented as mean ± SEM. An asterisk denotes a statistically significantly difference from uninhibited cells.

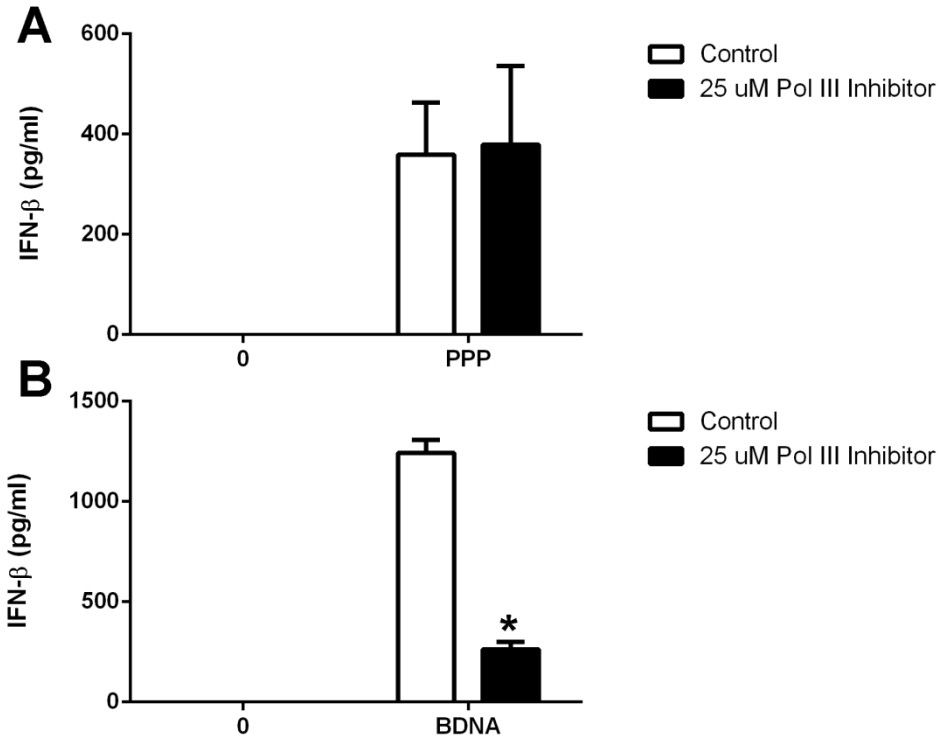

Figure 15: The presence of a RNA polymerase III inhibitor attenuates IFN-β protein secretion following transfection with the DAI ligand, B-DNA, but not the RIG-I ligand, PPP, in astrocytes. Primary murine astrocytes were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then untransfected or transfected with 4 ug/ml PPP (Panel A) or 3 ug/ml B-DNA (Panel B) and culture supernatants were collected at 6 hours post-transfection. IFN-β secretion was determined by specific capture ELISA. Data are presented as mean ± SEM. An asterisk denotes a statistically significantly difference from uninhibited cells.

