NEWLY DESCRIBED CYTOSOLIC PATTERN RECOGNITION RECEPTORS MEDIATE INFLAMMATORY GLIAL RESPONSES TO NEUROTROPIC VIRUSES

by

Samantha Rosa Lee Furr

A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biology

Charlotte

2012

Approved by:

_____
Dr. Ian Marriott

_____
Dr. Kenneth L. Bost

_____
Dr. Valery Z. Grdzelishvili

_____
Dr. Julie M. Goodliffe

_____
Dr. Andrew Harver

ABSTRACT

SAMANTHA ROSA LEE FURR. Newly described cytosolic pattern recognition receptors mediate inflammatory glial responses to neurotropic viruses. (Under the direction of DR. IAN MARRIOTT)

The rapid onset of potentially lethal neuroinflammation is a defining feature of viral encephalitis. Glial cells are ideally positioned to respond to invading pathogens of the central nervous system (CNS) and produce key inflammatory mediators following viral infection. However, the mechanisms by which resident CNS cells perceive such challenges have not been defined. Recently, several cytosolic pattern recognition receptors, including retinoic acid-inducible gene I (RIG-I) and DNA-dependent activator of IFN regulatory factors (DAI), have been described that appear to function as intracellular sensors of RNA and DNA viruses, respectively. Interestingly, recent studies suggest that RIG-I may also be able to recognize DNA pathogens in a polymerase III-dependent manner. However, little is known regarding the expression of these novel intracellular viral sensors or their role in the CNS. In the present study, we demonstrate that microglia and astrocytes constitutively express detectable levels of both RIG-I and DAI and their downstream adaptor molecules. In addition, we show that expression of RIG-I and DAI by glial cells is elevated following infection with the model neurotropic RNA virus, vesicular stomatitis virus (VSV), and/or DNA viruses including the neurotropic virus herpes simplex virus-1 (HSV-1), or specific synthetic ligands for these viral sensors. Importantly, these specific ligands elicit inflammatory mediator production by both microglia and astrocytes, and targeted knockdown of RIG-I or DAI attenuates such responses following RNA or DNA virus exposure, respectively, and limits the

production of soluble neurotoxic mediators by virally challenged cells. Interestingly, glial inflammatory responses to the DNA virus HSV-1 were also dependent on the expression of RIG-I and the activity of polymerase III, while glial responses to the RNA virus VSV required the expression of RIG-I but were DAI and polymerase III independent. These studies demonstrate that RIG-I and DAI play a critical role in the recognition of viral pathogens by resident CNS cells and suggest that these novel intracellular pattern recognition receptors may underlie the damaging inflammation and neuronal cell death associated with acute neurotropic viral infections.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Ian Marriott, for his guidance, ideas, and funding that made my graduate career both productive and stimulating, and also for tolerating my many opinions, eccentricities, and commas. I am deeply grateful to Dr. Valery Grdzelishvili for his detailed and constructive comments, and for teaching me everything I ever wanted to know about viruses. I am also forever indebted to my committee members, Dr. Kenneth Bost, Dr. Julie Goodliffe, and Dr. Andrew Harver, for their time, invaluable guidance, and letters of recommendation. I wish to thank my colleague and friend, Vinita, for her good advice, support, and friendship. They have been priceless on both an academic and a personal level. I would also like to thank my fellow lab members, past and present, for their assistance and camaraderie over the last five years. I would like to thank the students whom I have been privileged to teach throughout the years, and from whom I have also learned much. I also gratefully acknowledge the funding sources that made my Ph.D. research possible.

Lastly, I would like to express my appreciation to my friends and family for all of their unwavering support and encouragement, without which this dissertation would not be possible. Especially to my parents, who raised me with a love for education and have always supported me in my academic pursuits. And finally, to Dustin, whose faithful love, support, and patience during the final stages of my Ph.D. are irreplaceable. Thank you.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS viii

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER ONE: INTRODUCTION 1

1.1 Glial cells play a critical role in the initiation of virally-induced neuroinflammation 6

1.2 TLRs mediate glial responses to extracellular or endosomal viral motifs 8

1.3 RIG-like receptors can mediate protective immune responses and damaging CNS inflammation following RNA virus infection 14

1.4 Newly discovered cytosolic viral sensors may mediate glial responses to DNA viruses 17

1.5 PRRs act in a cooperative manner to induce glial responses to viral challenges 20

1.6 Hypothesis and present study 22

1.7 Figures 24

CHAPTER TWO: MATERIALS AND METHODS 27

CHAPTER THREE: A ROLE FOR DNA-DEPENDENT ACTIVATOR OF INTERFERON REGULATORY FACTOR IN THE RECOGNITION OF HERPES SIMPLEX VIRUS TYPE 1 BY GLIAL CELLS 38

3.1 Rationale 38

3.2 Results 40

3.3 Conclusions 43

3.4 Figures 48

CHAPTER FOUR: RIG-I MEDIATES NONSEGMENTED NEGATIVE-SENSE RNAVIRUS-INDUCED INFLAMMATORY RESPONSES OF PRIMARY HUMAN ASTROCYTES 53

4.1 Rationale 53

4.2 Results 55

4.3 Conclusions 59

4.4 Figures 64

CHAPTER FIVE: RIG-I IS AN ESSENTIAL COMPONENT IN MURINE GLIAL RESPONSES TO NEUROTROPIC RNA AND DNA VIRUSES 71

5.1 Rationale 71

5.2 Results 73

5.3 Conclusions 76

5.4 Figures 81

CHAPTER SIX: SUMMARY AND CONCLUSIONS 86

6.1 DAI plays an important role in inflammatory and neurotoxic responses of glial cells to DNA viral pathogens 86

6.2 RIG-I plays an important role in inflammatory and neurotoxic responses to RNA viral pathogens in human astrocytes 89

6.3 RIG-I is essential for the recognition of both RNA and DNA viral pathogens in the CNS 91

6.4 Potential for future studies 94

REFERENCES 96

APPENDIX: PUBLICATIONS 111

LIST OF ABBREVIATIONS

5’ppp-ssRNA	5’ triphosphate single-stranded RNA
AIM2	Absent in melanoma 2
BMDM	Bone marrow-derived macrophages
CARD	Caspase activation and recruitment domain
CNS	Central nervous system
DAI	DNA-dependent activator of interferon-regulatory factor
dsRNA	Double-stranded RNA
dsDNA	Double-stranded DNA
HSV	Herpes simplex virus
IFN	Interferon
IL	Interleukin
IPS-1	Interferon promoter stimulator-1
IRF	Interferon regulatory factor
JE	Japanese encephalitis virus
MDA5	Melanoma differentiation-associated gene 5
MHV	Murine gammaherpesvirus
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor-κB
NOD2	Nucleotide-binding oligomerization domain-containing protein 2

NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
poly(I:C)	Polyinosine-deoxycytidylic acid
PRR	Pattern recognition receptor
RABV	Rabies virus
RIG-I	Retinoic acid inducible gene-I
RIP	Receptor-interacting protein
RLR	RIG-I-like receptor
STING	Stimulator of interferon genes
TANK	TRAF family member-associated NF-κB activator
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-receptor associated factor
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
WNV	West Nile virus
ZBD	Z-DNA binding domain

LIST OF TABLES

TABLE 1: Both RNA and DNA viruses are causative agents of encephalitis. 3

LIST OF FIGURES

FIGURE 1: RLRs detect viral RNA motifs in the cytoplasm to trigger innate immunity and inflammation. 24

FIGURE 2: Recognition of DNA viruses by DAI. 25

FIGURE 3: Possible pathways for viral detection by glial cells. 26

FIGURE 4: Cultured primary murine astrocytes and microglia constitutively express the cytoplasmic viral sensor DAI and associated effector molecules, and its expression is elevated following viral challenge. 48

FIGURE 5: *Ex vivo* glial cells express DAI constitutively and/or inducibly following *in vivo* HSV-1 infection. 49

FIGURE 6: A specific ligand for DAI induces inflammatory cytokine production by isolated cultures of microglia and astrocytes. 50

FIGURE 7: DAI knockdown attenuates HSV-1-induced inflammatory cytokine production by murine glial cells. 51

FIGURE 8: HSV-1 induces the production of soluble factor(s) by glial cells that elicits neuronal cell damage in a DAI dependent manner. 52

FIGURE 9: Primary human astrocytes constitutively express the cytoplasmic viral RNA sensor, RIG-I, and such expression is elevated following viral challenge. 64

FIGURE 10: RIG-I expression and subsequent VSV-induced elevations in such expression are restricted to the cytoplasm. 65

FIGURE 11: VSV induces the activation of RIG-I associated signaling pathways in primary human astrocytes. 66

FIGURE 12: 5’ triphosphate single stranded RNA, a specific ligand for RIG-I, upregulates RIG-I expression but not IPS-1 in primary human astrocytes. 67

FIGURE 13: 5’ triphosphate single stranded RNA, a specific ligand for RIG-I, induces NF-κB activation and inflammatory cytokine production in primary human astrocytes. 68

FIGURE 14: RIG-I knockdown attenuates VSV-induced inflammatory cytokine production by primary human astrocytes. 69

FIGURE 15: VSV induces the production of a soluble factor(s) by human astrocytes that elicit neuronal cell death in a RIG-I dependent manner. 70

FIGURE 16: 5’ppp-ssRNA induces RIG-I expression in primary murine glial cells. 81

FIGURE 17: Dissimilar motifs induce RIG-I expression in primary murine glial cells. 82

FIGURE 18: Single stranded RNA induces inflammatory mediator production by primary murine glial cells. 83

FIGURE 19: RIG-I knockdown abolishes VSV-induced inflammatory cytokine production by primary glia. 84

FIGURE 20: Both RIG-1 and DAI are involved in cellular responses to the DNA virus, HSV-1. 85

FIGURE 21: Proposed model by which glia recognize neurotropic DNA viruses such as HSV-1 and elicit inflammatory CNS damage. 88

FIGURE 22: Proposed model by which astrocytes recognize neurotropic RNA viruses and elicit inflammatory CNS damage. 90

FIGURE 23: Proposed pathways involved in glial inflammatory responses to neurotropic viruses. 93

CHAPTER ONE: INTRODUCTION

Microbial and viral infections of the brain and spinal cord result in inflammation that underlies wide ranging symptoms including fever, headache, confusion, and in extreme cases, stroke, seizure, or even death. The resulting disease is classified based on the area of the central nervous system (CNS) affected and can include the membranes that surround the brain (meningitis), spinal cord (myelitis), or inflammation within the brain parenchyma (encephalitis). Of these, encephalitis tends to be associated with more significant alterations in the level of consciousness, cognitive defects, seizures, and focal neurological deficits than meningitis, although it should be noted that clinical presentation of these conditions often overlap. The incidence of encephalitis in the United States has been estimated at approximately 20,000 new cases annually according to the Centers for Disease Control, and an average incidence of 1.9 cases per 100,000 inhabitants has been reported in France for non-HIV patients (Stahl et al., 2008). While encephalitis and meningitis can be caused by bacterial or fungal pathogens, viruses are the major causative agents of CNS infection worldwide, with a review of worldwide prospective studies yielding a minimum incidence of 10.5 per 100,000 acute encephalitis cases in children and 2.2 per 100,000 in adults, with an overall incidence of 6.34 per 100,000 for all ages (Fidan et al., 2008). However, it must be noted that these numbers are likely to be underestimations given the problems associated with diagnosis, particularly in developing countries (Sejvar, 2006).

Viral CNS infections can be very difficult to treat due to the rapid onset of severe disease symptoms that can occur even in otherwise healthy individuals. Such infections trigger broad activation of glial cells including microglia and astrocytes, and the concomitant release of an array of proinflammatory molecules that can elicit both innate and adaptive immune responses. While host responses limit viral replication and dissemination and can lead to infection resolution, overzealous or sustained inflammation within the CNS can result in significant neurological damage.

One of the major challenges in the study and treatment of viral CNS infections is the wide range of etiological agents (Tunkel et al., 2008). Both RNA and DNA viruses can infect the CNS and cause meningitis or encephalitis (as reviewed in Table 1), although an etiological diagnosis is established in less than 30% of cases (Stahl et al., 2008). While historically important RNA viruses that can cause encephalitis such as measles, mumps, and rubella have seen steep decreases in incidence as a result of immunization (Koskiniemi et al., 1997), rabies virus (RABV) remains an endemic cause of viral encephalitis in the United States and a serious public health concern. RABV is transmitted from animals to humans and although rare, CNS infection results in uncontrolled virus replication, a prominent pro-inflammatory response, and death (Theerasurakarn et al., 1998). Encephalitis due to this zoonotic virus is 100% fatal once neurological symptoms develop and accounts for several thousand deaths per year in Asian countries. Interestingly, new RABV variants that can cause lethal encephalitis have recently been reported in the United States (as reviewed in Cliquet et al., 2004) and some evidence suggests that the incidence of RABV disease may be increasing due to the changing epidemiology of infection in animal populations (Whitley et al., 1999).

TABLE 1: Both RNA and DNA viruses are causative agents of encephalitis.

Virus	**Type**	**Mortality rate**	**Incidence**	**Major molecules released**
WNV	RNA	3-15%	No reliable estimates	TNF-α, IL-1β, IFN
RABV	RNA	> 99%*	55,000/yr	TNF-α, IL-1β, IL-6 IFNs
JE	RNA	25-50%	30-50,000/yr Local incidence rates 1-10/100,000 persons; Can reach more than 100/100,000 persons during outbreaks. Travel-associated risk: 1/5,000–20,000 per week of travel	IL-1β, IL-8, IL-6 TNF-α, IFNs
HSV-1	DNA	30-70%	Western country populations: 0.2/100,000 persons Neonatal HSV infection: 2-3/10,000 live births	TNF-α, IL-1β, IL-6
VZV	DNA	25-100%	1/2000 infected persons	

* Following development of neurological symptoms

In addition, emerging RNA viral pathogens such as West Nile virus (WNV) (Gubler, 2008), Japanese encephalitis virus (JE), Australian bat Lyssavirus (Samaratunga et al., 1998), retroviruses, and Nipah virus (McCormack et al., 2002) have become increasingly important causes of encephalitis. Nipah virus was first identified in 1999 and the mortality rate associated with acute encephalitis caused by this virus has been estimated to be between 40% and 75%, with a 2001 outbreak resulting in a 63% mortality rate (as reviewed in Tyler, 2009). While most survivors make a full recovery, 20%

demonstrate neurological sequelae including persistent convulsions and personality changes, and a small number subsequently relapse or develop delayed-onset encephalitis.

Worldwide, the flavivirus JE virus is the most common cause of arthropod-borne encephalitis with over 50,000 cases reported per year in China, Southeast Asia, and India (Whitley et al., 1999). Epidemics due to this arbovirus result in a mortality rate that ranges from 30-50% with death usually occurring within the first week (Roos et al., 1999; Soloman et al., 2003), and the development of sustained neurological deficits in approximately half of the survivors (Ghosh et al., 2009). Another flavivirus, WNV, is also transmitted by *Culex* spp. mosquitoes and can cause fatal encephalitis or long-term neurological sequelae. Once inside the CNS, JE and WNV infect neurons (Koh et al., 2005; Shrestha et al., 2003; Wang et al., 1997) leading to neuronal apoptosis (Liao et al., 1998; Parquet et al., 2001) and causing severe immunopathology (Licon Luna et al., 2002). WNV has been associated with over 12,000 cases of encephalitis and/or meningitis in the US with a mortality rate of 9.5% as reported by the CDC, and is especially life-threatening in susceptible individuals such as the elderly (Debiasi and Tyler, 2006).

DNA viral agents that can infect the CNS include varicella zoster virus (VZV) and several viruses in the human herpesvirus family that can cause encephalitis, including human herpesvirus 6 (Isaacson et al., 2005), herpes simplex virus 1 (HSV-1), and herpes simplex virus 2 (HSV-2) (Baringer., 2008). VZV is a medically important cause of encephalitis, particularly in children younger than 16 years of age (Stahl et al., 2008), characterized by up to 25% mortality and rates close to 100% in immunocompromised patients. However, HSV is considered to be an endemic cause of encephalitis in the

United States. HSV-1 is the most frequent cause of this disease in adults (Stahl et al, 2008) while HSV-2 is the most significant etiological agent of severe encephalitis in children accounting for approximately 10% of all cases in the U.S. (Bingham, 2000; Roos, 1999). Neonatal HSV-2 encephalitis, with treatment, has a mortality of 14% (compared to 85% without treatment) and severe neurological dysfunction is observed in 50 to 70% of surviving individuals. HSV-1 encephalitis in older children represents the most common cause of sporadic fatal viral encephalitis (Baringer, 2008). Untreated patients with HSV-1 encephalitis have a 70% mortality rate, while patients who receive early treatment have a 40% chance of recovering with no significant neurological deficits. However, despite appropriate diagnosis and therapy, the mortality rate remains at 30% (Baringer, 2008; Xu et al., 2006). HSV encephalitis may follow primary infection but most cases are the due to the reactivation of latent virus in the olfactory bulb or trigeminal ganglia and subsequent retrograde axonal transport into the CNS (as reviewed in Conrady et al., 2010).

The complexity seen in viral encephalitis due to the array of causative agents, clinical presentations, and severity has made defining the mechanisms that underlie disease pathology challenging. Ideally, host responses following CNS infection should result in rapid neutralization of the invading pathogen with minimal collateral damage to the sensitive and poorly regenerating neural tissue. However, viral CNS infections are often associated with inadequate antiviral responses and/or the rapid and severe onset of damaging inflammation (as reviewed in Savarin and Bergmann, 2008). While the brain has traditionally been viewed as a "victim organ" of infiltrating leukocytes, it has become increasingly apparent that specialized resident glial cells, such as microglia and astrocytes,

play an essential role in regulating the permeability of the blood-brain barrier, promoting the recruitment of leukocytes, and the activation of such cells following infiltration.

1.1 Glial cells play a critical role in the initiation of virally-induced neuroinflammation

Microglia and astrocytes are well known to play an important role in homeostasis within the CNS and to support neuronal cell function. However, these cell types are increasingly recognized as key players in the development of protective immune responses or the progression of damaging inflammation during CNS disease states (Bauer et al., 1995; Stoll et al., 1999; Fischer et al., 2001; Dong and Benveniste, 2001). Microglia are resident myeloid immune cells of the CNS and, similar to other myeloid cells such as macrophages and dendritic cells, these cells are facultative phagocytes and express antigen presenting MHC class II molecules (Hickey and Kimpura., 1988). Importantly, microglia produce key pro-inflammatory mediators such as interleukin (IL)-1β (Martin et al, 1993), tumor necrosis factor (TNF)-α (Streit et al., 1998), IL-6 (Kiefer et al., 1993), and bioactive IL-12 p70 (Suzumura et al., 1998; Aloisi et al., 1997) following activation. Astrocytes are the major glial cell type in the brain and may also function as an immune effector cell (Dong and Benveniste, 2001). Stimulated astrocytes can express an array of inflammatory cytokines and chemokines that can initiate leukocyte migration across the blood-brain barrier and promote effector functions in these infiltrating cells (Dong and Benveniste, 2001). As such, these cells are ideally situated to detect and respond to invading pathogens such as neurotropic viruses.

Viral infections in the CNS are known to broadly trigger glial activation and the release of pro-inflammatory molecules. For example, it is known that pro-inflammatory cytokines, IFN-γ and TNF-α, are markedly increased in CNS tissues during HIV-1

infection in the brain and microglia have been shown to respond to HSV-1 by secreting pro-inflammatory and chemotactic molecules including TNF-α, IL-1β, IL-6, IL-12, CCL7, CCL8, CCL9, CXCL1, CXCL2, CXCL4, and CXCL5 (Aravalli et al., 2005; Lokensgard et al., 2001). Human microglia have also been shown to respond to RNA viruses including WNV by producing cytokines and chemokines (Cheeran et al., 2005). Elevated levels of IL-1β are readily detectable in neural tissue from WNV encephalitis patients and cultured human glia produce this potent inflammatory cytokine in response to WNV challenge (van Marle et al., 2007). We have also documented the ability of primary cultured microglia and astrocytes to respond to several RNA and DNA viruses, including vesicular stomatitis virus (VSV), Sendai virus, and murine gammaherpesvirus (MHV)-68, by producing inflammatory mediators such as IL-6, TNF-α, and IL-1β (Chauhan et al., 2010; Rasley et al., 2004). Importantly, we confirmed that *in vivo* VSV administration results in viral infection of both glial cell types *in situ*, while earlier studies demonstrated that microglia and astrocytes respond to VSV by proliferating, producing inducible nitric oxide synthase (iNOS), and increasing the expression of cell surface MHC class II molecules (Bi et al., 1995), responses that are likely to set the stage for subsequent inflammatory damage.

An immune response to a virus in the CNS requires the recognition of this pathogen and the mechanisms by which innate immune cells achieve this have only recently become apparent with the discovery of a variety of cell surface and cytosolic molecules that serve as sensors for viral components. Such pattern recognition receptors (PRRs) are present on and within immune sentinel cells and appear to bind conserved viral structures known as pathogen-associated molecular patterns (PAMPs). These

PAMPs have several features that make them ideal targets for innate immune recognition. First, PAMPs are unique to microbes and viruses allowing for 'self ' versus 'non-self' recognition by the innate immune system. Second, PAMPs are often conserved among similar pathogens allowing a relatively small number of PRRs to detect a broad array of challenges. In addition, PAMPs tend to be essential for microbial/viral survival making pathogen evasion by mutation or deletion unlikely.

PRR engagement by PAMPs can precipitate the production of anti-viral type I interferons (IFN) and/or the release of chemotactic and inflammatory molecules that can then direct a subsequent adaptive immune response. Three families of PRRs have been defined: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid inducible gene (RIG)-I-like receptors (RLRs). As such, these molecules represent important mechanisms by which a protective host response or potentially damaging inflammation could be initiated (Kawai and Akira, 2007; Yoneyama et al., 2010).

1.2 TLRs mediate glial responses to extracellular or endosomal viral motifs

Toll was first identified as a gene critical for fruit fly embryogenesis (Hashimoto et al., 1988) but its products were subsequently shown to play an important role in *Drosophila's* immunity to fungal infections (Lemaitre et al., 1996). Interestingly, similar gene products were found in mammalian cells and these Toll-like receptors have since been shown to play an important role in the initiation of innate immune responses to infection (Medzhitov et al., 1997). To date, at least 13 TLRs have been discovered in mammals and members of this family of cell surface receptors can elicit inflammatory and/or anti-viral mediator production and can serve to initiate or modify adaptive immune

responses (as reviewed in Kawai and Akira, 2008). Several TLRs have been demonstrated to specifically recognize viral motifs, including TLRs 2, 3, 7, 8, and 9. While TLR2 is best known to bind a variety of microbial cell wall component including lipoproteins, peptidoglycans, and lipoteichoic acid present in bacterial cell walls and zymosan, an integral yeast cell wall component, this sensor can also recognize yet-identified viral motifs. In contrast, endosomal TLRs such as TLR3 recognize viral double-stranded RNA (dsRNA) and its synthetic analog, polyinosine-deoxycytidylic acid (polyI:C), while TLR7 and TLR8 mediate responses to GU-rich single-stranded RNA (ssRNA) produced in virus-infected cells. Finally, TLR9 detects bacterial and viral DNA with unmethylated CpG motifs.

Importantly, studies from our group and others have demonstrated that both microglia and astrocytes express these cell surface/endosomal receptors for viral motifs, including TLR3 (Bsibsi et al., 2006; Jack et al., 2005) TLR7 (Jack et al., 2005), and TLR9 (Jack et al., 2005), either constitutively or following activation. Upon ligand binding, TLRs dimerize and undergo conformational changes precipitating a complex cascade of intracellular signaling events that ultimately result in activation of transcription factors including nuclear factor-κB (NF-κB), and interferon regulatory factors (IRF) 3 and 7 (as reviewed in Wilkins and Gale, 2010; Kawai and Akira, 2008). NF-κB is a critical transcription factor in the regulation of inflammatory cytokine production, while IRF3 and IRF7 activation stimulates expression of antiviral genes including IFN-β. Hence, activation of cells via these receptors can initiate the repertoire of defense mechanisms used by the innate immune system against viral pathogens.

TLR3 is perhaps the most widely studied PRR in the perception of viral pathogens by glial cells. Isolated murine wild-type microglia are known to respond to the TLR3 ligand polyI:C by secreting TNF-α and IL-6 (Melton et al., 2003). In line with these findings, intracerebral administration of poly(I:C) has been shown to elicit microglial (Melton et al., 2003, Town et al., 2006) and astrocyte (Melton et al., 2003) activation associated with neuronal loss, a response that is nearly absent in TLR3 knockout animals (Town et al., 2006). Circumstantial evidence for the involvement of TLR3 in encephalitis following RABV infection comes from the finding that the expression of this PRR is upregulated prior to the onset of lethal neuroinflammation (McKimmie et al., 2005).

Several studies have also implicated TLR3 activation in WNV-associated pathogenicity. Double stranded RNA, a ligand for TLR3, is produced by WNV during infection and has been shown to trigger the release of cytokines and chemokines from human microglia (Cheeran et al., 2005). Seemingly in keeping with these observations, TLR3 deficient mice were found to be resistant to lethal WNV-associated encephalitis. The fulminate CNS inflammatory response seen in wild type mice, characterized in part by activated microglia, was markedly reduced or absent in knockout animals following intraperitoneal WNV infection (Wang and Fikrig, 2004). However, this reduced CNS disease occurs despite increased peripheral viral loads and reduced systemic production of antiviral and proinflammatory cytokines (Wang et al., 2004). These results suggest that decreased systemic inflammation in the absence of TLR3 expression may maintain blood-brain-barrier integrity and prevent WNV access into the CNS (Wang et al., 2004). Such a hypothesis is supported by the demonstration that direct intracerebral

administration of WNV leads to identical outcomes in wild type and TLR3 deficient mice (Wang et al., 2004). However, it must be noted that at least one study appears to contradict this finding in that neuronal WNV replication was increased in TLR3 knockout animals following subcutaneous infection, and the reason for this apparent disparity is presently unclear (Daffis et al., 2008).

Another endosome-associated PRR, TLR7 also appears to play an important role in inflammatory and/or protective glial responses to viral pathogens. Intracerebral administration of the TLR7 agonist imiquimod leads to pronounced neuroinflammation, with upregulated expression of TNF-α, IL-1β, IL-6, and IL-12 as well as the chemokines CCL2, CCL3, CXCL1, CXCL9, and CXCL10 (Butchi et al., 2008). Interestingly however, this effect appears to be predominantly due to the activation of astrocytes rather than microglia (Butchi et al., 2008). Upregulation of TLR7 has been demonstrated in the brains of infected mice prior to lethal RABV-induced inflammation (McKimmie et al., 2005). Furthermore, mice lacking the expression of myeloid differentiation primary response gene (MyD) 88, a critical downstream effector molecule of TLRs including TLR7, have increased susceptibility to an attenuated RABV strain and TLR7 deficient mice exhibited a phenotype with deficits in both the development of peripheral immunity and rabies virus clearance from the CNS (Li et al., 2011). As such, these data indicate a possible role for TLR7 in viral clearance from infected brain tissue. In contrast, another report has shown that macrophages isolated from TLR7 deficient animals had reduced IL-12 and IL-23 responses following *in vitro* WNV infection and TLR7 knockout mice demonstrated increased susceptibility to lethal WNV encephalitis (Town et al., 2009).

It has recently been shown that TLR2 contributes to detrimental inflammatory cytokine production following intracranial administration with HSV-1, with the absence of TLR2 expression leading to decreased peripheral (Kurt-Jones et al., 2004) and CNS (Aravalli et al., 2005) inflammatory responses and increased survival in mice. In addition, multiple reports have shown that maximal in vitro microglial and astrocyte responses to HSV-1 are dependent on the presence of TLR2 (Aravalli et al., 2005; Lokensgard et al., 2001; Schachtele et al., 2010; Wang et al., 2012) and HSV-1-infected microglia derived from TLR2 deficient mice have been demonstrated to elicit less neuronal oxidative damage and death in mixed neural cell cultures in comparison to infected wild-type microglia (Schachtele et al., 2010). However, it should be noted that these studies differ in their assessments of the relative importance of this PRR in such responses, and marked elevations in serum IL-6 concentrations have been found in TLR2 deficient mice following HSV-1 infection (Kurt-Jones et al., 2004). Together, these data suggest while TLR2 may play a major role in HSV-induced pathology and mortality, other possibly redundant mechanism(s) are likely to be involved.

In addition to the detection of HSV virion motifs by TLR2, genomic HSV DNA can be recognized by TLR9 and at least one study suggests that this sensor might play a more significant role in lethal HSV-1 encephalitis than TLR2 (Lima et al., 2010). In contrast, others have shown that the absence of TLR9 alone does not impact survival, type I IFN levels, or viral replication in the brain following HSV infection (Wang et al., 2012). However, it appears likely that these sensors could act in a cooperative or synergistic manner, as TLR2/9 double knockout mice show significantly greater

susceptibility to HSV-1 infection than mice deficient in either TLR2 or TLR9 alone (Lima et al., 2010).

Taken together, this body of work suggests that TLR activation is a significant contributor to sustained glial activation, inflammatory mediator production, and neuronal loss during viral CNS infections. However, it is important to note that TLRs can also mediate beneficial effects. For example, the activation of glial cells via TLR2, 3, or 9 has been shown to initiate the production of type I IFNs (Conrady et al., 2010; de Diego et al., 2010; Zhou et al., 2009) which limit viral replication through the induction of proteins such as RNase L and IFN-induced dsRNA-activated protein kinase, resulting in mRNA degradation and cessation of translation, respectively (Samuel et al., 2001). However, the mechanisms that keep the balance between damaging and protective glial responses have not been identified and are likely to include a multitude of variables including the number of infectious organisms, the kinetics of glial activation and immune mediator production, the specific identity of the responsive glial cell, and the direct influence of the pathogen itself on the cellular response. Moreover, evidence is accumulating that TLR expression is not limited to glial cells and that neurons can also express such PRRs under certain conditions (Cameron et al., 2007; Ma et al., 2006). However, the functional relevance of TLR expression in neurons has not been resolved.

While cell-surface receptors like TLRs appear to play an important role in glial responses to extracellular pathogens or those internalized into intracellular compartments, the ability of such cells to respond to pathogens in the absence of TLR expression suggests that additional mechanisms must be present. Furthermore, our recent studies employing the model neurotropic RNA virus, VSV, demonstrate that viral replication is

required to elicit robust immune responses by infected microglia and astrocytes, and indicate that mechanisms exist to sense the presence of replicative viral products within infected cells. We have shown that heat inactivated wild-type VSV or a host-range VSV mutant that is replication impaired (Grdzelishvili et al., 2005) elicit glial immune responses that are an order of magnitude smaller than those induced by wild type viral particles (Chauhan et al., 2010). These results suggest that VSV-induced glial responses are not predominantly mediated by cell surface and/or endosomal PRRs and suggest that active viral replication is a critical requirement for such responses.

The recent demonstration that microglia and astrocytes express members of the newly described RLR family of PRRs may provide a mechanism underlying the replication-dependent nature of glial responses (Furr et al., 2008). RLRs such as RIG-I and melanoma differentiation-associated gene 5 (MDA5) have been shown to serve as intracellular PRRs for viral nucleic acid motifs (Takeuchi and Akira, 2008; Kato et al., 2006), and their involvement in inflammatory and antiviral responses in other cell types raises the intriguing possibility that these cytosolic sensors could play an important role in glial responses to viral infection. However, the role of cytosolic PRRs in CNS inflammation has yet to be determined.

1.3 RIG-like receptors can mediate protective immune responses and damaging CNS inflammation following RNA virus infection

In contrast to cell-surface TLRs, RLRs detect viral RNA motifs or processed self-RNA in the cytoplasm to trigger innate immunity and inflammation. These intracellular sensors are responsible for the production of type I IFN in most cell types in response to RNA virus infection (Saito et al., 2007; Kato et al., 2006; Lui et al., 2007; Sumpter et al., 2005), a notable exception being plasmacytoid dendritic cells. In addition to such

antiviral responses, RLR engagement also elicits robust inflammatory cytokine production (Takeuchi and Akira, 2010). Helicase interaction with viral RNA induces the recruitment of downstream effector molecules including interferon promoter stimulator (IPS)-1 (also known as VISA, Cardif or MAVS) which localizes to the mitochondrial membrane and/or to peroxisomes (Dixit et al., 2010), and TNF receptor associated factor (TRAF)3. This leads to the subsequent activation of mitogen activated protein (MAP) kinases, and the IKK-related kinases, TRAF family member-associated NF-κB activator (TANK) binding kinase 1 and IκB kinase (IKK)-I (as shown in Figure 1). These kinases activate the transcription factors NF-κB and IRF3, which translocate into the nucleus and activate transcription of IFN stimulated genes and inflammatory cytokines. RLRs are comprised of three domains: N-terminal caspase activation and recruitment domains (CARDs), a helicase domain, and a C-terminal domain (CTD). Although RIG-I and MDA5 may share similar signaling features (Yoneyama et al., 2005) and structural homology (Yoneyama et al., 2004), it is now known that the two helicases discriminate among different ligands to trigger the innate immune response to RNA viruses.

Signaling by RIG-I is triggered during infection by a number of RNA viruses and by the presence of synthetic RNA transcribed in vitro (Saito et al., 2007; Kato et al., 2006; Lui et al., 2007; Sumpter et al., 2005). More recently, RIG-I has been implicated in the recognition of single stranded or double stranded RNA moieties that harbor 5′ triphosphate ends (5'ppp-ssRNA and 5'ppp-dsRNA, respectively) (Hornung et al., 2006; Pichlmair et al., 2006) or the detection of RNAs that assume complex secondary structures (Saito et al., 2007; Sumpter et al., 2005). Other recent studies have elucidated the structural basis for activation of RIG-I by such RNA ligands. Inactive RIG-I has an

open conformation in which the CARD domains are sequestered by a helical domain inserted between the helicase moieties. When activated, ATP and RNA binding induce a major rearrangement in RIG-I to a closed conformation in which the helicase and CTD bind the blunt end 5'ppp-dsRNA with perfect complementarity (Kowalinski, 2011).

In contrast, MDA5 has been reported to be more selective and is triggered during picornavirus infections or in the presence of poly(I:C) (Gitlin et al., 2006; Kato et al., 2006; Loo et al., 2008). The CTD domains of MDA5 and RIG-I involved in RNA binding have been suggested to be responsible for the differences in RNA binding affinity seen between these RLRs. Each have a similar fold and a similar basic surface but distinct RNA binding loops exist within the CTD for RIG-I and MDA5, and conformation of this RNA binding loop seems to be responsible for sensitivity to dsRNA or 5'ppp-ssRNA. Mutation studies of the basic surface and the RNA binding loop support this conclusion, as well as other RLR structural studies (Takahasi et al., 2009; Li et al., 2009).

We have recently demonstrated that the expression of RIG-I and MDA5 is discernable in uninfected mouse brain tissue, and both show elevated expression following intranasal administration of VSV (Furr et al., 2008). VSV is a neurotropic negative-sense single-stranded RNA virus that closely resembles rabies virus. In mice, intranasal VSV infection results in a severe encephalitis with rapid activation and proliferation of microglia and astrocytes (Huneycutt et al., 1993; Bi et al., 1995). Our lab has previously demonstrated that isolated murine microglia and astrocytes constitutively express both RIG-I and MDA5 transcripts and protein, as well as the critical downstream effector molecule, IPS-1, and has shown that expression is elevated following challenge

with VSV (Furr et al., 2008). Furthermore, the expression of these novel viral sensors has been confirmed in human astrocytoma cells (Yoshida et al., 2007).

Some studies suggest that IPS-1 is essential for triggering protective innate and adaptive immunity against WNV in the CNS. In these studies, mice deficient in the RLR adaptor molecule IPS-1 exhibited increased susceptibility to WNV infection, characterized by enhanced inflammation, viral replication and rapid dissemination into the CNS (Suthar et al., 2010). However, it is important to note that glial cells were not the focus of this study and IPS-1 dependent IFN responses and limiting of viral replication was attributed to effects on systemically generated immune cells and cortical neurons, perhaps via changes in the numbers and/or function of regulatory T-cells (Suthar et al., 2010). As such, these data suggest an innate/adaptive immune interface mediated through RLR signaling that regulates the balance of the immune response to WNV infection. Together, these findings raise the exciting possibility that RLR molecules may play important roles in the detection of viral CNS pathogens, and depending upon the host cell type or particular viral pathogen, the initiation of protective immune responses or, alternatively, the progression of damaging inflammation within the brain. However, the functional role of these molecules in glial responses to RNA viral pathogens within the CNS has not been explored

1.4 Newly discovered cytosolic viral sensors may mediate glial responses to DNA viruses

Recently, novel cytosolic DNA sensors have been discovered that include DNA-dependent activator of interferon-regulatory factors (DAI; also known as Z-DNA binding protein 1) (Takaoka et al., 2007) and a DNA sensing inflammasome consisting of the HIN200 protein, absent in melanoma 2 (AIM2) (Schroder and Tschopp, 2010). Acting in

concert with TLRs, these receptors may provide a diverse repertoire of mechanisms to alert the cell to viral and microbial DNA, leading to the activation of the innate immune system in a similar manner to that seen with RLR-mediated responses to viral RNA. DAI reportedly senses cytosolic DNA using two N-terminal Z-DNA binding domains (ZBDs) and a third putative DNA binding domain located next to the second ZBD. The crystal structure of the DAI/Z-DNA complex reveals that DAI adopts an unusual binding mode for Z-DNA recognition (Ha et al., 2008). The DAI ZBDs bind DNA and both must be bound for full B-to-Z conversion and so it conceivable that the binding of two DAI proteins to each cytoplasmic dsDNA elicits DAI dimerization and subsequent innate immune activation (Wang et al., 2008; Ha et al., 2008).

Importantly, DAI has been shown to recognize double-stranded DNA in its canonical B helical form (B-DNA) (Takaoka et al., 2007) and elicit type-I IFN and inflammatory cytokine production in a TLR9-independent manner (Ishii et al., 2006; Stetson et al., 2007) in other cells types. DAI elicits such immune functions via activation of the IRF and NF-κB transcription factors. DAI-induced IRF activation depends on TANK-binding kinase 1 (TBK1). In contrast, DAI-induced NF-κB activation is mediated through interactions between two receptor-interacting protein (RIP) homotypic interaction motifs (RHIMs) in the DAI protein and RHIM-containing kinases, RIP1 and RIP3 (Rebsamen et al., 2009; Kaiser et al., 2008) (as shown in Figure 2), as shown by the demonstration that knockdown of either RIP1 or RIP3 inhibits DAI-induced NF-κB activation. Overexpression of DAI in mouse fibroblasts enhances DNA-mediated type I IFN production while DAI knockdown attenuates responses to viral DNA (Takaoka et al., 2007). In an additional study, DNA-mediated activation of NF-κB and

the induction of NF-κB-dependent genes and their products were also significantly inhibited in cells following DAI knockdown (Rebsamen et al., 2009). As such, these data support a significant role for DAI in DNA-mediated activation of the NF-κB pathway and viral recognition. However, it is interesting to note that DAI-mediated NF-κB activation appears to facilitate replication of HIV-1 although the mechanisms underlying this effect remain unclear (Hayashi et al., 2010). The recent description of a molecule, stimulator of interferon genes (STING), that is essential for the recognition of intracellular DNA provides the possibility of a role for this protein in DAI-induced responses. Interestingly, loss of STING renders mice susceptible to lethal infection with the DNA virus HSV-1 (Ishikawa and Barber, 2008).

Furthermore, unlike the RNA sensor RIG-I, DAI detects a structure common to both self and non-self DNA. This suggests that the discrimination between these types of DNA is based on its subcellular localization, and may likely be a function of the amount and the length of the DNA present in the cytosol rather than a specific chemical feature of the ligand (as reviewed in Keating et al., 2011). Interestingly, a recent study has shown that HSV-1 DNA, mislocated from the viral capsid, is readily detectable in the cytoplasm of infected RAW264.7 macrophages, bone marrow-derived macrophages (BMDMs), and THP-1 cells (Unterholzner et al., 2010). However, it remains to be determined how detection of cellular DNA in the cytoplasm by this sensor is avoided.

DAI has been reported to be constitutively expressed in the lymph nodes and spleen, although detectable levels are also found in circulating leukocytes and other tissues including bone marrow and small intestines (Rothenburg et al., 2002). To date, the

functional expression of DAI by microglia and astrocytes and its involvement in innate immune responses to DNA viruses in the CNS have not been explored.

1.5 PRRs act in a cooperative manner to induce glial responses to viral challenges

While the preceding sections have focused on the roles of specific individual PRRs in the detection of viral pathogens by CNS cells, this narrow approach is fraught with peril. It is highly likely that glial responses represent the sum of the inputs from disparate cell surface, endosomal, and cytosolic viral sensors. It has been demonstrated that the level of expression of a particular TLR, NLR, or RLR by microglia and astrocytes can be regulated by ligands for dissimilar PRRs (Bowman et al., 2003; Jack et al., 2005; Carpentier et al., 2005). Furthermore, mechanisms of positive and negative regulation of RLR signaling have been identified that include signaling crosstalk between RLR, NLR and DNA-sensing pathways, and caspase networks. For example, it has been shown that mice deficient in the expression of the DNA-sensing downstream effector molecule STING (as shown in Figure 2) are highly susceptible to infection by negative-stranded viruses including VSV suggesting a role for this signaling molecule in both DAI and RIG-I mediated cell activation (Ishikawa and Barber, 2008). Immunoprecipitation studies indicate STING associates with RIG-I complexes in close proximity with endoplasmic reticulum associated mitochondria or possibly peroxisomes (Ishikawa et al., 2009; Dixit et al., 2010) (as shown in Figure 1). Furthermore, a number of RNA viruses including yellow fever virus, dengue virus, and hepatitis C virus, encode proteins with significant structural homology to STING and at least one of these proteins produced by the dengue virus has been shown to inhibit STING function (Ishikawa et al., 2009). As

such, these observations suggest a role for STING in antiviral host responses to both DNA and RNA viruses.