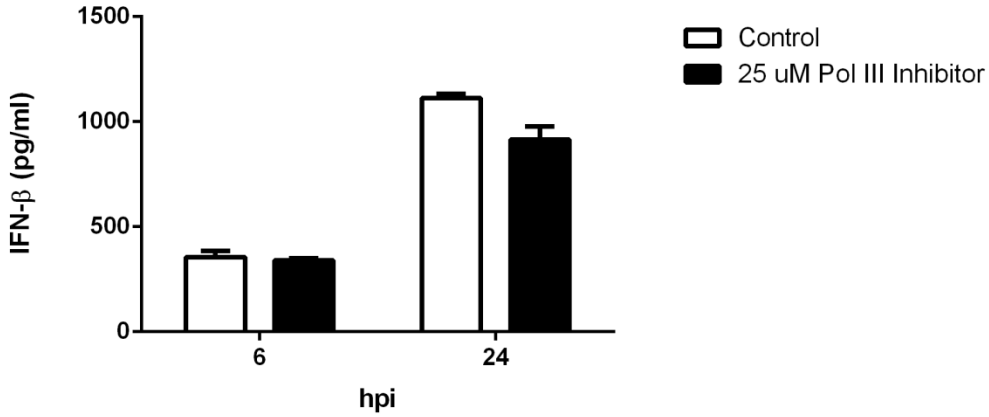

Figure 16: IFN-β secretion by EOC 13.31 is not attenuated in the presence of a RNA polymerase III inhibitor following infection with WT VSV. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with WT VSV at MOI 1. Culture supernatants were collected at 6 and 24 h.p.i. and IFN-β secretion was determined by specific capture ELISA. Data are presented as mean ± SEM.

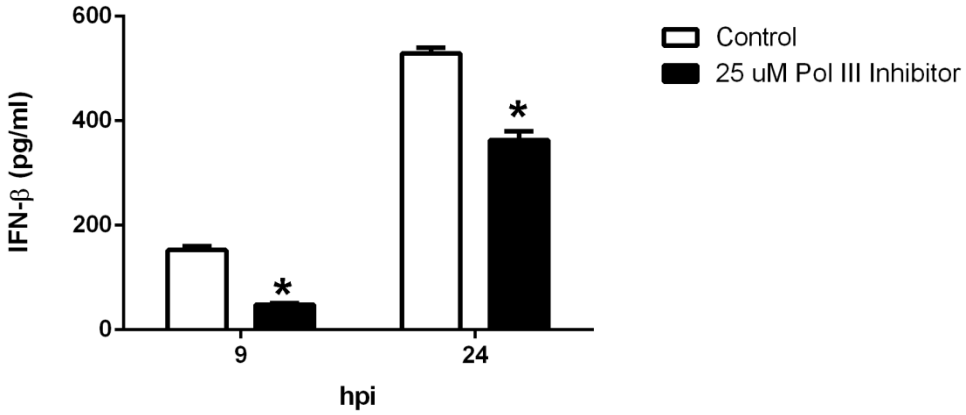

Figure 17: IFN-β secretion by EOC 13.31 is attenuated in the presence of a RNA polymerase III inhibitor following infection with HSV-1. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with HSV-1 at MOI 1. Culture supernatants were collected at 9 and 24 h.p.i. and IFN-β secretion was determined by specific capture ELISA. Data are presented as mean ± SEM. Asterisks denote a statistically significantly difference from uninhibited cells.

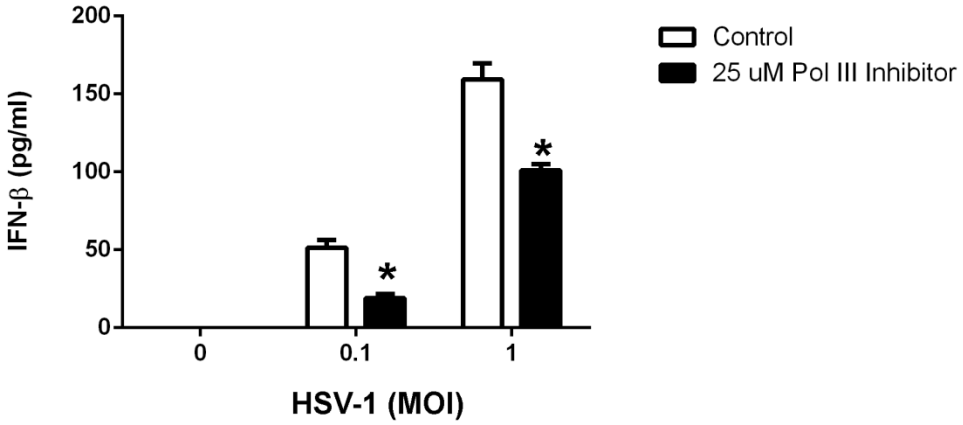

Figure 18: IFN-β secretion by astrocytes is attenuated in the presence of a RNA polymerase III inhibitor following infection with HSV-1. Primary murine astrocytes were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with HSV-1 at MOI 0.1 and 1. Culture supernatants were collected at 6 h.p.i. and IFN-β secretion was determined by specific capture ELISA. Data are presented as mean ± SEM. Asterisks denote a statistically significantly difference from uninhibited cells.

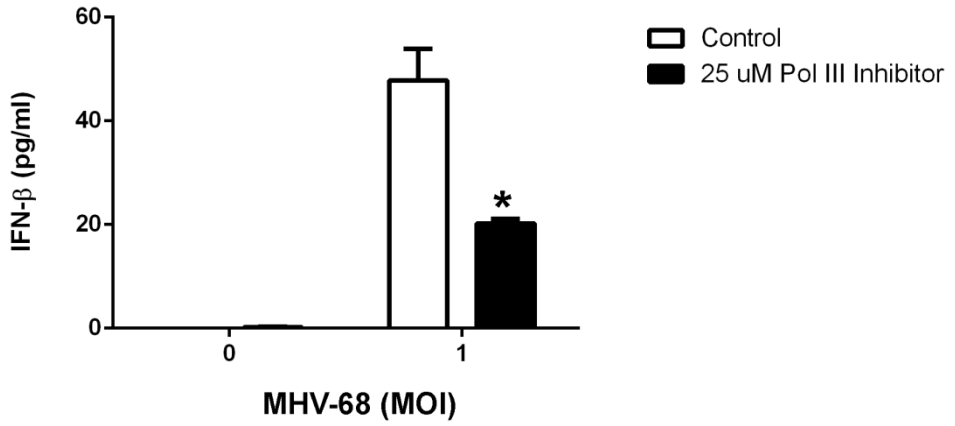

Figure 19: IFN-β secretion by EOC 13.31 is attenuated in the presence of a RNA polymerase III inhibitor following infection with MHV-68. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with MHV-68 at MOI 1. Culture supernatants were collected at 9 h.p.i. and IFN-β secretion was determined by specific capture ELISA. Data are presented as mean ± SEM. The asterisk denotes a statistically significantly difference from uninhibited cells.

3.4 RNA Polymerase III Inhibition Permits Enhanced DNA, but not RNA, Viral Replication.

Finally, we have performed experiments to determine whether RNA polymerase III activity is essential for glial responses that limit viral replication. EOC13.31 microglia-like cells were untreated or pretreated with a RNA polymerase III inhibitor prior to challenge with GFP-expressing VSV, wild type VSV, wild type HSV-1, or wild type MHV68. RNA polymerase III inhibition does not influence intracellular GFP-VSV replication as assessed by fluorescence intensity (Figure 20). Similarly, absence of RNA polymerase III functionality does not affect the production of wild type VSV virion proteins as assessed by western immunoblot (Figure 21). In contrast, inhibition of RNA polymerase III activity significantly increased intracellular levels of the HSV-1 product VP16 (Figure 22) and the expression of mRNA encoding the MHV68 product ORF65 (Figure 23). Together, these data support the contention that indicates RNA polymerase III activity is required to limit the replication of DNA, but not RNA viruses.