Interestingly, there have been recent reports of a role for the well-characterized bacterial sensor, NOD2, in the recognition of viral infections and the activation of IRF3 via IPS-1 (Sabbah et al., 2009). NOD2 is known to signal through the adaptor molecule RIP2 to trigger NF-κB activation following recognition of muramyl dipeptide, a component of bacterial cell walls. In their study, Sabbah and colleagues (2009) suggested that this sensor was important in the detection of ssRNA derived from respiratory syncytial virus. However, it is currently unknown whether NOD2 is directly activated by this viral ligand, or is activated secondarily following association with other PRRs or RNA binding factors.

Another example of crosstalk between viral sensing pathways is the recent demonstration that RIG-I may be important in the production of type I IFNs by cells in response to DNA viral challenge in a DNA-dependent RNA polymerase III manner (Ablasser et al., 2009; Chui et al., 2009). In this described pathway, AT-rich double-stranded DNA serves as a template for RNA polymerase III and is transcribed into RNA containing a 5'-triphosphate, the ligand for RIG-I. Subsequent activation of RIG-I by this ligand has been shown to induce type I IFN production and to activate NF-κB, and studies employing RNA polymerase III knockdown or pharmacological inhibitors of this enzyme have shown that this pathway is important in cell responses to the gammaherpesvirus Epstein-Barr virus (Ablasser et al., 2009; Chui et al., 2009). These studies indicate that the transcription of cytosolic viral DNA by RNA polymerase III and subsequent RNA recognition by RIG-I may provide an additional mechanism for the

perception of DNA viruses by host cells. Furthermore, it remains to be determined whether such interactions between other PRRs and RIG-I-associated signaling components occur in glial cells or whether they play a significant role during viral CNS infections.

1.6 Hypothesis and present study

In the present study, we have tested the hypothesis that the activation of microglia and astrocytes in response to viral pathogens of the CNS is mediated by novel intracellular pattern recognition receptors, RIG-I and DAI. As shown in Figure 3, the current body of knowledge concerning the sensors involved in pathogen recognition, the signaling pathways that result from activation, and the outcomes following viral infection in glial cells is very limited.

Here, we demonstrate that primary murine microglia and murine/human astrocytes constitutively express detectable levels of the intracellular pattern recognition receptor, RIG-I, and show that such expression is elevated following exposure to a model neurotropic RNA virus, VSV. Evidence for the functional nature of RIG-I expression in these cells comes from the observation that this molecule associates with its downstream effector molecule, IPS-1, following VSV infection and from the finding that a specific ligand for RIG-I, 5'ppp-ssRNA, elicits significant inflammatory cytokine responses. Importantly, RIG-I knockdown significantly reduces this inflammatory cytokine production by VSV-infected human glia and inhibits the production of soluble neurotoxic mediators by these cells. These findings directly implicate RIG-I in the initiation of inflammatory immune responses by glial cells and provide a potential mechanism underlying the neuronal cell death associated with acute viral CNS infections.

In addition, we show that isolated microglia and astrocytes constitutively express DAI and its effector molecules RIP3 and STING, and show that such expression is upregulated following DNA virus challenge. We demonstrate that these resident CNS cells express DAI *in situ*, and show that its expression is similarly elevated in a murine model of HSV-1 encephalitis. Importantly, we show B-DNA transfection can elicit inflammatory cytokine production by isolated glial cells and DAI knockdown can significantly reduce microglial and astrocyte responses to HSV-1. Finally, we demonstrate that HSV-1 challenged microglia and astrocytes release neurotoxic mediators and show that such production is significantly attenuated following DAI knockdown. Therefore, the functional expression of DAI by microglia and astrocytes may represent an important innate immune mechanism underlying the rapid and potentially lethal inflammation associated with neurotropic DNA virus infection.

Interestingly, we show that glial inflammatory responses to the DNA virus HSV-1 were also dependent on the expression of RIG-I and the activity of polymerase III, while glial responses to the RNA virus VSV required the expression of RIG-I but were DAI and polymerase III independent. Collectively, these studies provide the first evidence that both RIG-I and DAI play a critical role in the recognition of viral pathogens by resident CNS cells and suggest that these novel intracellular pattern recognition receptors may underlie the damaging inflammation and neuronal cell death associated with acute neurotropic viral infections.

1.7 Figures

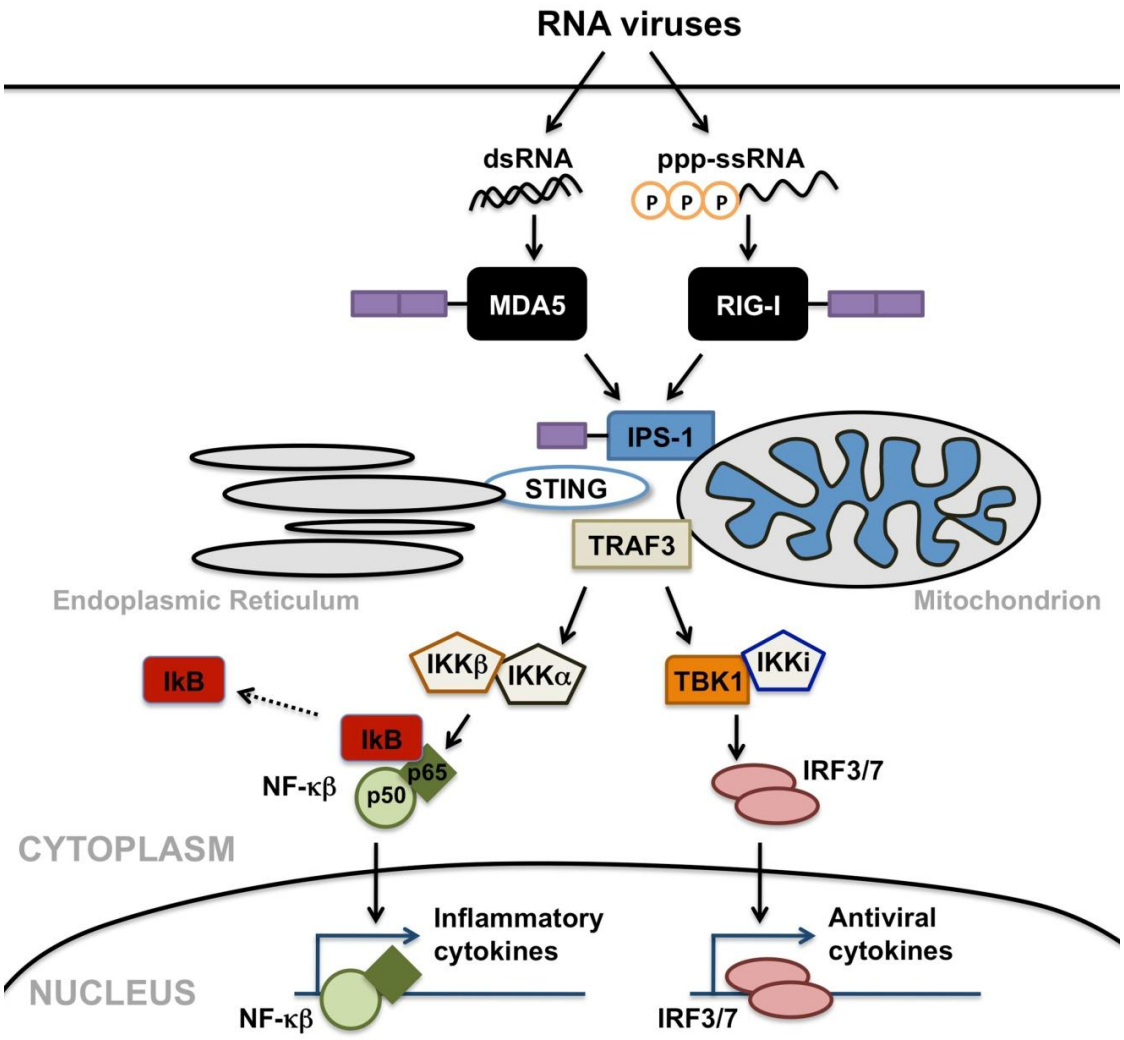

FIGURE 1: RLRs detect viral RNA motifs in the cytoplasm to trigger innate immunity and inflammation. MDA5 recognizes double-stranded RNA moieties (dsRNA), while RIG-I perceives short single or double-stranded RNAs with 5′ triphosphate ends (ppp-ssRNA). Helicase interaction with these viral RNAs induces the recruitment of downstream effector molecules including mitochondrial- or peroxisomal-associated IPS-1 (also known as VISA, Cardif or MAVS), TRAF3, and/or STING, leading to the activation of IKK-related kinases, TBK1, and IKK-I. These kinases activate the transcription factors NF-κB and IRF3, which translocate into the nucleus and activate transcription of antiviral and inflammatory cytokines.

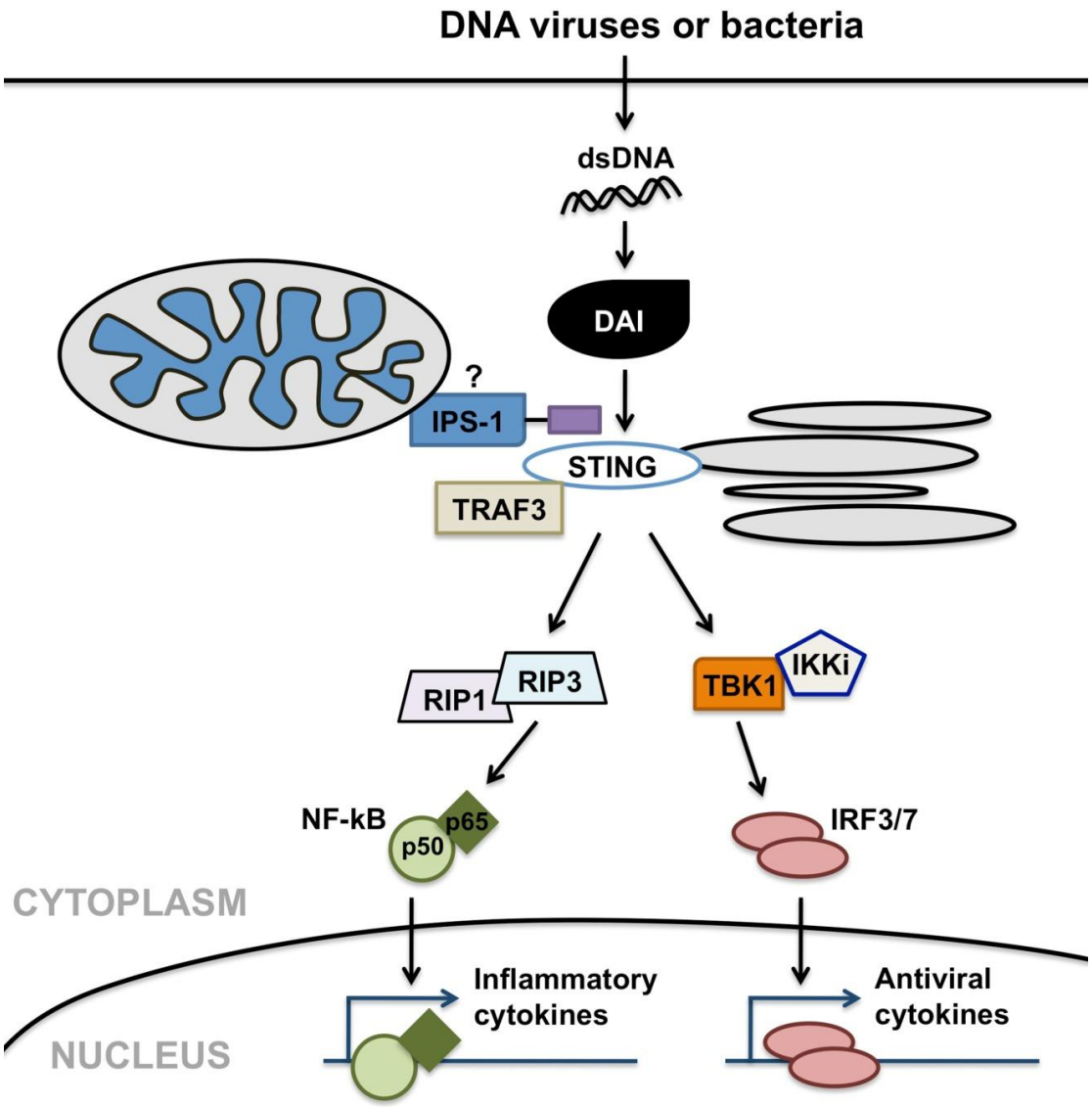

FIGURE 2: Recognition of DNA viruses by DAI. DAI has been shown to detect viral or microbial double-stranded DNA (dsDNA) in the cytoplasm of infected cells, and elicit antiviral and inflammatory cytokine production. DAI elicits such immune functions via possible interactions with mitochondrial- or peroxisomal-associated IPS-1, and/or STING, facilitating the activation of the IRF and NF-kB transcription factors. DAI-induced IRF activation is dependent on TANK-binding kinase 1 (TBK1). In contrast, DAI-induced NF-κB activation is mediated through interactions involving RIP1 and RIP3.

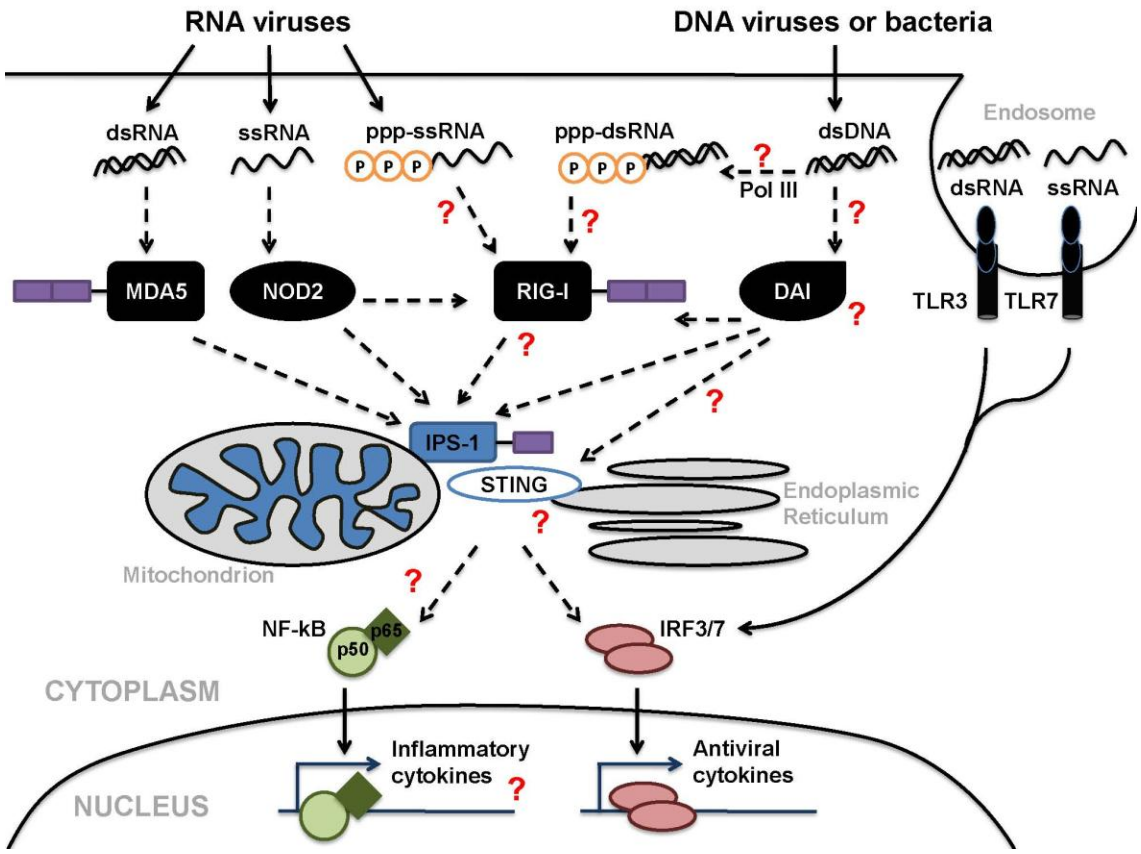

FIGURE 3: Possible pathways for viral detection by glial cells. While these pathways have been explored and/or suggested in other cell types, it is currently unknown if these receptors, including RIG-I and DAI, are expressed or functional in glial cells. In addition, the possibility that glial cells express newly discovered critical downstream adaptors such as STING and recently described pathways involving RNA polymerase III (Pol III) could provide additional mechanisms for the perception of DNA viruses by glial cells. Finally, it remains to be determined whether the activation of these possible pathways results in damaging or protective immune responses, including the production of inflammatory cytokines.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Isolation of neonatal brain microglia and astrocytes

Briefly, six to eight neonatal C57 mouse brains per preparation were dissected free of meninges and large blood vessels and finely minced with sterile surgical scissors. The minced tissue was then forced through a wire screen and briefly incubated with 0.01% trypsin–0.005% EDTA for 5 minutes. The cell suspension was then washed and this mixed glial culture was maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and gentamicin for 2 weeks. A microglia culture was obtained from this mixed glial culture by shaking flasks for 2 hours at 200 rpm in an orbital shaker (New Brunswick Scientific, Edison, NJ) and allowing the transferred dislodged cells to adhere to new tissue culture vessels for 1 hour. The media were then removed and fresh RPMI 1640 with 10% FBS and 20% conditioned medium from LADMAC cells (ATCC, CRL-2420) as a source of colony stimulating factor-1 was added to maintain microglia cultures for 1 week, while astrocytes were cultured in RPMI 1640 containing 10% FBS. Cells isolated in this manner were >95% and >97% authentic microglia and astrocytes, respectively, as assessed by their characteristic morphology and by expression of the microglial/macrophage markers CD11b and F4/80 or the astrocyte marker glial fibrillary acidic protein (GFAP) as determined by immunofluorescent microscopy. All studies were performed in accordance with relevant federal guidelines and institutional policies regarding the use of animals for research purposes (IACUC Protocol # 11-010).

2.2 Primary human astrocyte and human neuronal cell culture

Primary astrocytes isolated from human brain tissue were purchased from ScienCell (Carlsbad, CA). Cells were maintained in a specially formulated medium supplemented with FBS, growth supplement, and penicillin/streptomycin solution, purchased from the supplier. These commercially available cells have been characterized as authentic astrocytes by the vendor according to their expression of GFAP as determined by immunofluorescent analyses. The human cortical neuron cell line, HCN-1A, was obtained from ATCC (CRL-10442; Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's media containing 10% FBS with penicillin and streptomycin.

2.3 Preparation of viral stocks

VSV viral stocks were prepared by infecting monolayer cultures of baby hamster kidney epithelial cells (BHK-21; ATCC, CCL-10) with wild type VSV (Indiana strain) at a low multiplicity of infection (MOI) of 0.05 plaque-forming units (PFU) per cell and incubated for 48 hours at 34^{o}C in SFM4MegaVir protein-free medium (Thermo Scientific Hyclone, Waltham, MA). The cell-free medium containing released viruses was collected and viral titers were quantified using a standard plaque assay of serial dilutions of VSV on BHK-21 cells at 34^{o}C. VSV-GFP is VSV (Indiana strain) encoding green fluorescent protein as an extra gene between G and L genes (Das et al., 2006), and was kindly provided by Dr. Asit K. Pattnaik (University of Nebraska). VSV-GFP was utilized to verify the absence of infectious particles in conditioned medium following filtration. HSV-1 viral stocks were prepared by infecting monolayer cultures of Vero cells (ATCC; CCL-81) with HSV-1 (MacIntyre strain from a patient with encephalitis;

ATCC, VR-539) at a low multiplicity of infection (MOI) of 0.05 plaque-forming units (PFU) per cell and incubated for 48 hours at 34^{o}C in SFM4MegaVir protein-free medium (Thermo Scientific Hyclone, Waltham, MA). Cells were removed with trypsin and pulse sonicated (Vibra Cell; Sonics & Materials Inc., Newtown, CT) to release intact virions. The sonicated material was centrifuged to remove unwanted cellular debris and the viral titers in the cell-free supernatant was quantified using a standard plaque assay of serial dilutions of HSV-1 on Vero cells at 34^{o}C. MHV-68 viral stocks were prepared by infecting monolayer cultures of BHK-21 cells (ATCC, CCL-10) at a low viral MOI of 0.1 PFU per cell. After 24 hours, the cells were removed with trypsin and pulse sonicated to release intact virions. The sonicated material was centrifuged to remove unwanted cellular debris and the supernatant containing virus was aliquoted and stored at −80°C. The medium containing released virus was collected and viral titers were quantified using a standard plaque assay of threefold serial dilutions on NIH-3T3 cells at 34^{o}C.

2.4 *In vitro* HSV-1, MHV-68, and VSV infections

Microglia and astrocytes were infected with HSV-1, MHV-68, or VSV at MOIs of 0.01, 0.1, 1, and 10 PFU per cell and the viruses were allowed to adsorb for 1 hour prior to washing to remove nonadherent viral particles. Cultures were maintained for 12 or 24 hours prior to collection of culture supernatants or preparation of whole cell protein isolates.

2.5 *In vivo* HSV-1 infection

HSV-1 (MacIntyre strain) was administered to 4-6 week-old female C57BL/6 mice (Jackson Laboratories) via intranasal (i.n.) infection essentially as described by our laboratory with other viral pathogens (Elsawa et al., 2003). Anesthetized animals were

untreated or received i.n. HSV-1 administration (1×10^5 - 1×10^6 PFU) in PBS (final volume of 20 ul). Animals were euthanized at 4 days post-infection and protein isolates were prepared from whole brain tissue homogenates or glial cells acutely isolated from infected and uninfected animals by flow cytometry as described below. All studies were performed in accordance with relevant federal guidelines and institutional policies regarding the use of animals for research purposes (IACUC Protocol # 08-027).

2.6 Acute isolation and cytometric analysis of CNS cells

Mixed CNS cells were acutely isolated from infected and uninfected animals using a protocol modified from Campanella and coworkers (2003) as previously described (Chauhan et al., 2008). Whole brains were rapidly removed and mechanically disrupted in a glass homogenizer, washed, and resuspended in PBS/30% Percoll (Fluka, Sigma Aldrich, St. Louis, MO) solution. This was overlaid on a gradient containing 37% and 70% Percoll solutions and centrifuged at 600 X G for 20 minutes at room temperature. Glial cells were then collected from the interface and washed with PBS. Microglia/macrophages and astrocytes and were isolated from the mixed glial preparation by flow cytometry using an R-phycoerythrin conjugated monoclonal antibody directed against mouse CD11b (BD Biosciences, clone M1-70) and an AlexaFluor488-conjugated monoclonal antibody directed against mouse GFAP (Invitrogen, Eugene, OR, clone 131-17719), respectively. Protein isolates were prepared and analyzed for the presence of DAI, STING, RIP3, and viral gG1 by immunoblot analysis.

2.7 Isolation of RNA and semi-quantitative reverse transcribed PCR

Poly(A) + RNA was isolated from glial cells and reverse transcribed as previously described (Bowman et al., 2003). Polymerase chain reactions (PCR) were performed to

determine the expression of mRNA encoding RIG-I. Positive and negative strand PCR primers used, respectively, were 5'-GTGCAAAGCCTTGGCATGT-3' and 5'-TGGCTTGGGATGTGGTCTACTC-3' to amplify mRNA encoding human RIG-1 (115 bp fragment). Primers were designed by using OligoCalc (Kibbe, 2007: www.basic.northwestern.edu/biotools/oligocalc.html) based on their location in different exons of the genomic sequence and their lack of significant homology to sequences present in GenBank (MacVector Sequence analysis software, IBI, New Haven, CT). The identity of the PCR amplified fragments was verified by size comparison with DNA standards (Promega). The input RNA was normalized to the expression of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

2.8 Nuclear and cytoplasmic protein extractions

Nuclear and cytoplasmic protein extracts were prepared from human astrocytes by suspension in lysis buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM HEPES, 0.5 mM dithiothreitol (DTT), and protease inhibitor cocktail for 20 minutes at 4^oC. The nuclei and other fragments were then separated by centrifugation and supernatants were retained as cytoplasmic fractions. Nuclei were lysed by exposure to a high salt buffer containing 420 mM NaCl, 15 mM $MgCl_2$, 20 mM HEPES, 0.2 mM EDTA, 20% glycerol, 0.5 mM phenylmethylsulphonylfluoride (PMSF), 1 mM DTT, 1% NP-40, and 2.5 ug/ml leupeptin for 20 minutes at 4^oC. Samples were then cleared of cellular debris by centrifugation and supernatants containing the nuclear fraction were collected.

2.9 Western blot analyses for RIG-1, IPS-1, NF-κB p65 (RelA), DAI, RIP3, STING, and the HSV-1 gene product glycoprotein G-1 (gG1)

Western blot analyses for the presence of RIG-1, IPS-1, NF-κB, DAI, RIP3, and STING in glial cells or whole brain homogenates were performed as described previously

by our laboratory (Bowman et al., 2003). After incubation with a rabbit polyclonal antibody against RIG-1 (Abgent, San Diego, CA), IPS-1 (Abcam, Cambridge, MA), the p65 (RelA) subunit of NF-kB (Millipore, Billerica, MA), DAI (Abcam, Cambridge, MA), RIP3 (Abcam, Cambridge, MA), STING (Abcam, Cambridge, MA), or a mouse monoclonal antibody against gG1 (Abnova, Taipei, Taiwan) for 24 hours at 4°C, blots were washed and incubated in the presence of an horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Cell Signaling, Danvers, MA) or an HRP-conjugated anti-mouse antibody (Cell Signaling, Danvers, MA), respectively. Bound enzyme was detected with the Super Signal system (Thermo Scientific, Rockford, IL). To assess total protein loading in each well, immunoblots were reprobed with a goat anti-mouse β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the expression level of an unidentified non-specific protein present in Coomassie blue stained membranes was determined. Immunoblots shown are representative of at least three separate experiments.

2.10 Co-immunoprecipitation

Briefly, human astrocytes (2×10^6) infected with VSV were washed with ice-cold PBS and lysed at 4°C at 1 hour following infection in Tris-buffered saline with EDTA (150 mM NaCl, 5 mM EDTA, 20 mM Tris, pH 7.5) plus 1% Brij-97 (Sigma-Aldrich) and 10 units/mL aprotinin (Calbiochem, San Diego, CA), 1 mM PMSF, and 1 ug/mL pepstatin A. The lysates were incubated with protein A agarose beads (Pierce Endogen) conjugated with antibodies directed against IPS-1 for 18 hours at 4°C. The immunoprecipitated material was subsequently subjected to immunoblot analysis for the

presence of RIG-I. To assess total protein loading in each well, immunoblots were probed for the presence of total IPS-1 content in the immunoprecipitated material.

2.11 *In vitro* stimulation of glial cells with the RIG-I ligand, 5'-triphosphate single stranded RNA

To generate 5'-triphosphate single stranded RNA (5'ppp-ssRNA), we created a linear template for run-off in vitro transcription by digesting the pGEM-SeV-NP plasmid (previously described in Curran and Kolakofsky, 1991) with EcoRV. EcoRV cuts pGEM-SeV-NP at a single site allowing a 342 base RNA to be generated when using the SP6 promoter. RNA was generated using the MAXIscript In Vitro Transcription Kit (Ambion) at 37^{o}C for 2.5 hours in a final volume of 200 ul containing approximately 1 ug of linearized pGEM-SeV-NP, all four NTPs, transcription buffer, and 200 U of SP6 enzyme mix. The reaction was then separated into two fractions and one was twice treated with 2 U calf intestinal alkaline phosphatase (CIAP; Promega) at 37^{o}C for 15 minutes to dephosphorylate the RNA for use as a negative control in our studies. Both CIAP-treated and untreated RNAs were then incubated at 37^{o}C for 20 minutes in the presence of 10 U Turbo DNase (Ambion) to remove the plasmid template. Following addition of EDTA (35 mM), the samples were phenol/chloroform extracted and ethanol precipitated. Precipitated RNA was resuspended in RNase free water to a final concentration of 200-250 ng/ul. The final RNA products were analyzed by electrophoresis on a 2.5% agarose gel.

5'ppp-ssRNA or dephosphorylated ssRNA was introduced intracellularly into human astrocytes using Tfx-20 transfection reagent (Promega) or into murine glial cells using FuGENE HD transfection reagent (Promega) at concentrations of 4 ug/ml and 8

ug/ml for one hour. For comparison purposes, cells were exposed to transfection reagent alone or 5'ppp-ssRNA in the absence of transfection reagent.

2.12 *In vitro* stimulation of glial cells with the DAI ligand, B-DNA

Poly(dA:dT) double-stranded B-DNA (InvivoGen, San Diego, CA) was directly introduced into microglia and astrocytes at concentrations of 3 ug/ml and 6 ug/ml using FuGENE HD Transfection Reagent (Promega, Madison, WI) according to the manufacturer's instructions. At 6 and 12 hours following transfection, culture supernatants and whole cell protein isolates were collected for analysis.

2.13 siRNA-mediated knockdown of RIG-I and DAI

Three validated Stealth RNAi™ siRNA duplexes targeting human or murine RIG-I, as well as universal negative control siRNA not homologous to anything in the vertebrate transcriptome, were purchased from Invitrogen (Carlsbad, CA). Glial cells were transfected with each siRNA duplex individually or in concert using Tfx-20 Reagent (Promega) or FuGENE HD transfection reagent (Promega) according to the manufacturer's instructions. Antibiotic-free media was replaced with complete media at 6 hours following transfection. At 24, 48 and 72 hours after transfection, whole cell lysates were collected for immunoblot analyses to confirm RIG-I expression knockdown.

Three validated Stealth RNAi™ siRNA duplexes targeting murine DAI, in addition to a universal negative control siRNA that was not homologous to anything in the vertebrate transcriptome, were purchased from Invitrogen (Carlsbad, CA). Microglia and astrocytes were transfected with the siRNA duplexes using FuGENE HD transfection reagent. Antibiotic-free media was replaced with complete media at 6 hours following transfection. At 72 hours post-transfection, DAI knockdown was confirmed in whole cell

lysates by immunoblot analysis. In some studies, double knockdown was achieved in glial cells by transfecting with both RIG-I and DAI siRNA duplexes described above, using FuGENE HD transfection reagent.

2.14 Quantification of IL-6 and TNF-α secretion in glial cell culture supernatants

Specific capture ELISAs were performed to quantify concentrations of IL-6 and TNF-α. A commercially available ELISA kit was used to measure murine TNF-α (R&D Systems, Minneapolis, MN) secretion while murine IL-6 secretion was measured using a rat anti-mouse IL-6 capture antibody (Clone MP5-20F3) and a biotinylated rat anti-mouse IL-6 detection antibody (Clone MP5-C2311) (BD Pharmingen, San Diego, CA). A commercially available ELISA kit (Ready-Set-Go!; eBioscience, San Diego, CA) was used to assay human TNF-α levels according to the manufacturer's instructions. A pair of capture and detection anti-human cytokine antibodies (BD Biosciences, San Diego, CA) was used to quantify IL-6 (clone MQ2-13A5 and biotinylated clone MQ2-39C3). Bound antibody was detected by addition of streptavidin-horseradish peroxidase (BD Biosciences). After addition of TMB substrate and H_2SO_4 stop solution, absorbances were measured at 450 nm. A standard curve was constructed using varying dilutions of recombinant cytokines (BD Biosciences) and the cytokine content of culture supernatants determined by extrapolation of absorbances to the standard curve.

2.15 Assessment of soluble neurotoxic mediator production by infected glial cells

Transfected or untransfected microglia and astrocytes were uninfected or infected with VSV or HSV-1 (MOI of 1 PFU per cell) for 1 hour prior to washing to remove non-adherent viral particles. At 24 hours following infection, the conditioned medium was collected and filtered using a 0.1-um syringe filter (Sterlitech, Kent,WA) to remove

residual VSV (0.1 to 0.4 mm in length) or HSV-1 particles (180 – 200 nm in diameter) prior to addition to resting HCN-1A human neuronal cells or CATH.a murine neuron-like cell cultures (ATCC #CRL-11179). Our ability to remove all infectious viral particles from the conditioned medium was verified in parallel experiments by demonstrating the exclusion of the smaller VSV (0.1-0.4 nm in length) engineered to express green fluorescent protein. At 4, 8, and 12 hours following filtered conditioned medium addition, the numbers of adherent HCN-1A or CATH.a cells were counted in ten microscopy fields and viability was assessed by trypan blue exclusion.

2.16 RNA polymerase III inhibition

Glial cells were pre-treated with 10 U (300 pmol) of Tagetin™ RNA Polymerase inhibitor (Epicentre Biotechnologies) for 10 hours. Following treatment, cells were either infected with VSV (MOI of 10) for 12 hours or transfected with siRNA against DAI for 72 hours, then infected with HSV-1 (MOI of 10) for 12 hours.

2.17 Densitometric analyses

Densitometric analyses of immunoblots were performed using ImageJ (obtained from the NIH Web site http://rsbweb.nih.gov/ij/download.html). Results are presented as mean values of arbitrary densitometric units corrected for background intensity normalized to the expression of β-actin or irrelevant proteins, or as fold increases over levels in unstimulated cells.

2.18 Statistical analyses

Results of the present studies were tested statistically by ANOVA and Tukey's post hoc test using commercially available software (SAS Institute, Cary, NC). In all experiments,

results were considered statistically significant when a P-value of less than 0.05 was obtained.

CHAPTER THREE: A ROLE FOR DNA-DEPENDENT ACTIVATOR OF INTERFERON REGULATORY FACTOR IN THE RECOGNITION OF HERPES SIMPLEX VIRUS TYPE 1 BY GLIAL CELLS

3.1 Rationale

The neurotropic DNA virus HSV-1 is capable of causing severe necrotizing encephalitis and accounts for 95% of all fatal cases of sporadic viral encephalitis (Xu et al., 2006). Untreated HSV-1 encephalitis has a 70% mortality rate and patients who receive early treatment have only a 40% chance of recovery without significant neurological deficits. Furthermore, the overall mortality rate of HSV-1 encephalitis in the US remains at 30% despite improvements in diagnosis and therapy (Baringer, 2008). It has recently been shown that HSV-1-mediated cytokine and chemokine production contributes to CNS damage following *in vivo* infection suggesting that an overzealous host response is a major contributor to the neuropathology associated with acute viral encephalitis (Nakajima et al., 2001; Rosler et al., 1998; Wildemann et al., 1997). However, the mechanisms underlying the onset of such damaging neuroinflammation have not been defined.

The rapid onset of inflammation following CNS infection suggests that resident glial cells play a pivotal role in the initiation and progression of encephalitis. Microglia and astrocytes are resident cells of the CNS cells and are susceptible to HSV-1 infection (Aravalli et al., 2006). Both of these cell types are now recognized to have innate

immune functions and respond to invading pathogens by producing soluble mediators that can promote inflammation and leukocyte recruitment across the blood-brain barrier (Konat et al., 2006; Carpentier et al., 2005; Falsig et al., 2008). Importantly, microglia have been shown to produce significant levels of the proinflammatory cytokines TNF-α and IL-6 in response to HSV-1 infection (Lokensgard et al., 2007). While host immune cells have been shown to recognize HSV-1 or HSV-2 via cell-surface or endosomal pattern recognition receptors including TLR2 and TLR9 (Lund et al., 2003; Kurt-Jones et al., 2004), the means by which resident CNS cells perceive DNA virus infection and initiate inflammatory cytokine production have not been defined.

Recently, the cytosolic protein, DAI (also known as Z-DNA-binding protein 1 (ZBP1)), has been reported to function as an innate sensor of intracellular viral DNA (Fu et al., 1999; Ishii et al., 2006; Takaoka et al., 2007; Wang et al., 2008). This molecule has been shown to recognize double-stranded DNA in its canonical B helical form (B-DNA) (Takaoka et al., 2007; Wang et al., 2008) and elicit type-I IFN production in a TANK-binding kinase 1 and IRF3 dependent, but TLR9-independent, manner (Ishii et al., 2006; Stetson et al., 2006). Importantly, this cytosolic sensor has been reported to mediate type I IFN and inflammatory cytokine production by HSV-1-infected murine fibroblasts (Takaoka et al., 2007). To date, DAI expression has not been reported in microglia or astrocytes, and a role for this putative viral sensor in the innate immune responses of resident CNS cells to viral challenge has not been explored.

In the present study, we provide the first demonstration of constitutive and inducible expression of DAI and its downstream effector molecules by glial cells both *in vitro* and *in situ*. Importantly, we confirm the functional status of DAI in primary

microglia and astrocytes and demonstrate that this viral sensor plays a significant role in the inflammatory and neurotoxic responses of resident CNS cells to HSV-1 challenge.

3.2 Results

To determine whether resident CNS cells express DAI, we have assessed the expression of this cytoplasmic viral sensor in isolated primary murine microglia and astrocyte cultures. As shown in Figure 4, both microglia and astrocytes constitutively express detectable levels of DAI as determined by immunoblot analysis although it is noteworthy that resting astrocytes demonstrated higher expression levels than that seen in equal numbers of microglia. Interestingly, HSV-1 elicited an upregulation in microglial DAI expression by up to 23.8 fold over that seen in resting cells (Figure 4A). Similarly, despite robust constitutive expression, HSV-1 exposure was able to further increase DAI expression in astrocytes with a maximal increase of 2.2 fold over that seen in unstimulated cells (Figure 4B). Interestingly, the ability of viral challenge to augment DAI expression by primary glial cells was not limited to this neurotropic alphaherpesvirus as the lymphotropic (Flano et al., 2002) gammaherpesvirus, MHV-68, was also capable of eliciting robust increases in microglial DAI expression (Figure 4A) and causing modest increases in the expression of this molecule in astrocytes (7.3-fold) (Figure 4B).

Finally, to begin to determine whether DAI is functional in glial cells we have investigated whether these cells express RIP3 and STING, two critical downstream effector molecules in DAI signaling (Rebsamen et al., 2009; Ishikawa et al., 2009; Ishikawa and Barber, 2008). As shown in Figure 4C, both microglia and astrocytes

constitutively express robust levels of RIP3 and STING that were not significantly enhanced in either cell type following viral challenge.

To determine if glial cells express DAI *in situ*, we first assessed the expression of this viral sensor in microglia/macrophages and astrocytes acutely isolated from brain tissue of uninfected mice. As shown in Figure 5A, whole brain homogenates constitutively expressed robust levels of DAI. Interestingly, both GFAP+ astrocytes and CD11b+ microglia/macrophages isolated from uninfected brain tissue demonstrated only very low DAI expression levels (Figure 5B). This apparent discrepancy suggested that other major CNS cell types, such as neurons, might constitutively express DAI and this hypothesis was supported by the observation that resting CATH.a neuron-like cells expressed high levels of this molecule (Figure 5A).

Furthermore, we assessed glial DAI expression following *in vivo* HSV-1 infection. To confirm that intranasal HSV-1 administration results in *in situ* infection of glial cells, we first determined the expression of HSV-1 glycoprotein G1 (gG1) in microglia and astrocytes acutely isolated from infected and sham-infected mice. As shown in Figure 5C, robust levels of this HSV-1 gene product was readily detectable in glial cells isolated from the brains of infected animals, with astrocytes exhibiting higher levels of protein than microglia/macrophages, while it was absent in cells derived from sham infected mice. Importantly, *in vivo* HSV-1 challenge elicited marked increases in DAI expression in both microglia/macrophages (24.8 fold) and astrocytes (8.5 fold) acutely isolated from the brains of infected animals (Figure 5B) in the absence of significant increases in levels of this viral sensor in total brain protein isolates (Figure 5A). Finally, we have assessed the expression of the DAI downstream effector molecules

RIP3 and STING in glia isolated from HSV-1 infected and sham infected mice. As shown in Figure 5D, microglia/macrophages and astrocytes acutely isolated from uninfected brain tissue showed detectable levels of RIP3 and STING. Consistent with our *in vitro* findings, *in vivo* HSV-1 infection failed to elicit significant changes in RIP3 expression by either cell type. Interestingly, and in contrast to our studies in cultured cells, STING expression was significantly elevated in both microglia/macrophages and astrocytes following in vivo HSV-1 infection (Figure 5D).

To begin to determine whether DAI is functional in glial cells, we have assessed the sensitivity of isolated cultures of astrocytes and microglia to intracellular administration of B-DNA, a DAI ligand. As shown in Figure 6, B-DNA administration induced production of the inflammatory cytokines TNF-α and IL-6 by primary microglia or astrocytes as rapidly as 6 hours post-transfection at levels that matched or exceeded those elicited following 24-hour HSV-1 challenge.

To confirm the functional status of DAI in glial cells and to begin to determine the relative importance of this innate immune sensor in their responses to neurotropic DNS viruses we have assessed the effect of DAI knockdown on inflammatory cytokine production by HSV-1 challenged microglia and astrocytes. As shown in Figure 7A, siRNA directed against DAI specifically and markedly inhibited HSV-induced TNF-α production by murine microglia. Surprisingly, such an approach was not as effective in reducing cytokine production by astrocytes and only elicited a statistically significant reduction in TNF-α production at the highest viral MOI used (Figure 7B).

To begin to establish a role for DAI in the inflammatory CNS damage associated with neurotropic DNA viral infections, we have assessed the effect of DAI knockdown

on the production of soluble neurotoxic mediators by microglia and astrocytes following HSV-1 infection. As shown in Figure 8A, HSV-1 induced the production of soluble mediators by microglia that decreased CATH.a neuron-like cell viability as assessed by changes in cell attachment and trypan blue exclusion in an MOI and time dependent manner. Importantly, virally-induced neurotoxic mediator production was significantly attenuated in cells transfected with siRNA targeting DAI (-DAI) as compared to control cells that were transfected with scrambled non-specific siRNA (+DAI) (Figure 8A). Interestingly, HSV-infected primary astrocytes also produced a substance that elicited neuronal detachment and death, and production of such neurotoxic mediators was similarly attenuated in cells transfected with siRNA targeting DAI (Figure 8B). Together, these data point to a key role for DAI in the neurotoxic immune responses of microglia and astrocytes to DNA virus infection and may represent an important component in the inflammation and damage associated with viral encephalitis.