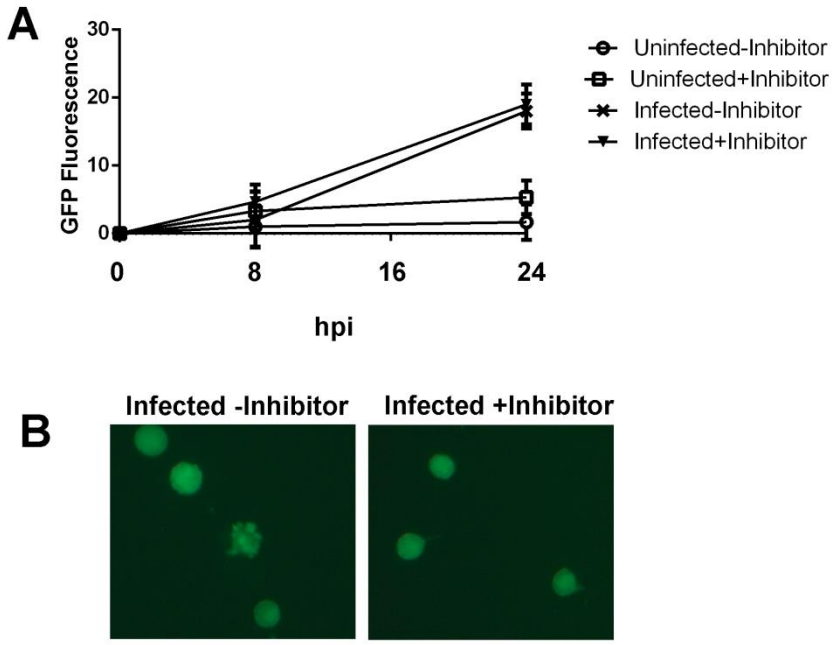

Figure 20: The presence of a RNA polymerase III inhibitor does not influence VSV-ΔM51-GFP replication in EOC13.31. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with VSV-ΔM51-GFP at MOI 1. Fluorescence readings were obtained at 0, 8 and 24 h.p.i. and fluorescence microscopy images were taken at 24 h.p.i. Data are presented as mean ± SEM.

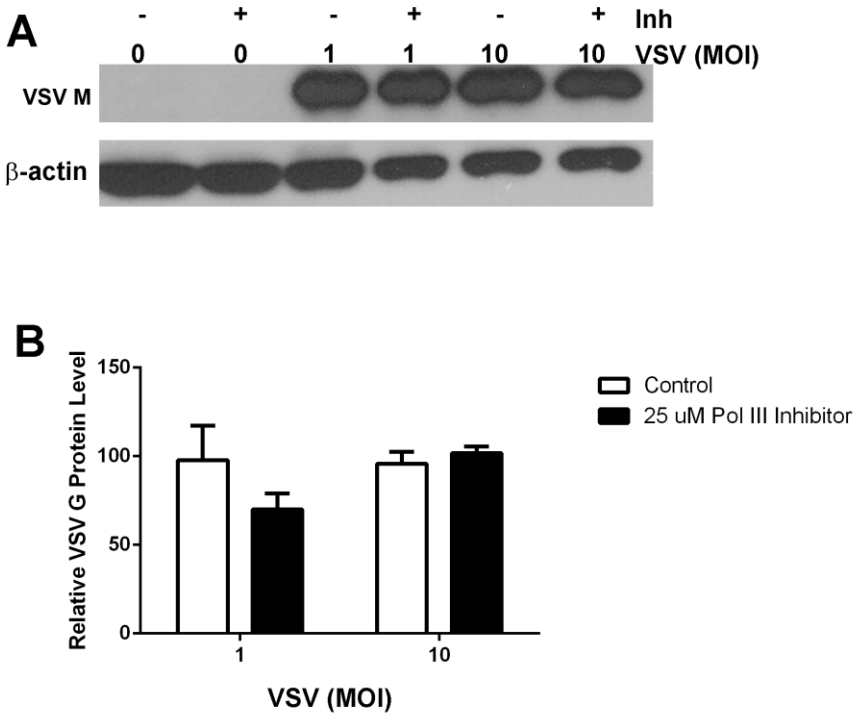

Figure 21: The presence of a RNA polymerase III inhibitor has no effect on VSV protein expression in EOC 13.31. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with WT VSV at MOI 1 and 10. At 24 h.p.i., whole-cell isolates were collected and VSV virion protein expression was determined by immunoblot assay. Densitometric analysis data is presented as mean expression ± SEM, normalized to β-actin.