3.3 Conclusions

Several members of the herpesvirus family including human herpesvirus 6, HSV-1, and HSV-2 can elicit damaging CNS inflammation (Baringer, 2008; Isaacson et al., 2005). Acute HSV -1 infection or the reactivation of latent virus in the trigeminal nerve can lead to the development of severe encephalitis that is associated with a high degree of morbidity and mortality. While acyclovir is currently employed in the treatment of HSV encephalitis, drug-resistant strains of HSV-1 are beginning to emerge (Whitley et al., 1986; Kimberlin et al., 2007) and, despite improvements in diagnosis, such infections are associated with a 30% mortality rate and 62% of survivors recover with severe neurological deficits (Whitley et al., 1986; Raschilas et al., 2002; Hjalmarsson et al.,

2007). The treatment of HSV-1 associated encephalitis is especially challenging due to the rapid onset of disease and development of irreversible neurological damage in otherwise healthy individuals. These characteristics suggest that the innate immune responses of resident CNS cells play a pivotal role in disease progression, a notion that is supported by the ability of human microglia to produce key inflammatory mediators in response to *in vitro* HSV challenge or following *in vivo* infection (Lokensgard et al., 2001; Marques et al., 2004; Marques et al., 2008). However, much of our current understanding of HSV-1 encephalitis pathogenesis comes from rodent models in which intranasal HSV-1 administration results in an acute necrotizing encephalitis that closely resembles human disease (Mori et al., 2005; Ikemoto et al., 1995; Esiri et al., 1995; Mansur et al., 2005; Nair et al., 2007). In these models, HSV-1 infects both neurons and glial cells and elicits inflammatory mediator production that precedes leukocyte infiltration into the CNS (Mori et al., 2005; Beers et al., 1995). Such findings are consistent with the recognized ability of other viral pathogens to induce inflammatory cytokine production by microglia (Mariani et al., 2009), and the susceptibility and responsiveness of astrocytes to productive HSV-1 infection (Aravalli et al., 2006; Dix et al., 1992). However, the mechanisms by which resident CNS cells recognize DNA viral pathogens such as HSV-1 have not been fully defined.

We and others have demonstrated that microglia and astrocytes express an array of cell surface and endosomal innate pattern recognition receptors, including TLR2 (Konat et al., 2006), TLR3 (Bsibsi et al., 2006; Jack et al., 2005), TLR7 (Jack et al., 2005), and TLR9 (Jack et al., 2005), that are capable of recognizing viral motifs (as reviewed in Kumar et al., 2011). Importantly, roles for each of these cell surface sensors

have been described in the perception of HSV by a variety of cell types (Lund et al., 2003; Kurt-Jones et al., 2004; Zhang et al., 2007; Wilkins et al., 2010). However, it is becoming increasingly apparent that cells such as glia possess intracellular sensors that can detect when the cytosolic compartment has been compromised. Our lab has previously demonstrated that murine cells functionally express RIG-I and MDA5 (Furr et al., 2008), two members of the RIG-I-like family of helicases that have been shown to function as intracellular pattern recognition receptors for replicative viral RNA motifs (Kumar et al., 2011; Wilkins et al., 2010). It is possible that such receptors may also indirectly serve as sensors for viral and/or bacterial DNA via the actions of RNA polymerase III (Ablasser et al., 2009) although a role for this pathway in HSV recognition by immune cells remains controversial (Melchjorsen et al., 2010). Interestingly, a number of cytosolic proteins including DAI have been described that can directly mediate cellular responses to dsDNA (Takaoka et al., 2007; Wilkins et al., 2010). It has therefore been suggested that such sensors could play a critical role in the perception of viral DNA and this notion has been supported by the report that DAI mediates immune molecule production by HSV-1-infected murine fibroblasts (Takaoka et al., 2007).

In the present study, we provide the first evidence that glial cells express DAI. Resting cultures of primary microglia expressed low levels of this intracellular viral sensor, a finding that is consistent with the very low DAI expression observed in acutely isolated *ex vivo* microglia. In contrast, astrocytes in culture constitutively expressed robust levels of this molecule, although it should be noted that this might be attributable, in part, to our *in vitro* culture conditions, as astrocytes acutely isolated from uninfected

mice expressed somewhat lower DAI levels. Importantly, DAI expression was significantly elevated in microglia and astrocytes following either *in vitro* or *in vivo* HSV-1 challenge. Such upregulation was not specific to this neurotropic alphaherpesvirus as the leukotropic gammaherpesvirus, MHV-68, was also capable of elevating DAI expression by both cell types. As such, it is possible that glial perception of DNA viruses via this sensor could promote further DAI expression in a feed-forward manner.

The constitutive expression of this viral sensor by glial cells and its upregulation following viral challenge provide circumstantial evidence of a role for DAI in glial responses to DNA viral pathogens. This notion is further supported by the robust constitutive expression in microglia and astrocytes of IPS-1 (Furr et al., 2008), STING, and RIP3, molecules reported to serve as downstream effector molecules for DAI (Rebsamen et al., 2009; Ishikawa and Barber, 2009). Importantly, we have confirmed that DAI is functional in primary murine glia by showing that cytoplasmic administration of the DAI ligand, B-DNA, is a potent stimulus for the production of key inflammatory mediators by both microglia and astrocytes. Finally, we have demonstrated a major role for this intracellular viral sensor in the immune responses of primary murine microglia to a clinically relevant neurotropic DNA virus by demonstrating that DAI knockdown specifically and significantly inhibits HSV-1-induced cytokine production by this CNS sentinel cell. Interestingly, while B-DNA administration elicits robust inflammatory cytokine production by astrocytes comparable to similarly stimulated microglia, DAI knockdown was far less effective in attenuating HSV-induced cytokine production by astrocytes. While the reason for this difference is presently unclear, it is possible that the

siRNA approach employed did not sufficiently reduce the high DAI levels seen in cultured astrocytes. However, this appears unlikely due to the ability of siRNA directed against DAI to significantly reduce the production of neurotoxic substance(s) by astrocytes. Alternatively, these results could be due to the presence of redundant viral sensor mechanisms in astrocytes, but not microglia, that are capable of perceiving this DNA virus.

Host responses to viral CNS infections are increasingly recognized to play a major role in disease pathology and the neuroinflammation elicited by HSV-1 has been suggested to underlie the neurological damage associated with infection (Nakajima et al., 2001; Rosler et al., 1998; Wildemann et al., 1997). However, it not clear whether this inflammatory damage is mediated primarily by infiltrating leukocytes or by the responses of the resident cells of the CNS themselves. In support of the latter possibility, activated glial cells are known to be capable of producing toxic mediators that can cause widespread CNS damage (Marques et al., 2008). Furthermore, the rapidity with which HSV-1 travels from the initial site of infection to the brain all but assures escape from recognition by adaptive immune cells. Based on these observations, it appears likely that the production of cytotoxic substances by HSV-1 challenged glial cells could play a significant role in neuronal cell dysfunction and/or loss and contribute to the neuropathology associated with HSV-1 encephalitis. In the present study, we have demonstrated that soluble factor(s) released by HSV-infected microglia and astrocytes elicit neuronal cell damage/death and that the production of this factor(s) is dependent, at least in part, on the expression of DAI.

3.4 Figures

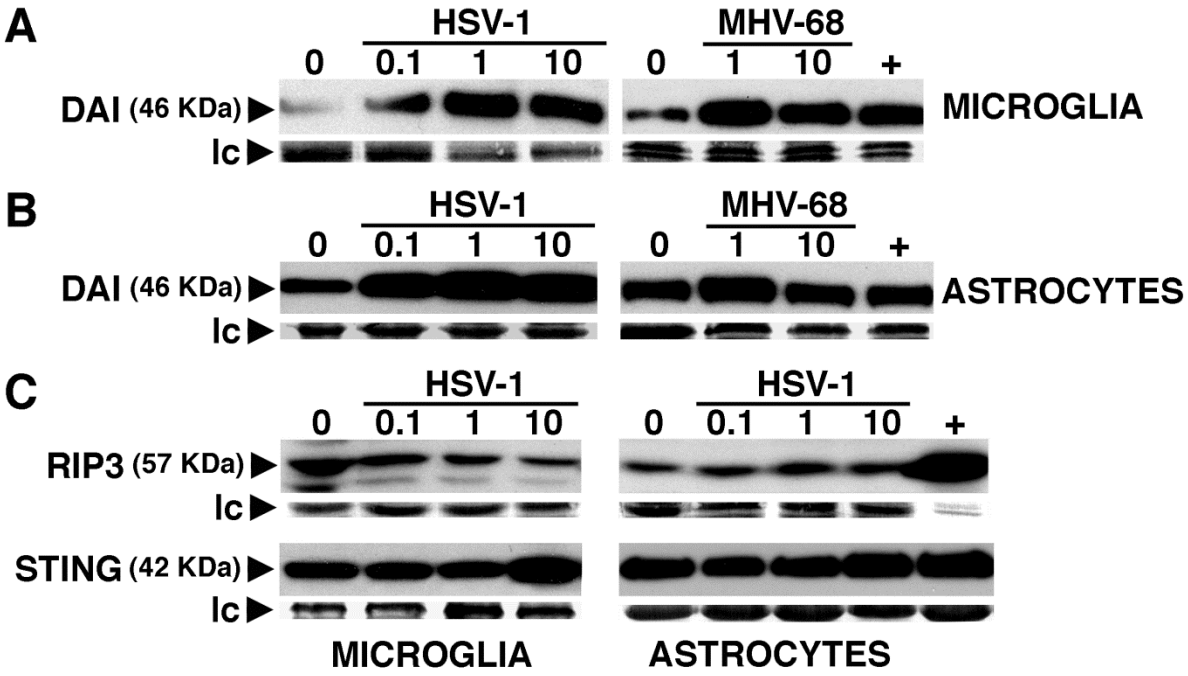

FIGURE 4: Cultured primary murine astrocytes and microglia constitutively express the cytoplasmic viral sensor DAI and associated effector molecules, and its expression is elevated following viral challenge. Cultured microglia (Panels A and C) or astrocytes (Panels B and C) were untreated (0) or infected with HSV-1 (MOI of 0.1, 1 and 10) or MHV-68 (MOI of 1 and 10). At 24 hours post-infection expression of DAI (Panels A and B), RIP3 and STING (Panel C) were determined in whole cell lysates by immunoblot analysis. Representative results are shown for one of three separate experiments.

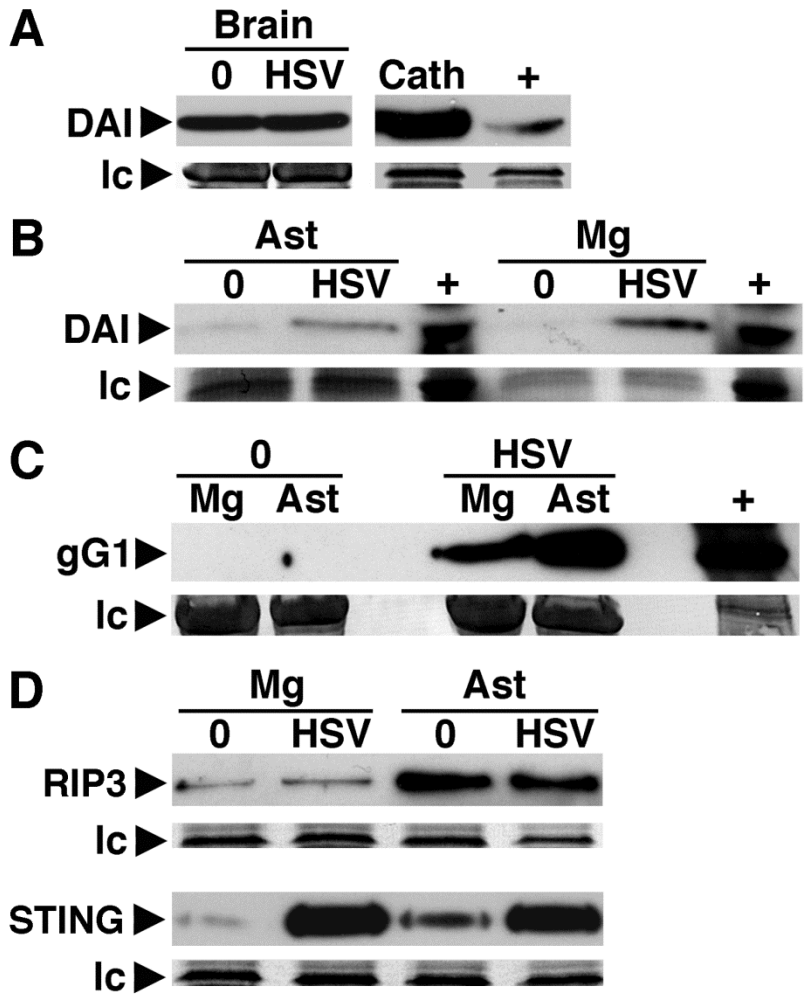

FIGURE 5: *Ex vivo* glial cells express DAI constitutively and/or inducibly following *in vivo* HSV-1 infection. Mice were sham-infected (0) or infected with HSV-1 (HSV: 1 x 10^5 PFU, i.n.). At 4 days post-infection protein isolates were prepared from whole brain tissue (Brain), or microglia (Mg) or astrocytes (Ast) acutely isolated by flow cytometry, and analyzed for the presence of DAI (Panels A and B), viral gG1 (Panel C), RIP3 and STING (Panel D) by immunoblot analysis. For comparison purposes, DAI expression in the murine neuron-like cell line CATH.a (Panel A: Cath) and murine small intestine tissue (Panels A and B: +), or viral gG1 expression in HSV-1 infected Vero cells (Panel C: +) is shown. Immunoblots shown are representative of three separate experiments.

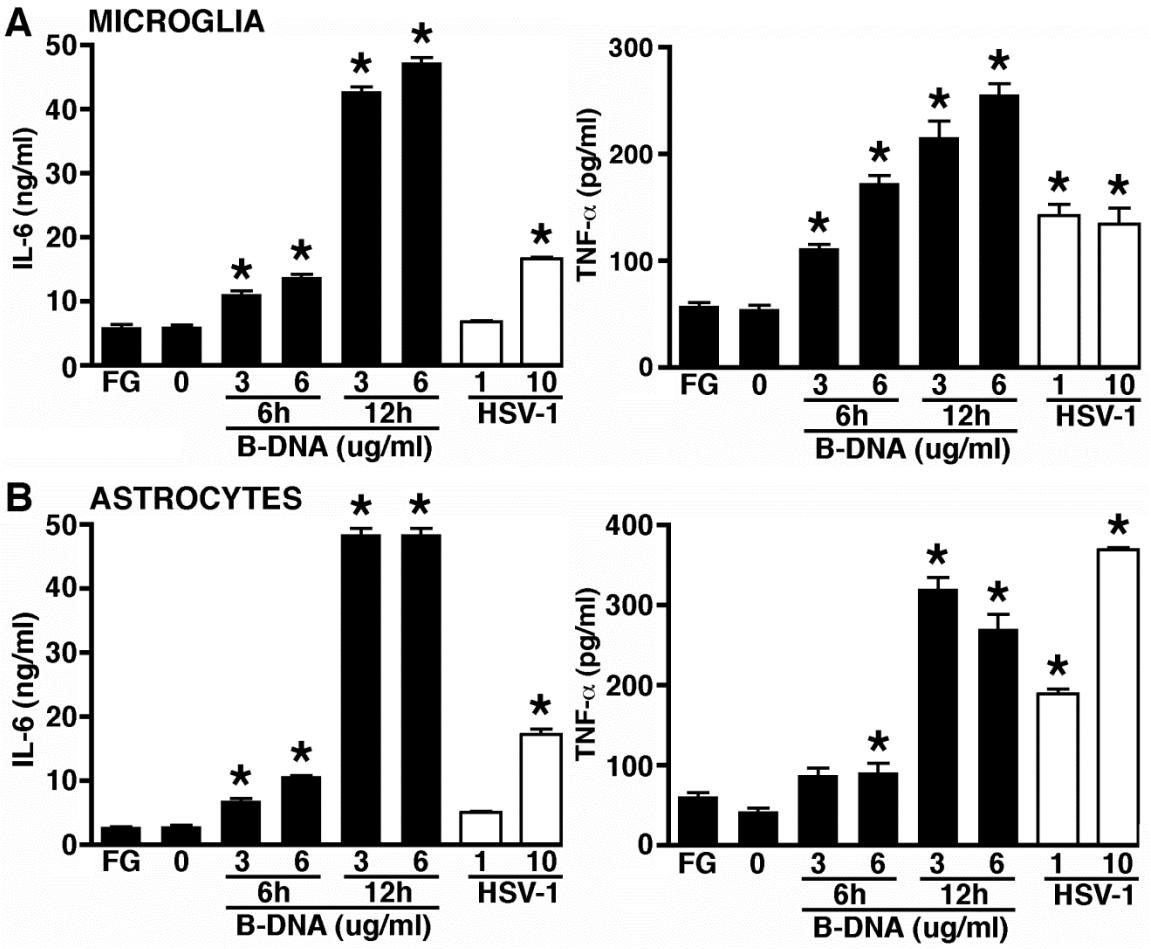

FIGURE 6: A specific ligand for DAI induces inflammatory cytokine production by isolated cultures of microglia and astrocytes. Microglia (Panel A) and astrocytes (Panel B) were untreated (0), treated with transfection reagent alone (FG), or transfected with the DAI ligand B-DNA (3 or 6 ug/ml). At 6 and 12 hours following transfection culture supernatants were collected and IL-6 and TNF-α content was assessed by specific capture ELISA. For comparison purposes inflammatory cytokine production was assessed at 24 hours following HSV-1 infection (MOI of 1 and 10). Data is expressed as mean +/- SEM (n = 6) and an asterisk indicates a statistically significant difference from levels produced by unstimulated cells ($p < 0.05$).

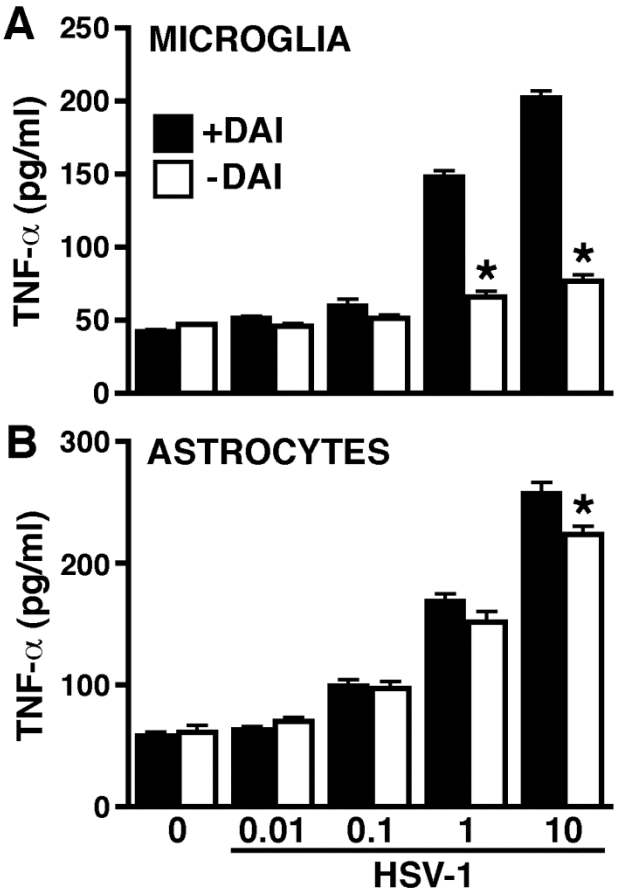

FIGURE 7: DAI knockdown attenuates HSV-1-induced inflammatory cytokine production by murine glial cells. Microglia (Panel A) and astrocytes (Panel B) were untreated (0), or transfected with siRNA targeting DAI (-DAI) or scrambled siRNA (+DAI). At 72 hours following transfection, cells were exposed to HSV-1 (MOI of 0.01, 0.1, 1, and 10) and levels of TNF-α in the culture supernatants were assessed at 24 hours post-infection. Data is expressed as mean +/- SEM (n = 3) and an asterisk indicates a statistically significant difference from levels produced by cells treated with scrambled siRNA ($p < 0.05$).

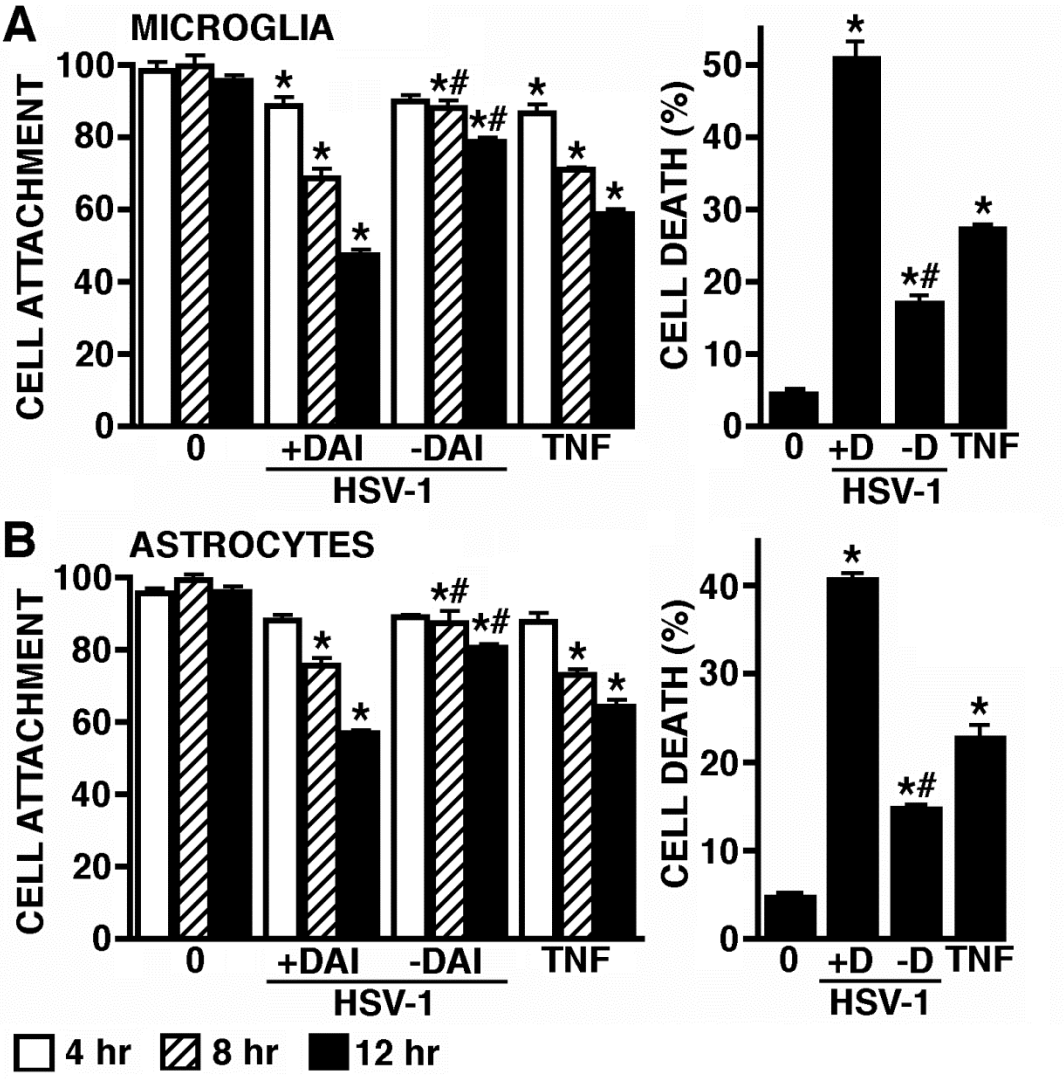

FIGURE 8: HSV-1 induces the production of soluble factor(s) by glial cells that elicits neuronal cell damage in a DAI dependent manner. Primary microglia (Panel A) or astrocytes (Panel B) were untreated (0) or transfected with siRNA targeting DAI (-DAI) or scrambled siRNA (+DAI). At 72 hours following the transfection protocol, cells were uninfected or infected with HSV-1 (MOI of 1) and cultured for a further 24 hours. Filtered conditioned media from these cells was placed on CATH.a cells and the number of attached cells was monitored at 4, 8 and 12 hours post-infection prior to assessment of cell death by trypan blue exclusion at 12 hours post-infection. Data is expressed as mean +/- SEM (n = 3). Asterisks indicate a statistically significant difference in the number of attached cells or degree of cell death from that seen in unstimulated cells while pound symbols indicate a statistically significant difference in these parameters between cells treated with siRNA directed against DAI and those that received scrambled siRNA ($p < 0.05$).

CHAPTER FOUR: RIG-I MEDIATES NONSEGMENTED NEGATIVE-SENSE RNAVIRUS-INDUCED INFLAMMATORY RESPONSES OF PRIMARY HUMAN ASTROCYTES

4.1 Rationale

The order *Mononegavirales* includes numerous etiological agents of important human viral diseases, including rabies, Ebola, Marburg, Nipah and Hendra viruses. Interestingly, members of this order include medically important pathogens with CNS involvement such as RABV and measles virus (Fishbein et al., 1993). Virally induced CNS inflammation can be initiated by the elevated expression of key soluble inflammatory mediators including two cytokines, TNF-α and IL-6. Importantly, elevated TNF-α (Nuovo et al., 2005; Solanki et al., 2009) and IL-6 (Phares et al., 2006) expression in the CNS has been reported following RNA virus infection and TNF-α has been implicated as a key factor in the pathogenesis of such infections (Camelo et al., 2000; Nuovo et al., 2005). Like RABV, VSV is a negative-sense single-stranded RNA virus belonging to the *Rhabdoviridae* family. VSV has the ability to infect neurons and generate acute encephalitis in mice (Huneycutt et al., 1993) and has therefore proven to be a useful model for this and other non-segmented, negative-sense RNA viruses. As described in work by Dr. Carol Reiss and others (Bi et al., 1995; Lundh et al., 1987; 1990; Miyoshi et al., 1971), intranasal inoculation of VSV in mice leads to infection of the olfactory bulb via the olfactory neurons and subsequently spreads throughout the CNS. This infection is associated with acute encephalitis, breakdown of the blood-brain

barrier, and a high degree of mortality (Honeycutt et al., 1993). Interestingly, VSV-induced encephalitis appears to be T-cell independent, having been observed in athymic mice after viral administration (Frei et al, 1989). As such, it is likely that the innate immune functions of resident CNS cells play an important role in the rapid inflammatory response following VSV infection.

There is growing appreciation that astrocytes can initiate and augment inflammation following infection (as reviewed in Dong and Benveniste, 2001). These glial cells are activated at the site of challenge and assume immune effector functions, including the production of TNF-α and IL-6 (Streit et al., 1998). Various RNA viruses have been reported to infect human and murine astrocytes in vitro (Ray et al., 1997) and in vivo (Garg et al., 2008; Wong et al., 2009), and the presence of RABV and Hendra virus antigens have been observed in astrocytes following in vivo infection (Jogai et al., 2000; Wong et al., 2009). Similarly, we have recently demonstrated that VSV can infect and replicate within cultured primary murine astrocytes (Chauhan et al., 2010). Astrocytes appear to respond to VSV as the encephalitis caused by this virus is associated with proliferation of this cell type and increased MHC class II molecule expression on their cell surface (Bi et al., 1995). Furthermore, we have demonstrated that astrocytes express TNF-α and IL-6 following challenge with VSV, and that such cytokine production is dependent on the active replication of this virus within these cells (Chauhan et al., 2010). While the ability of astrocytes to be infected by and respond to non-segmented negative-sense RNA viruses has been established, the mechanisms underlying the subsequent activation of this resident CNS cell type have not been defined.

Recently, a newly described group of molecules have been shown to function as intracellular sensors for replicative viral RNA (Takeuchi and Akira, 2007; Yoneyama et al, 2004; Yoneyama and Fujita, 2007). A member of the retinoic acid-inducible gene (RIG)-I like receptor (RLR) family, RIG-I is a soluble protein found in the cytosol of many cell types and has been shown to mediate innate immune responses to viral RNA. Responses initiated via this cytosolic sensor include the production of antiviral cytokines as well as proinflammatory cytokines such as TNF-α and IL-6 (as reviewed in Meylan and Tschopp, 2006, and Rehwinkel and Reis e Sousa, 2010). Genomic RNA from several viruses in the order *Mononegavirales*, including VSV, has been demonstrated to specifically activate immune responses via RIG-I (Kato et al., 2006). Importantly, our lab has previously demonstrated that murine astrocytes express RIG-I in conjunction with its downstream effector molecule, IPS-1 (Furr et al., 2008). In the present study, we demonstrate that primary human astrocytes express RIG-I and we show that such expression is inducible. Importantly, we show that this cytosolic viral sensor is functional in these glial cells by demonstrating that a reported RNA ligand for this receptor can initiate inflammatory mediator production. Finally, we have utilized silencing RNA (siRNA) techniques to begin to define the functional significance of RIG-I expression in primary human astrocytes and its role in encephalitis caused by non-segmented, negative-sense RNA viruses.

4.2 Results

To determine whether primary human astrocytes express this newly described viral pattern recognition receptor, we have assessed the level of RIG-I mRNA expression in cultures of these cells at rest and following challenge with VSV. Human astrocytes

were untreated or exposed to VSV at varying MOIs prior to RNA isolation at 4 and 8 hours post-infection (p.i.). Semi-quantitative RT-PCR was performed to assess the presence of mRNA encoding RIG-I. As shown in Figure 9A, astrocytes constitutively express mRNA encoding RIG-I. Interestingly, such expression is rapidly elevated at 4 hours p.i. but such elevations are transient and are not apparent at 8 hours p.i. (Figure 9A).

To determine whether the presence of mRNA encoding this RLR in human astrocytes translates to protein expression, immunoblot analyses were performed to assess RIG-I protein levels. As shown in Figure 9B and as consistent with RIG-I mRNA expression, human astrocytes constitutively express detectable levels of RIG-I protein. Furthermore, exposure of astrocytes to VSV elicits elevations in the expression of this receptor as rapidly as 6 hours p.i with a maximal 3.3 fold induction at a MOI of 10 PFU/cell as determined by densitometric analysis. Importantly, constitutive RIG-I expression and subsequent VSV-induced elevations in such expression are restricted to the cytoplasm, and there is an absence of any detectable RIG-I protein in the nucleus of these cells (Figure 10) consistent with its role as a cytoplasmic viral pattern recognition receptor.

To begin to determine whether RIG-I is functional in human astrocytes, we have investigated whether these cells express IPS-1, a critical downstream effector molecule in RIG-I signaling (Kumar et al, 2006). As shown in Figure 11A, while astrocytes constitutively express robust levels of IPS-1, VSV does not significantly elevate IPS-1 expression at any MOI of VSV at 12 hours (Figure 11A) p.i. Next, we examined whether RIG-I interacts with IPS-1 following viral challenge using co-immunoprecipitation

techniques. As shown in Figure 11B, infection of human astrocytes with VSV induces association of RIG-I with IPS-1 within one hour of infection.

Interaction of RIG-I with IPS-1 has been previously demonstrated to elicit activation of the pivotal inflammatory transcription factor NF-κB in other cell types (Lui et al., 2008). We have therefore correlated the observed interaction of RIG-I and IPS-1 with activation of NF-κB in human astrocytes as assessed by the nuclear translocation of the NF-κB p65 (RelA) subunit. As shown in Figure 11C, infection of human astrocytes with VSV elicits nuclear translocation of RelA at 1-2 hours p.i. Such rapid responses are consistent with the constitutive expression of both RIG-I and IPS-1 in these primary glial cells as demonstrated in Figures 9 and 11A.

Viral genomes have been reported to serve as the major trigger for RIG-I in cells infected with negative-sense single-stranded RNA viruses (Rehwinkel et al., 2010) confirming earlier suggestions that ssRNAs bearing 5′-ppp generated in vitro, perhaps with base-pairing at the 5'-end, are effective agonists for RIG-I (Hornung et al., 2006 and Pichlmair et al., 2006). Consistent with this notion, treatment with calf intestinal phosphatase, which removes 5′-phosphates, has been shown to abolish stimulatory activity (Hornung et al., 2006 and Pichlmair et al., 2006). To confirm the functional nature of RIG-I expression in primary human astrocytes, we have investigated the effects of intracellular administration of in vitro generated uncapped 5'ppp-ssRNA. As shown in Figure 12A, administration of 5'ppp-ssRNA elicits significant increases in levels of mRNA encoding RIG-I. Similarly, this ligand induces a modest but discernable elevation in RIG-I protein levels over those seen in cells that received only the transfection reagent (1.6 fold), an effect that was not seen in cells transfected with a

control dephosphorylated ssRNA (Figure 12B). Interestingly, and in agreement with the effects of VSV (Figure 11A), 5'ppp-ssRNA fails to consistently elicit increases in IPS-1 expression (Figure 12C). While IPS-1 levels decreased with increasing concentrations of 5'ppp-ssRNA, this effect does not appear to be specific as dephosphorylated ssRNA similarly decreased IPS-1 with increasing concentrations (Figure 12C).

Furthermore, we have determined that 5'ppp-ssRNA can elicit inflammatory immune responses in primary human astrocytes. As shown in Figure 13A, 5'ppp-ssRNA induces nuclear translocation of NF-κB (RelA) in a dose dependent manner. Such NF-κB activation is consistent with the ability of this ligand to induce the production of the key inflammatory cytokines IL-6 (Figure 13B) and TNF-α (Figure 13C). Importantly, these effects were significantly attenuated when the ligand was dephosphorylated (Figures 13B and C), consistent with the specific recognition of 5'ppp-ssRNA by RIG-I. Taken together, these data indicate that RIG-I is functionally expressed in primary human astrocytes and recognizes replicative viral RNA motifs.

To begin to establish the relative importance of RIG-I in the initiation of VSV-mediated astrocytes immune responses, we employed silencing RNA techniques to knock down the expression of this viral sensor. As shown in Figure 14A, the use of a combination of three siRNA duplex oligomers effectively knocked down the expression of RIG-I in a time dependent manner with a maximal reduction observed at 72 hours post-transfection. We then optimized our knockdown protocol by selecting the most effective siRNA duplex (DDX58-HSS177513; Invitrogen) for use in our subsequent studies (Figure 14B; duplex 3). Importantly, we determined that transfection of human astrocytes with this siRNA duplex significantly inhibited VSV-induced inflammatory

cytokine production relative to that produced by cells transfected with non-specific scrambled siRNA (Figure 14C). Together, these data indicate that RIG-I is an important component in the initiation of inflammatory immune responses of human astrocytes to negative sense single-stranded RNA virus challenge.

To begin to establish a role for RIG-I in the inflammatory CNS damage associated with neurotropic non-segmented, negative-sense RNA viral infections, we have assessed the effect of RIG-I knockdown of the production of soluble mediators that can elicit neuronal cell death by VSV-infected human astrocytes. As shown in Figure 15A, VSV induces the production of soluble mediators by primary human astrocytes that can elicit decreases in the viability of HCN-1A human neuronal-like cells, as assessed by changes in cell attachment and trypan blue exclusion, in a MOI and time dependent manner. Importantly, the virally-induced production of such neurotoxic mediators was significantly attenuated in cells transfected with siRNA targeting RIG-I (-R) as compared to control cells that were transfected with scrambled non-specific siRNA (+R) (Figure 15B). Together, these data point to a key role for RIG-I in the inflammatory immune responses of human astrocytes to non-segmented, negative-sense RNA viral infection and may represent a key component in the inflammation and damage associated with virally induced encephalitis.

4.3 Conclusions

The order *Mononegavirales* consists of viruses containing a non-segmented, negative-sense RNA genome and includes the causative agents for a number of established (rabies, measles, mumps) and emerging (Ebola, Borna, Hendra, Nipah) human diseases (Pringle, 1997). Some members of this order, such as RABV and

Newcastle disease virus, have the ability to circumvent the blood-brain barrier to establish CNS infection resulting in a severe and often fatal inflammation of brain tissue (Leung et al., 2007; Seal et al., 2000). VSV has proven to be a useful model for neurotropic non-segmented, negative-sense RNA viruses that cause human CNS infections due to its ability to gain access to the CNS and to elicit acute encephalitis (Honeycutt et al., 1993). VSV-associated CNS inflammation is rapid, occurring within days of intranasal inoculation (Honeycutt et al., 1993), and this rapid onset is indicative of an innate immune response that is likely to involve resident CNS cells. Such a hypothesis is supported by the observation that astrocytes exhibit immune functions in situ following VSV infection (Bi et al., 1995; Honeycutt et al., 1993; Leung et al., 2007). In a recent study we have demonstrated that isolated murine astrocytes produce key soluble inflammatory mediators including the cytokines TNF-α and IL-6 following exposure to VSV, and that such production is dependent upon the ability of this virus to replicate within the cytoplasm of these cells (Chauhan et al., 2010). The recent description of RLRs, cytosolic DExD/H box RNA helicases that can recognize early viral replicative intermediates (Takeuchi and Akira, 2007), raises the intriguing possibility that such molecules could play an important role in the detection of RNA viruses by astrocytes.

In the present study, we describe the expression of RIG-I, an RLR that serves as an intracellular viral sensor, in primary cultures of human astrocytes. We show that these cells constitutively express robust levels of mRNA encoding RIG-I. Importantly, we demonstrate detectable levels of these proteins in resting astrocytes and we show that such expression is restricted to the cytoplasm of these cells. These data are in agreement

with a recent study demonstrating the expression of mRNA encoding RIG-I and the detection of RIG-I protein in human astrocytoma cells, and the presence of RIG-I protein in uninfected primary human astrocytes as determined by immunocytochemical techniques (Yoshida et al., 2007). In addition, the present studies are consistent with our recent demonstration that primary murine astrocytes constitutively express this RLR (Furr et al., 2008). Interestingly, we show that the expression of RIG-I in primary human astrocytes is induced following infection with the model RNA virus, VSV. Levels of mRNA encoding this RLR and the expression of this receptor protein in the cytoplasm are elevated following VSV infection. Importantly, the present study provides the first demonstration that intracellular administration of in vitro generated 5'ppp-ssRNA, a reported ligand for RIG-I, induces RIG-I expression in primary human astrocytes. As such, the present demonstration of robust levels of RIG-I in resting human astrocytes is consistent with the ability of these cells to mount rapid inflammatory immune responses following viral challenge. Furthermore, the inducible nature of such expression by intact viral particles and a specific ligand for RIG-I suggests that astrocytes may become sensitized to the presence of intracellular viral motifs generated during viral replication in a feed-forward manner.

Perhaps more importantly, in the present study we provide the first demonstration that this RLR plays an important role in the inflammatory immune responses of astrocytes to infection with a non-segmented, negative-sense RNA virus. First, we have demonstrated that primary human astrocytes constitutively express robust levels of IPS-1, a critical downstream adaptor molecule for RIG-I signaling. The caspase activation and recruitment domains, or CARDs, of RIG-I interact with IPS-1 to induce type I interferon

gene expression in mouse embryonic fibroblasts via TRAF3 and IRF3/IRF-7 pathways (Kumar et al., 2006), and activate NF-κB in a caspase-8 and 10-dependent manner to induce inflammatory cytokine production in immune cells such as macrophages (Hiscott, 2007). Consistent with this mechanism of action, we have demonstrated that exposure of human astrocytes to VSV initiates an association between RIG-I and IPS-1, and we have shown that viral challenge induces the translocation of the RelA subunit of NF-κB to the nucleus in this cell type. Furthermore, we have demonstrated that 5'ppp-ssRNA delivered intracellularly into primary human astrocytes similarly elicits NF-kB activation and inflammatory cytokine production. We have confirmed the specificity of the actions of this RIG-I ligand by demonstrating that dephosphorylated RNA oligomers fail to elicit such marked cytokine responses. Finally, we have confirmed a role for this intracellular viral sensor in the immune responses of primary human astrocytes to this model neurotropic rhabdovirus by demonstrating that RIG-I knockdown specifically and significantly inhibits VSV-induced cytokine production.

Neuronal loss has been reported in the CNS in human cases of RABV (Juntrakul et al., 2005) Hendra virus (Wong et al., 2009), and measles virus (Garg et al., 2008; McQuaid et al., 1997) infection. However, at least one study has indicated that rhabdoviruses do not have an intrinsic ability to induce apoptosis in isolated neuron-like cells (Baloul and Lafon, 2003) suggesting that these viruses elicit neuronal cell death in an indirect manner. Similarly, the pathogenesis of measles virus infection in the CNS has been suggested to result from neuronal apoptosis occurring either as a direct result of viral infection or as an indirect consequence of cytokine-mediated effects (McQuaid et al., 1997). Interestingly, the level of cytotoxic factors including TNF-α has been found

to correlate with alterations in neuronal function and CNS damage in a murine model of RABV infection (Marquette et al., 1996), and a high level of TNF-α has also been associated with poor prognosis in patients with other acute viral encephalopathies (Anlar et al., 2001; Ravi et al., 1997). In the present study, we have demonstrated that human astrocytes express marked levels of TNF-α in response to VSV, and such production occurs in a RIG-I dependent manner. Furthermore, we have shown that soluble factors released by VSV-infected human astrocytes elicits cell damage/death of a human neuronal cell line and that the production of these factors is dependent on the expression of RIG-I. These findings directly implicate RIG-I in the initiation of inflammatory immune responses by human glial cells and provide a potential mechanism underlying the neuronal cell loss associated with acute viral CNS infections.