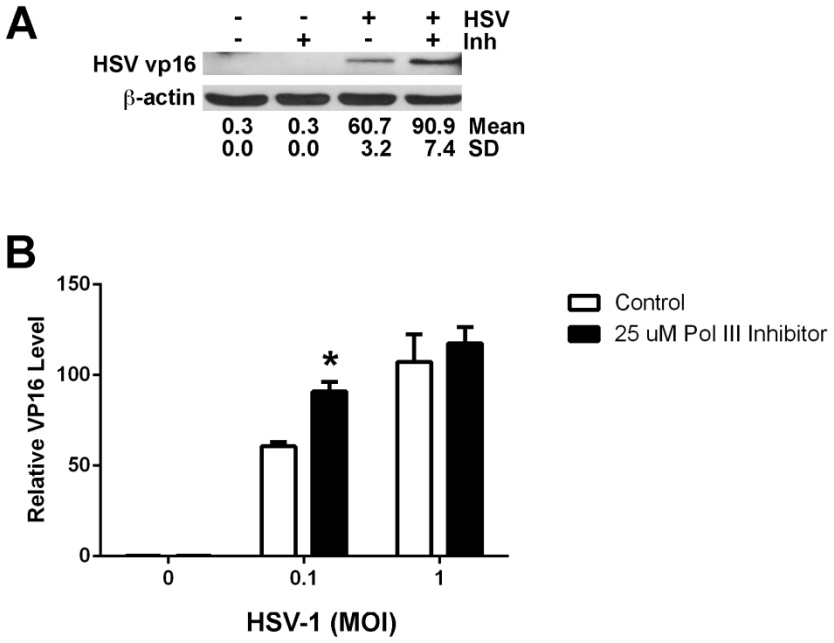

Figure 22: HSV-1 VP16 protein expression is enhanced in EOC 13.31 in the presence of a RNA polymerase III inhibitor. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with HSV-1 at MOI 0.1 and 1. At 24 h.p.i., whole-cell isolates were collected and VP16 protein expression was determined by immunoblot assay. Densitometric analysis data is presented as mean expression ± SEM, normalized to β-actin. The asterisk denotes a statistically significantly difference from uninhibited cells.

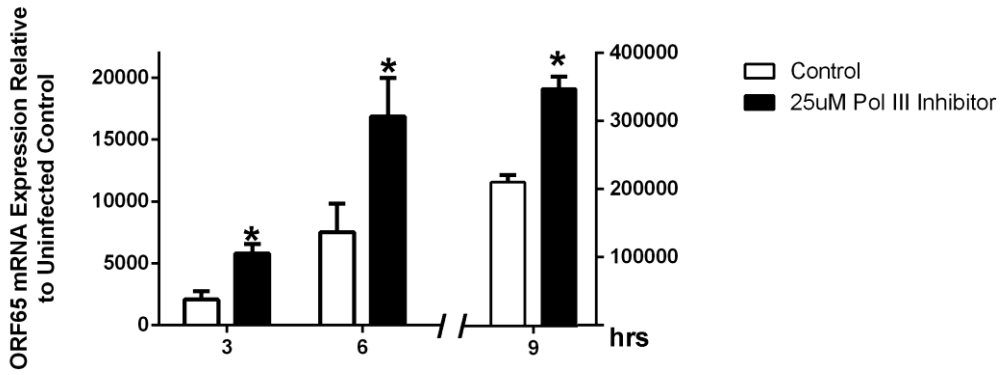

Figure 23: MHV-68 ORF65 expression is enhanced in EOC 13.31 in the presence of a RNA polymerase III inhibitor. EOC 13.31 were pre-treated with a 25 uM RNA polymerase III inhibitor solution or media alone for 24 hours and then uninfected or infected with MHV-68 at MOI 1. At 3, 6 and 9 h.p.i., total RNA was collected and expression of ORF65 and GAPDH was determined by qRT-PCR. Data are presented as mean fold induction ± SEM, normalized to GAPDH expression. Asterisks indicate a statistically significant difference from uninhibited cells.