4.4 Figures

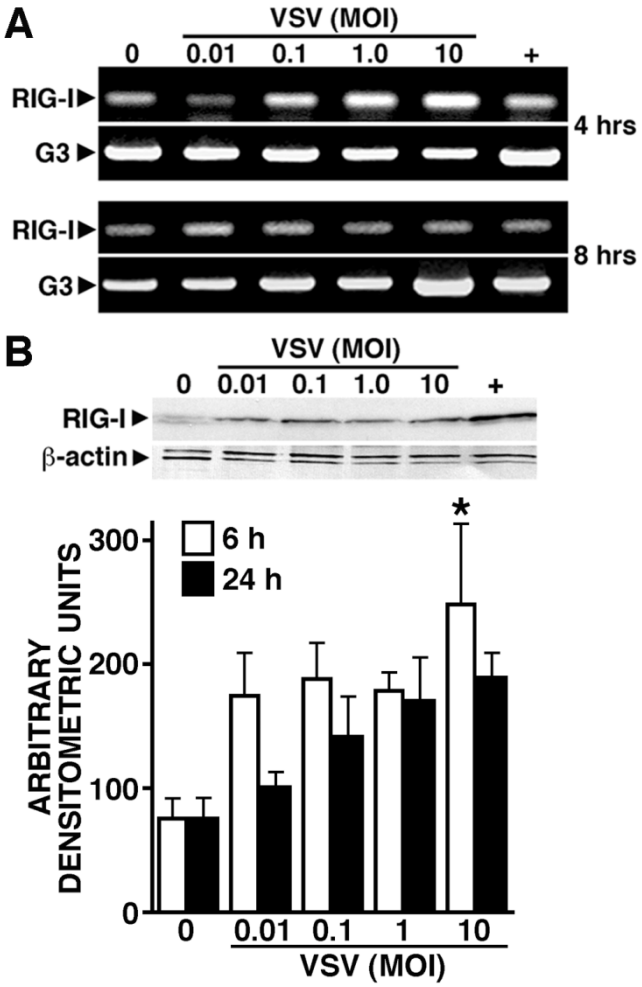

FIGURE 9: Primary human astrocytes constitutively express the cytoplasmic viral RNA sensor, RIG-I, and such expression is elevated following viral challenge. Panel A: Cultured astrocytes (2 X 10^6) were untreated (0) or infected with VSV (MOI of 0.01, 0.1, 1, and 10). At 4 and 8 hours p.i., RNA was isolated and semi-quantitative PCR was performed to determine the level of expression of mRNA encoding RIG-I or the housekeeping gene, G3PDH (G3). For comparison purposes, RIG-I mRNA levels in a similar number of HeLa cells are shown (+). Representative results of three separate experiments are shown. Panel B: Cultured astrocytes (2 X 10^6) were untreated (0) or infected with VSV (MOI of 0.01, 0.1, 1, and 10). At 6 and 24 hours p.i., RIG-I protein expression was determined in whole cell lysates by immunoblot analyses. A representative immunoblot of protein isolates prepared at 6 hours p.i. is shown in the upper panel, and the average densitometric values of three separate experiments at 6 and 24 hours p.i. normalized to b-actin expression is shown below. Data is expressed as mean +/- SEM and an asterisk indicates a statistically significant difference from uninfected cells ($p < 0.05$). For comparison purposes, RIG-I protein expression in a similar number of resting HeLa cells is shown in the representative immunoblot (+).

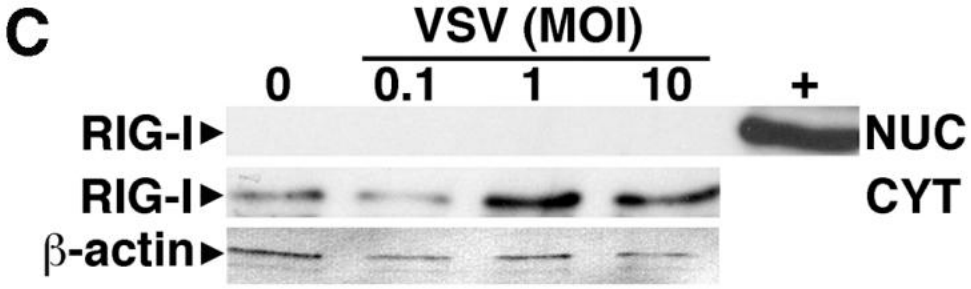

FIGURE 10: RIG-I expression and subsequent VSV-induced elevations in such expression are restricted to the cytoplasm. Cultured astrocytes (2 X 10^6) were untreated (0) or infected with VSV (MOI of 0.1, 1, and 10). At 12 hours p.i., nuclear and cytoplasmic extracts were prepared and RIG-I expression was assessed in each by immunoblot analysis. For comparison purposes, RIG-I protein expression in HeLa cells is shown (+). A representative blot of three separate experiments is shown.

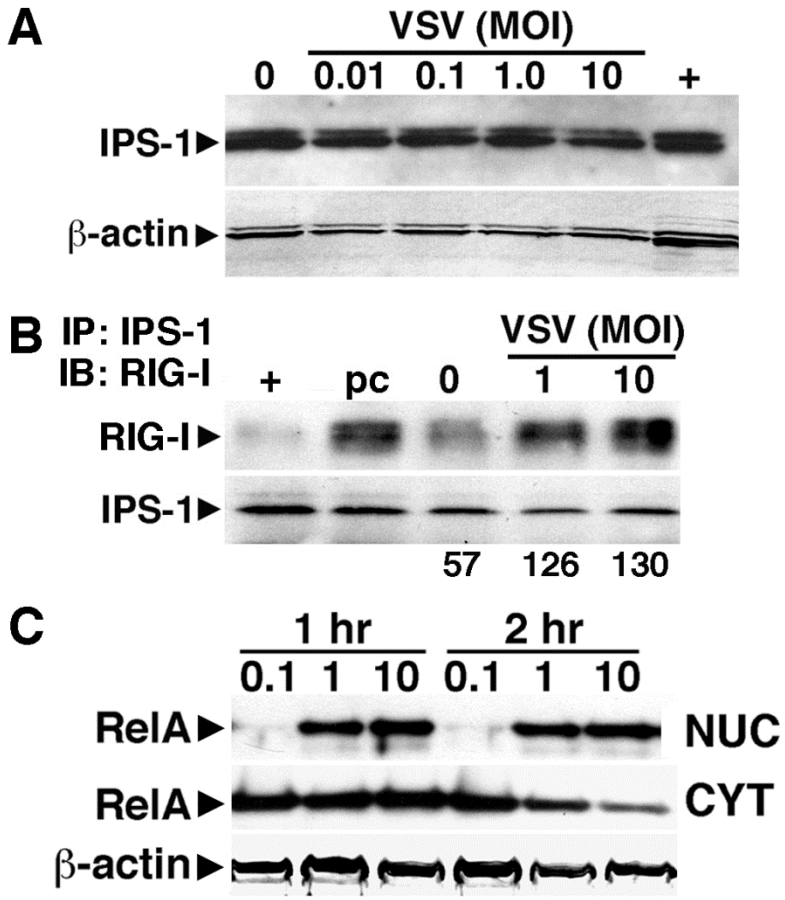

FIGURE 11: VSV induces the activation of RIG-I associated signaling pathways in primary human astrocytes. Panel A: Cells (2 X 10^6) were untreated (0) or infected with VSV (MOI of 0.01, 0.1, 1, and 10). At 12 hours p.i., expression of the RIG-I downstream effector molecule IPS-1, was determined in whole cell protein isolates by immunoblot analysis. IPS-1 expression in HeLa cells (+) is shown for comparison purposes. Panel B: Cells (2 X 10^6) were untreated (0) or infected with VSV (MOI of 1 and 10). At 1 hour p.i., whole cell protein isolates were prepared and immunoprecipitated using beads coated with antibodies directed against IPS-1 prior immunoblot blot analysis for the presence of RIG-I. RIG-I content in samples following the pre-clearing step (pc) is shown as a control and RIG-I expression in a similar number of resting HeLa cells is shown (+) for comparison purposes. Representative results are shown for one of three separate experiments. The relative level of RIG-I/IPS-1 complex formation in this experiment was determined by densitometric analysis normalized to total IPS-1 content in the precipitated material and is shown below the immunoblot. Panel C: Cells (2 X 10^6) were untreated (0) or infected with VSV (MOI of 0.1, 1, and 10). At 1 and 2 hours p.i., nuclear (NUC) and cytoplasmic (CYT) extracts were prepared and NF-kB expression was determined by immunoblot analysis. A representative blot of three separate experiments is shown.

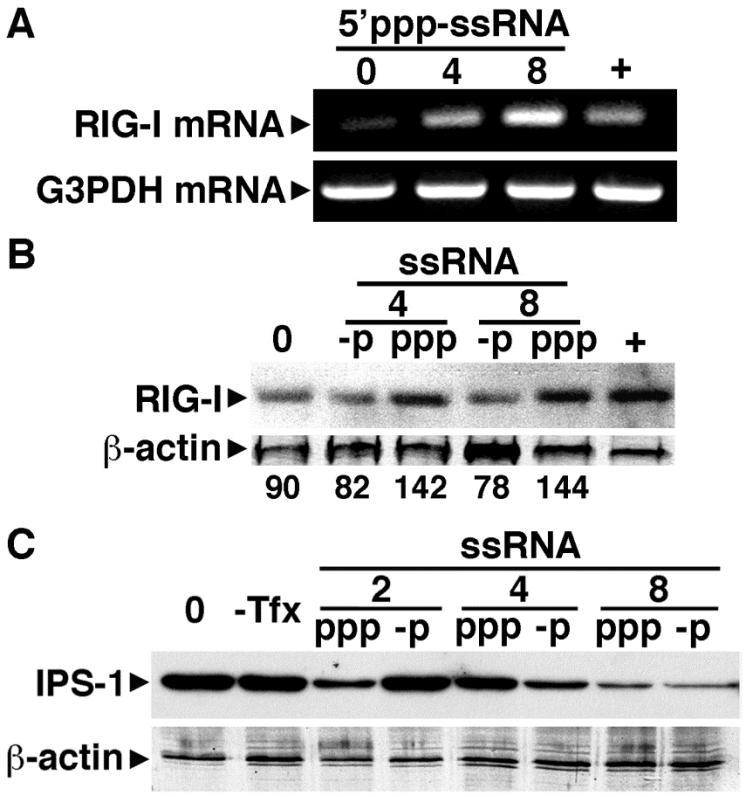

FIGURE 12: 5' triphosphate single stranded RNA, a specific ligand for RIG-I, upregulates RIG-I expression but not IPS-1 in primary human astrocytes. Panel A: Cells (2 X 10^6) were untreated (0) or exposed to 5' triphosphate ssRNA (5'ppp-ssRNA: 4 or 8 ug/ml) in the presence of Tfx-20 transfection reagent. At 2 hrs following transfection, RNA was isolated and PCR was performed to determine the level of expression of mRNA encoding RIG-I and G3PDH. For comparison purposes, RIG-I mRNA levels in a similar number of resting HeLa cells are shown (+). Representative results of three separate experiments are shown. Panel B: Cells (2 X 10^6) were treated with Tfx-20 transfection reagent alone (0) or exposed to 5' triphosphate ssRNA (ppp: 4 or 8 ug/ml) or unphosphorylated RNA (-p: 4 or 8 ug/ml). At 6 hours following transfection, RIG-I protein expression was assessed in whole cell lysates by immunoblot analysis. For comparison purposes, RIG-I protein expression in a similar number of resting HeLa cells is shown (+). Representative results are shown for one of three separate experiments and densitometric analysis of this experiment normalized to b-actin expression is shown below the immunoblot. Panel C: Cells (2 X 10^6) were untreated (0) or exposed to 5' triphosphate ssRNA (ppp: 2, 4, or 8 ug/ml) or unphosphorylated ssRNA (-p: 2, 4, or 8 ug/ml) as a control in the presence of Tfx-20 transfection reagent or without the addition of the transfection reagent (-Tfx). At 6 hours following transfection, IPS-1 protein expression was determined in whole cell lysates by immunoblot analyses. Representative results are shown of one of three separate experiments

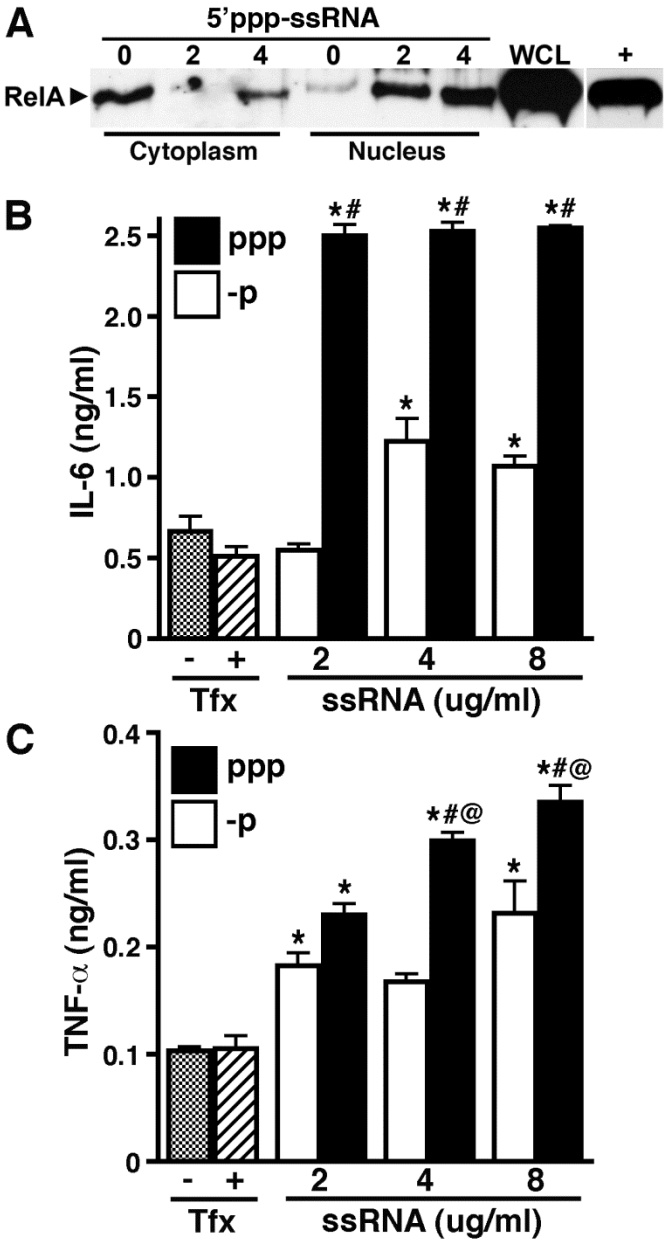

FIGURE 13: 5' triphosphate single stranded RNA, a specific ligand for RIG-I, induces NF-kB activation and inflammatory cytokine production in primary human astrocytes. Panel A: Cells (2 X 10^6) were untreated (0) or exposed to 5' triphosphate ssRNA (5'ppp-ssRNA: 2 or 4 ug/ml) in the presence of Tfx-20 transfection reagent. At 1 hour following transfection, nuclear and cytoplasmic extracts were prepared and RelA levels in each were assessed by immunoblot analysis. Panels B and C: Cells (2 X 10^6) were exposed to tri-phosphorylated (ppp) or dephosphorylated (-p) recombinant ssRNA (ssRNA; 2, 4, or 8 ug/ml) in the presence of Tfx-20 transfection reagent, or were unstimulated in the presence (+) or absence (-) of Tfx-20 alone. At 6 hrs following transfection, supernatants were collected and IL-6 (Panel B) and TNF-α (Panel C) levels were measured by ELISA analyses. Data is expressed as mean +/- SEM (n = 6). An asterisk indicates a statistically significant difference from unstimulated cells, pound symbol indicates a statistically significant difference from the equivalent concentration of dephosphorylated RNA, and asperand indicates a statistically significant difference from the preceding lower dose of each stimulus ($p < 0.05$).

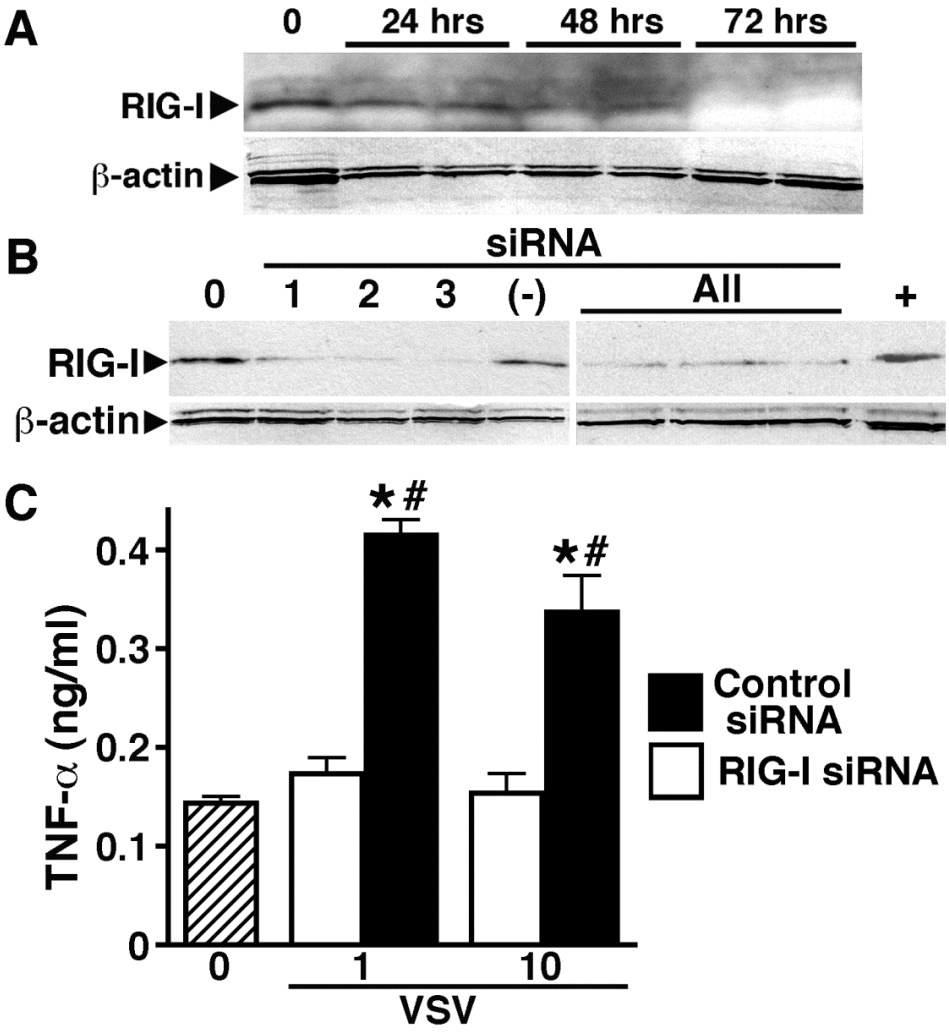

FIGURE 14: RIG-I knockdown attenuates VSV-induced inflammatory cytokine production by primary human astrocytes. Panel A: To determine knockdown efficiency, cells were untreated (0) or transfected with a combination of three different siRNA oligonucleotide pairs targeting RIG-I and cultured for 24, 48, or 72 hours prior to immunoblot analysis for the presence of RIG-I and the housekeeping gene product, β-actin. Panel B: To verify the effectiveness of the siRNA oligonucleotide pairs, cells were untreated (0) or transfected with each siRNA pair (1, 2, or 3), or a combination of the three (All), and cultured for 72 hours prior to immunoblot analysis for the presence of RIG-I and β-actin. The relative level of RIG-I in astrocytes transfected with scrambled siRNA (-) is shown for comparison purposes. Panel C: Cells were untreated (0), or transfected with one siRNA pair (pair 3) targeting RIG-I (RIGI siRNA) or scrambled siRNA (Control siRNA). At 72 hours following transfection, cells were infected with VSV (MOI of 0.01, 0.1, 1, and 10) and the levels of TNF-α in the culture supernatants were assayed by specific capture ELISA at 12 hours p.i. Data is expressed as mean +/- SEM (n = 3). An asterisk indicates a statistically significant difference from unstimulated cells; a pound symbol indicates a statistically significant difference between cells transfected with siRNA directed against RIG-I versus scrambled siRNA ($p < 0.05$).

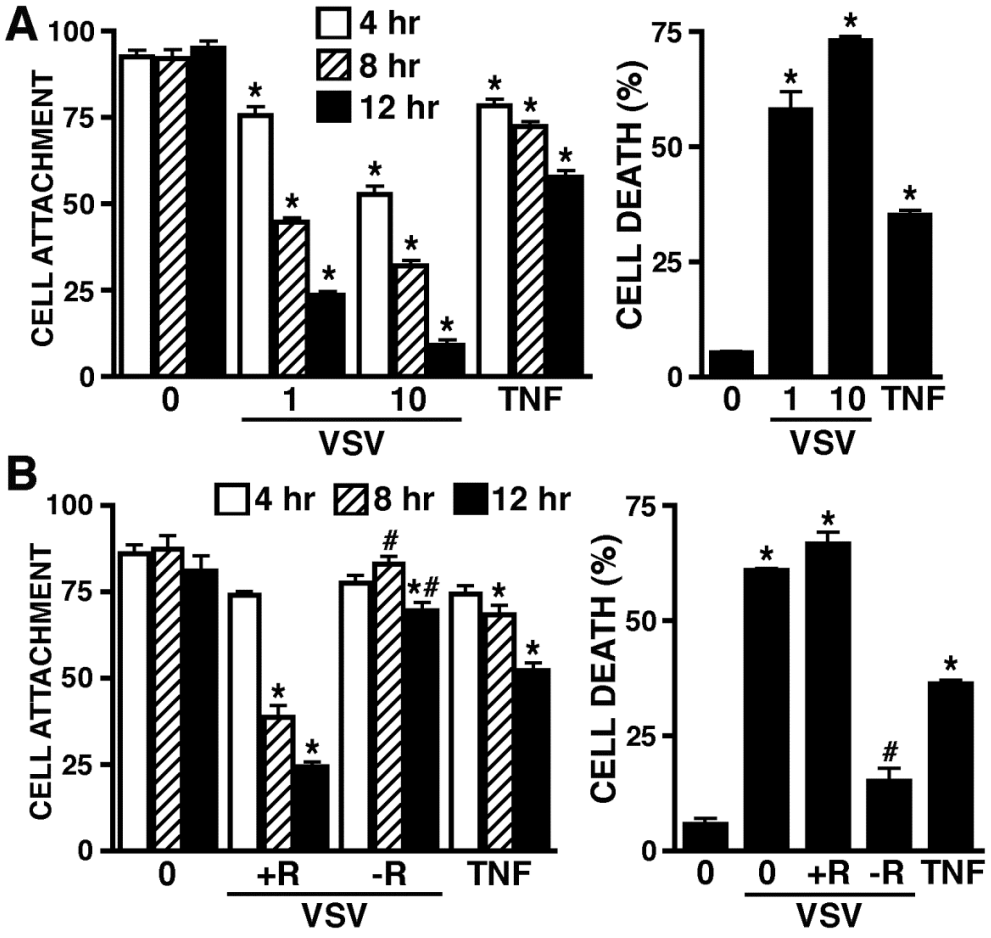

FIGURE 15: VSV induces the production of a soluble factor(s) by human astrocytes that elicit neuronal cell death in a RIG-I dependent manner. Panel A: Human astrocytes were untreated (0) or infected with VSV (MOI of 0.01, 0.1, 1, and 10). At 24 hours p.i., filtered conditioned media from these infected cells or media spiked with recombinant TNF-α (63 pg/ml; TNF) was placed on HCN-1A cells. Changes in viability of these human neuronal-like cells were assessed at 4, 8 and 12 hours by quantification of cell attachment and at 12 hours by trypan blue exclusion. Panel B: Human astrocytes were untreated (0) or transfected with siRNA targeting RIG-I (-R) or scrambled siRNA (+R). At 72 hours following the transfection protocol, cells were uninfected or infected with VSV (MOI of 1) and cultured for a further 24 hours. Filtered conditioned media from these cells was placed on HCN-1A cells. As a positive control, media was spiked with recombinant TNF-α (63 pg/ml; TNF) and placed on HCN-1A cells. Changes in viability of these human neuronal-like cells were assessed at 4, 8 and 12 hours by quantification of cell attachment and at 12 hours by trypan blue exclusion. For comparison purposes, cell death of HCN-1A cells was assessed following exposure to conditioned medium from untransfected astrocytes infected with VSV (1). Data is expressed as mean +/- SEM (n = 3). An asterisk indicates a statistically significant difference from unstimulated cells; a pound symbol indicates a statistically significant difference between cells transfected with siRNA directed against RIG-I versus scrambled siRNA ($p < 0.05$).