CHAPTER 4: DISCUSSION

It is increasingly apparent that glial cells possess intracellular sensors that can detect compromise of the cytosolic compartment. Most RNA viruses replicate in the cytoplasm and therefore allow detection of viral replicative motifs by RIG-I-like receptors (RLRs) present in the host cell. Our lab has previously demonstrated that primary murine microglia and astrocytes constitutively express RIG-I mRNA and protein (Furr et al., 2008). Challenge with the RNA virus, SeV, which is recognized by RIG-I led to a marked increase in RIG-I protein production by primary murine microglia (Furr et al., 2008). In former studies we have shown that microglia and astrocytes are permissive for VSV infection and that productive replication is required for inflammatory mediator production by these cells (Chauhan et al., 2010). Infection of primary murine astrocytes with VSV significantly enhanced RIG-I mRNA expression well above constitutive expression levels (Furr et al., 2008). Furthermore, we have previously shown that human astrocytes also express RIG-I and that expression levels of this cytosolic viral RNA sensor increase in human glial cells following challenge with the model neurotropic RNA virus, VSV (Furr et al., 2010). In addition to RIG-I expression, our lab has also demonstrated the functionality of the RIG-I receptor in primary human astrocytes. Infection of primary human astrocytes with VSV induced the association of RIG-I with its adaptor molecule, IPS-1, which is a prerequisite for promotion down the RIG-I signaling pathway and ultimately, the production of proinflammatory molecules and antiviral cytokines.

Similar to our findings with VSV, our lab has shown that intracellular administration of a 5'triphosphorylated RNA ligand for RIG-I results in enhanced RIG-I expression in primary murine microglia and astrocytes (Crill et al., Manuscript in Revision). Other groups have also demonstrated the expression of RIG-I in primary murine astrocytes and a murine microglia-like cell line, BV-2, which was upregulated following stimulation with RNA ligands and virus (de Rivero Vaccari et al., 2012; Jiang et al., 2014). As such, these findings support the functional status of RIG-I in both glial cell types in mice. In addition, the inducible nature of such expression by intact viral particles and a specific RIG-I ligand suggests the possibility that glial cells could become sensitized to the presence of intracellular viral moieties that are produced during viral replication in a feed-forward manner.

Perhaps more importantly, the present study demonstrates that this RLR plays an essential role in the inflammatory and antiviral immune responses of murine astrocytes and microglia to RNA virus infection. Similar to our findings in human astrocytes, intracellular delivery of a 5'triphosphorylated RNA ligand into primary murine astrocytes induces enhanced type I interferon mRNA expression and robust inflammatory cytokine production (Furr et al., 2010). Previously, we have shown that intracellular delivery of this RIG-I ligand induces increased expression of the inflammatory mediators, IL-6 and TNF-α, by both microglia and astrocytes. The current study demonstrates that 5'triphosphorylated RNA transfection significantly upregulates IFN-β mRNA expression in murine glial cells. Taken together, these results suggest that RIG-I promotes both antiviral and proinflammatory responses in glial cells. Additionally, our lab previously demonstrated that RIG-I plays a critical role in the

immune responses of microglia and astrocytes to a model neurotropic rhabdovirus by demonstrating that RIG-I knockdown specifically and almost totally abolishes VSV-induced cytokine production by either of these CNS cell types (Crill et al., Manuscript in Revision). This finding is consistent with several studies in glial cell lines demonstrating that inflammatory cytokine production by JEV infected BV-2 microglia-like cells and poly I:C-mediated chemokine release by U373MG human astrocytoma cells is decreased following RIG-I knockdown (Jiang et al., 2014; Yoshida et al., 2007). Furthermore, it is in agreement with the observations that a putative RLR inhibitor can attenuate RNA ligand-mediated astrocyte activation (de Rivero Vaccari et al., 2012). As expected, RIG-I knockdown promotes enhanced JEV virus proliferation in BV-2 microglia-like cells (Jiang et al., 2014).

In contrast to RNA viruses, many DNA viruses are known to replicate within the nucleus. In addition, specific recognition of viral DNA motifs is further complicated by the apparent detection by DAI of a structure common to both self and non-self DNA. This suggests that discrimination between these types of DNA is based on its subcellular localization and may be a function of the amount and length of the DNA present in the cytosol rather than a specific chemical feature of the ligand (Keating et al., 2011). A recent study has shown that HSV-1 DNA, dislocated from the viral capsid, is readily detectable in the cytoplasm of infected primary macrophages and monocyte/macrophage cell lines suggesting that viral DNA can be detected by receptors present in the cytosol (Unterholzner et al., 2010). The present study and our recent work support such a hypothesis since intracellular administration of the putative DAI ligand, B-DNA, is a potent stimulus for inflammatory cytokine production and type I interferon

expression by murine glial cells (Furr et al., 2011). Furthermore, previous demonstration that DAI knockdown significantly reduces HSV-1-induced cytokine production by microglia and astrocytes indicates that this cytosolic viral DNA sensor plays a significant role in the recognition of DNA viruses (Furr et al., 2011).

It has recently been proposed that RIG-I may also contribute to type I interferon and inflammatory cytokine production by cells in response to DNA virus challenge via the actions of DNA-dependent RNA polymerase III. In this model, AT-rich double-stranded DNA serves as a template for RNA polymerase III and is transcribed as RNA containing a 5'-triphosphate end, a ligand for RIG-I. Such a hypothesis is supported by the demonstration that cell responses to the gammaherpesvirus, Epstein-Barr virus, are attenuated following RNA polymerase III knockdown or treatment with pharmacological inhibitors of this enzyme (Ablasser et al., 2009; Chiu et al., 2009). The present finding that the DNA virus HSV-1, as well as a synthetic DNA ligand for DAI, can upregulate RIG-I expression by both astrocytes and microglia provides circumstantial evidence for the involvement of RIG-I in the perception of DNA pathogens. However, more direct evidence comes from the demonstration that both RIG-I knockdown and RNA polymerase III inhibition significantly attenuates inflammatory cytokine and type I interferon production by microglia and astrocytes in response to the DNA viruses HSV-1 and MHV-68, and a synthetic DAI ligand (Crill et al., Manuscript in Revision). Furthermore, we provide evidence that such a mechanism can contribute to the control of viral replication within glial cells by demonstrating that the pharmacological inhibition of RNA polymerase III results in the elevated expression of DNA viral products in infected microglia-like cells, but has no effect on VSV

product levels. Together, these data provide the first evidence that transcription of cytosolic viral DNA by RNA polymerase III and subsequent RNA recognition by RIG-I could serve as a mechanism for the perception of DNA viruses by CNS cells.

The present finding that both RIG-I and DAI are necessary to mount maximal glial cytokine responses to DNA pathogens is consistent with the observation that DAI knockdown reduces, but does not abolish, glial responses to HSV-1 (Crill et al., Manuscript in Revision). While the ability of a specific RNA polymerase III inhibitor to attenuate inflammatory cytokine production by HSV-1 infected, and B-DNA challenged, glial cells provides strong support for the notion that RIG-I detects RNA moieties transcribed from cytosolic viral DNA, we cannot discount the possibility that DAI and RIG-I signaling pathways interact in other ways in microglia or astrocytes. For example, the adaptor molecule STING has been shown to play a role in both RIG-I and DAI signaling pathways (Ishikawa and Barber, 2008). As such, the possibility exists that HSV-1-initiated RIG-I and DAI signaling pathways converge at this component to potentiate cellular activation. Alternatively, it is conceivable that RIG-I and DAI interact either directly or via STING to promote glial responses. In support of this possibility, immunoprecipitation studies indicate that STING associates with RIG-I complexes in close proximity to endoplasmic reticulum associated-mitochondria (Ishikawa et al., 2009; Dixit et al., 2010). However, it remains to be determined whether DAI associates with RIG-I, STING, or other RIG-I signaling components in glial cells. Finally, TLR3 and its associated adaptor molecule TRIF, have been associated with the control of HSV-1 in the CNS of human patients and mice and inflammatory processes that lead to CNS pathology following HSV-1 infection have been reported to be

associated with TLR2 (Zhang et al., 2007; Conrady et al., 2013; Kurt-Jones et al., 2004). While these studies appear to contrast with our findings, a possible explanation may lie in the ability of PRRs to act in a cooperative manner to promote glial inflammatory responses (Furr and Marriott, 2012). Clearly, further work will be required to resolve these issues.

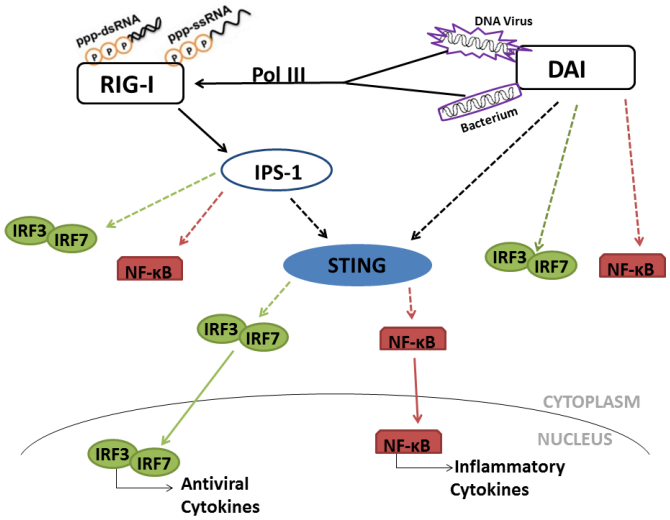

Figure 24: Proposed mechanisms of crosstalk between RIG-I and DAI pathways. The employment of RNA polymerase III permits RIG-I to indirectly detect DAI ligands. RIG-I and DAI signaling has also been proposed to converge at the STING adaptor molecule. Regardless of the pathway employed, we have shown that RIG-I signaling is required in order to elicit maximal immune responses against a DNA pathogen.

Taken in concert, the present study illustrates the functional significance of RIG-I expression in primary murine glial cells. Our data show that primary murine microglia and astrocyte responses to the RNA virus, VSV, are RIG-I dependent and independent of the expression of DAI or RNA polymerase III activity. In contrast, maximal glial inflammatory and antiviral responses to HSV-1 are dependent on the expression of both RIG-I and DAI, and require RNA polymerase III activity. Based upon our results we

propose the model shown in Figure 24. We suggest that neurotropic single-stranded RNA viruses such as VSV infect microglia and astrocytes, and replicate within them generating 5'triphosphorylated single-stranded RNA in the cytoplasm that acts as a ligand for RIG-I. RIG-I then associates with IPS-1, and perhaps STING, which subsequently activates NF-kB, IRF3 and IRF7. These transcription factors translocate to the nucleus and initiate the production of inflammatory mediators including TNF-α, IL-6 and type I interferons. In contrast, neurotropic double-stranded DNA viruses, such as HSV-1, infect glial cells and replicate within them generating genomic DNA. Viral DNA is recognized by DAI which activates downstream effector molecules including IPS-1 and STING, subsequently activating NF-κB and IRF3 and IRF7. In addition, RNA polymerase III in the cytosol transcribes viral DNA into double-stranded RNA containing a 5'triphosphate that is recognized by RIG-I and similarly initiates transcription factor activation via IPS-1 and potentially STING. Upon secretion, cytokines such as TNF-α, and IL-6 would be anticipated to promote inflammation, increase blood-brain barrier permeability, and facilitate leukocyte recruitment into the CNS, while type I interferons function to limit viral replication.

The findings of this study and previous studies by our laboratory support the notion that RIG-I may play a more ubiquitous role than formerly thought. The RNA polymerase III-mediated indirect recognition of foreign cytoplasmic DNA by RIG-I, a protective innate immune response during active viral infections, may also have implications in bacterial and protozoan infections. In accordance with this hypothesis, a study by Chiu and colleagues (2009) demonstrated that RNA polymerase III inhibition promotes enhanced growth of the lung pathogen, *Legionella pneumophila*, which is

thought to be a direct consequence of attenuated IFN-β production (Chiu et al., 2009). In conclusion, the current study and others by our laboratory suggest that RIG-I plays an important role in the perception of both RNA and DNA pathogens by microglia and astrocytes. However, it remains to be determined whether RIG-I-mediated glial responses assume a more protective role or contribute to the rapid and potentially lethal inflammation associated with CNS pathogens.

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