CHAPTER FIVE: RIG-I IS AN ESSENTIAL COMPONENT IN MURINE GLIAL RESPONSES TO NEUROTROPIC RNA AND DNA VIRUSES

5.1 Rationale

Viral infections in the CNS are known to broadly trigger glial cell activation and the subsequent release of proinflammatory molecules. For example, microglia have been shown to respond to DNA viruses such as herpes simplex virus (HSV)-I (Aravalli et al., 2005; Lokensgard et al., 2001) and RNA viruses including West Nile Virus (WNV) (Cheeran et al., 2005, van Marle et al., 2007), by secreting various proinflammatory and chemotactic molecules. However, the mechanisms underlying such glial responses to viruses have not been fully elucidated. Viruses are sensed almost exclusively by their nucleic acid genomes, typically as a result of their transcriptional and/or replicative activity. Two disparate PRRs, retinoic acid inducible gene (RIG)-I and DNA-dependent activator of interferon regulatory factors (DAI), have been identified that serve as sensors for different classes of viruses. RIG-I senses the 5'-triphosphate moiety of negative-sense single-stranded RNA (ssRNA) viruses (Hornung et al., 2006; Pichlmair et al., 2006) or short blunt-ended double-stranded RNA (dsRNA) (Takahasi et al., 2008). Subsequent ligation of RIG-I by either of these RNA moieties triggers downstream signaling and activation of the NF-κB and/or IRF3 pathway(s), which induce the production of proinflammatory cytokines and type I IFNs, respectively. In addition to viral RNA motifs, DNA is also a potent activator of innate immunity (Stetson & Medzhitov,

2006). DAI has been shown to bind synthetic dsDNA, B-DNA, and activate NF-κB and IRF3 to promote the transcription of inflammatory and antiviral genes (Takaoka et al., 2007; Ishii et al., 2006) similar to the RIG-I pathway. Our previous studies demonstrated that isolated murine microglia and astrocytes constitutively express inducible levels of RIG-I and DAI. In addition, we have shown that intracellular administration of the specific ligand for RIG-I elicits significant inflammatory mediator production, while targeted knockdown of RIG-I abolishes such responses following exposure to VSV and limits the production of soluble neurotoxic mediators by virally challenged human astrocytes. In murine glial cells, recent studies from our lab have also established a similar functional role for DAI-dependent inflammatory and neurotoxic responses to the DNA virus HSV-1. Together, this indicates a critical role for RIG-I and DAI in host responses to RNA viruses and DNA viruses in the CNS, respectively.

Interestingly, recent studies suggest that RIG-I may also be able to recognize DNA pathogens in a polymerase III-dependent manner (Ablasser et al., 2009, Chui et al., 2009). In this pathway, AT-rich dsDNA serves as a template for RNA polymerase III, and is transcribed into RNA containing a 5'-triphosphate, the ligand for RIG-I. Activation of RIG-I by dsDNA has been shown to induce type I IFN production and NF-κB activation indicating that RNA polymerase III and RIG-I may provide another mechanism by which cells can sense viral DNA. In the present study, we provide the first demonstration that RIG-I is functional in murine microglia and astrocytes and plays an essential role in glial responses to the neurotropic RNA virus VSV. Furthermore, we have determined that this viral sensor and RNA polymerase III are essential for DAI-dependent inflammatory immune responses of glia to the DNA virus HSV-1. Taken

together, the present study indicates that RIG-I plays a significant role in the inflammatory responses of resident CNS cells to both RNA and DNA viral challenges.

5.2 Results

We have previously reported the ability of primary murine microglia and astrocytes to express the novel viral RNA sensor, RIG-I (Furr et al., 2008). Furthermore, we have also shown that RIG-I is elevated following challenge with disparate RNA viruses (Furr et al., 2008). To begin to establish the functional nature of RIG-I in murine glia (Furr et al., 2008) we have investigated the effects of the RIG-I specific ligand, uncapped 5'ppp-ssRNA (Hornung et al., 2006; Saito and Gale., 2008; Takahasi et al, 2008), on the level of expression of this receptor. As shown in Figure 16A, intracellular administration of 5'ppp-ssRNA elicits a significant increase in RIG-I protein levels in microglia over that seen in unstimulated cells. Similarly, this ligand induces a modest but discernable elevation in RIG-I protein levels in murine astrocytes (Figure 16A).

To determine whether RIG-I induction is restricted to RNA viruses and a RIG-I specific ligand, we investigated the effects of the DNA virus, HSV-1, on RIG-I expression. As shown in Figure 17B, RIG-I levels were significantly enhanced in microglia as early as 12 hours post-infection. HSV-1 also elevated RIG-I expression in murine astrocytes albeit with slower kinetics of induction (Figure 17C) with significant upregulation at all MOIs used at 24 hours following infection. Finally, we have assessed the effect of a DNA virus-associated nucleic acid on RIG-I expression by primary murine glial cells. B-DNA is a double-stranded DNA in its canonical B helical form, and is the putative ligand of DAI (Takaoka et al, 2007; Wang et al, 2008). As shown in Figure 17D, intracellular B-DNA administration resulted in marked elevations in RIG-I

expression in both microglia and astrocytes. Together, these data indicate that RIG-I expression is not only induced by its own ligand, but can also be upregulated via disparate viral sensors.

To further assess the functional role of RIG-I, we have determined the ability of 5'ppp-ssRNA to elicit inflammatory immune responses by both glial cell types. As shown in Figure 18A, intracellular 5'ppp-ssRNA administration induces significant IL-6 and TNF-α production as early as 6 hours following transfection in primary microglia. Similarly, murine astrocytes also produce these inflammatory cytokines in response to 5'ppp-ssRNA (Figure 18B). Taken together, these data indicate that specific activation of RIG-I elicits glial inflammatory responses.

To confirm the functional status of RIG-I in murine glia and to begin to determine the relative importance of this sensor in glial responses to RNA viruses, we have assessed the effect of RIG-I knockdown on inflammatory cytokine production by VSV-infected microglia and astrocytes using silencing RNA techniques. Similar to our previous findings in human astrocytes (Furr et al., 2010), virally-induced production of TNF-α was specifically and markedly inhibited in murine astrocytes transfected with siRNA targeting RIG-I as compared to control cells that were transfected with scrambled non-specific siRNA (Control) (Figure 19A). Importantly, RIG-I knockdown abolished inflammatory mediator release by VSV-challenged microglia (Figure 19B). The ability to inhibit cytokine production was specific for this RNA sensor, as transfection with siRNA directed against the DNA sensor, DAI, failed to significantly affect VSV-induced TNF-α release by either microglia or astrocytes (Figure 19). Furthermore, when cells were transfected with siRNA targeting both sensors, there was no additional effect on

cytokine production versus RIG-I knockdown alone (Figure 19). These results indicate that RIG-I activation provides a full and sufficient signal for glial responses to this neurotropic RNA virus.

To determine if RIG-I also plays a role in glial sensing of viral DNA and to begin to determine the relative importance of this innate immune sensor in microglial and astrocyte responses to DNA viral challenge, we have assessed the effect of RIG-I knockdown on glial production of TNF-α following infection with the DNA virus HSV-1. We have previously shown that DAI plays an important role in glial responses to this virus and this finding was confirmed here as transfection with siRNA directed against DAI attenuated cytokine production by HSV-1 challenged microglia or astrocytes by approximately 67% and 30% at the highest MOI, respectively (Figure 20). Surprisingly, RIG-I knockdown also inhibited such responses in both cell types and to the same degree as DAI knockdown (Figure 20). When transfected with siRNAs targeting both DAI and RIG-I, HSV-1-induced TNF-α production by microglia was reduced by approximately 80% to levels that were not significantly different from those produced by uninfected cells (Figure 20A), while cytokine production by astrocytes was also reduced albeit to a lesser extent (60%). Taken together, these data indicate that RIG-I may play a significant role in the perception of DNA viruses by glia, and that this RNA sensor may act in a cooperative manner with DAI to induce inflammatory immune responses by these cells.

To begin to determine the mechanisms underlying the involvement of the RNA sensor RIG-I in glial responses to this DNA virus we have investigated the involvement of RNA polymerase III in these responses. As shown in Figure 20, pre-treatment of cells with the selective RNA polymerase III inhibitor Tagetin (10 U/ml) (Epicentre

Biotechnologies) significantly reduced HSV-1-mediated TNF-α production by microglia and astrocytes to the same extent as RIG-I knockdown (approximately 62% and 30%, respectively). Furthermore, when the inhibitor was utilized in concert with DAI knockdown, microglial responses were reduced by approximately 85% and those of astrocytes by 50%, to levels equivalent to those observed in cells transfected with siRNA against both DAI and RIG-I (Figure 20). It is important to note that RNA polymerase III inhibitor treatment failed to significantly alter glial cytokine responses following VSV infection (Figure 19). As such, these findings suggest that RIG-I could play a cooperative role with DAI in the detection of DNA viruses following the generation of RNA ligands from cytosolic viral DNA by RNA polymerase III.

5.3 Conclusions

As aforementioned, viral infections in the CNS trigger glial activation and the subsequent release of proinflammatory cytokines and chemotactic molecules (Aravalli et al., 2005; Lokensgard et al., 2001; Cheeran et al., 2005; Marle et al., 2007). We have documented the ability of primary cultured microglia and astrocytes to respond to disparate RNA and DNA viruses, including VSV, Sendai virus, murine gammaherpesvirus (MHV)-68, and HSV-1, by producing inflammatory mediators such as IL-6, TNF-α, and IL-1β (Chauhan et al., 2010; Rasley et al., 2004). These characteristics suggest that the innate immune responses of resident CNS cells play a pivotal role in disease progression. However, the mechanisms by which these cells recognize viral pathogens have not been fully defined. It is becoming increasingly apparent that glia possess intracellular sensors that can detect compromise of the cytosolic compartment. While most RNA viruses replicate in the cytoplasm and

therefore allow detection of viral replicative motifs by RLRs present in the host cells, many DNA viruses are known to replicate in the nucleus. In addition, DAI detects a structure common to both self and non-self DNA. This suggests that the discrimination between these types of DNA is based on its subcellular localization, and may likely be a function of the amount and the length of the DNA present in the cytosol rather than a specific chemical feature of the ligand (as reviewed in Keating et al., 2011). However, a recent study has shown that HSV-1 DNA, mislocated from the viral capsid, is readily detectable in the cytoplasm of infected RAW264.7 macrophages, bone marrow-derived macrophages (BMDMs), and THP-1 cells (Unterholzner et al., 2010), suggesting that both viral RNA and DNA can be detected by receptors present in the cytosol.

We have recently demonstrated that murine and human glial cells constitutively express robust levels of RIG-I and MDA5 and that the expression of RIG-I in primary glial cells is induced following infection with the model RNA virus, VSV (Furr et al., 2008). Importantly, the present study shows that intracellular administration of in vitro generated 5'ppp-ssRNA, a reported ligand for RIG-I, induces RIG-I expression in primary murine astrocytes, which is consistent with our previous findings in human astrocytes. Furthermore, we have also provided the first evidence that RIG-I expression in murine microglia is inducible following transfection with 5'ppp-ssRNA. As such, the present demonstration of robust levels of RIG-I in resting microglia and astrocytes is consistent with the ability of these cells to mount rapid inflammatory immune responses following viral challenge. In addition, the inducible nature of such expression by intact viral particles and a specific ligand for RIG-I suggests that these cells may become

sensitized to the presence of intracellular viral moieties produced during viral replication in a feed-forward manner.

Perhaps more importantly, in the present study we provide the first demonstration that this RLR plays an important role in the inflammatory immune responses of murine astrocytes and microglia to viral infection. Similar to our findings in human astrocytes, we have demonstrated that 5'ppp-ssRNA delivered intracellularly into primary murine astrocytes elicits significant inflammatory cytokine production. Interestingly, primary microglia also produce significant amounts of IL-6 and TNF-α following transfection with this specific ligand. Finally, we have confirmed a role for this intracellular viral sensor in the immune responses of glial astrocytes to a model neurotropic rhabdovirus by demonstrating that RIG-I knockdown specifically and significantly inhibits VSV-induced cytokine production.

Recent studies have demonstrated that RIG-I may be important in the production of type I IFNs by cells in response to DNA viral challenge in a DNA-dependent RNA polymerase III manner (Ablasser et al., 2009; Chui et al., 2009). In this described pathway, AT-rich double-stranded DNA serves as a template for RNA polymerase III and is transcribed into RNA containing a 5'-triphosphate, the ligand for RIG-I. Subsequent activation of RIG-I by this ligand has been shown to induce type I IFN production and to activate NF-κB, and studies employing RNA polymerase III knockdown or pharmacological inhibitors of this enzyme have shown that this pathway is important in cell responses to the gammaherpesvirus Epstein-Barr virus (Ablasser et al., 2009; Chui et al., 2009). Indirect evidence for an ability of RIG-I to perceive DNA pathogens comes from the finding that the DNA virus HSV-1, as well as synthetic DNA

ligand reported to activate the cytosolic viral DNA sensor DAI, are able to elicit upregulation of RIG-I expression in astrocytes and microglia. More direct evidence for a role for RIG-I in the detection of DNA comes from our studies which indicate that inhibition of RNA polymerase III or knockdown of RIG-I can markedly inhibit the inflammatory responses of murine glial cells to HSV-1 infection. Collectively, these studies provide the first evidence that the transcription of cytosolic viral DNA by RNA polymerase III and subsequent RNA recognition by RIG-I may provide an additional mechanism for the perception of DNA viruses by CNS cells.

Interestingly, our findings suggest a dual role for RIG-I in the recognition of both RNA and DNA viruses in glial cells. The observation that both RIG-I and DAI are necessary to mount a maximal immune response to DNA pathogens is in line with our demonstration that DAI knockdown attenuates but does not abolish glial responses to HSV-1 (Figure 7). While the ability of a specific inhibitor of RNA polymerase III to attenuate inflammatory cytokine production by infected glial cells provides strong support that RIG-I detects RNA moieties transcribed from cytosolic viral DNA, we cannot discount the possibility that other interactions between the DAI and RIG-I signaling pathways exist 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 the RIG-I and DAI signaling pathways converge at this component to facilitate cellular activation. Alternatively, it is also conceivable that RIG-I and DAI could interact either directly or via STING to promote inflammatory responses. In support of this possibility, previous immunoprecipitation studies have indicated 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 components associated with RIG-I signaling in glial cells.

In the present study, we show that glial inflammatory responses to the DNA virus HSV-1 are dependent on the expression of DAI and RIG-I and the activity of polymerase III, while glial responses to the RNA virus VSV required the expression of RIG-I but were DAI and polymerase III independent. Collectively, these studies provide the first evidence that both RIG-I and DAI play a critical role in the recognition of viral pathogens by resident CNS cells and suggest that these novel intracellular pattern recognition receptors may underlie the damaging inflammation and neuronal cell death associated with acute neurotropic viral infections

5.4 Figures

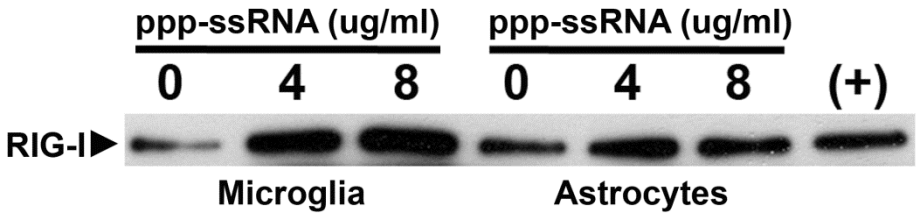

FIGURE 16: 5'ppp-ssRNA induces RIG-I expression in primary murine glial cells. Cells (2×10^6) were untreated (0) or exposed to 5' triphosphate ssRNA (ppp-ssRNA: 4 or 8 ug/ml) in the presence of FuGENE transfection reagent. At 6 hours following transfection, RIG-I protein expression was assessed in whole cell lysates by immunoblot analysis. For comparison purposes, RIG-I protein expression in a similar number of resting HeLa cells is shown (+). Representative results are shown for one of three separate experiments.

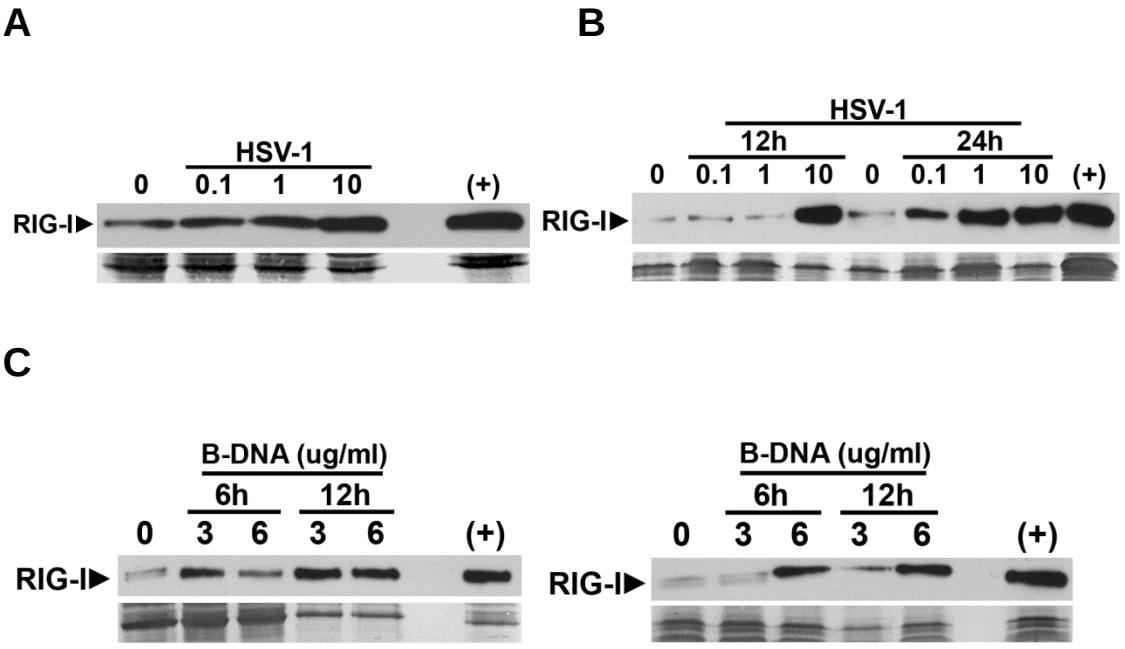

FIGURE 17: Dissimilar motifs induce RIG-I expression in primary murine glial cells. Panel A: Microglia (2 X 10^6) were untreated (0) or infected with HSV-1 (MOI of 0.1, 1, and 10). At 12 hours p.i., expression of RIG-I was determined in whole cell protein isolates by immunoblot analysis. Panel B: Astrocytes (2 X 10^6) were untreated (0) or infected with HSV-1 (MOI of 0.1, 1, and 10). At 12 and 24 hours p.i., expression of RIG-I was determined in whole cell protein isolates by immunoblot analysis. Panel C: Cells (2 X 10^6) were untreated (0) or exposed to B-DNA (3 or 6 8 ug/ml). At 6 hours following transfection, RIG-I protein expression was determined in whole cell lysates by immunoblot analyses. Representative results are shown of one of three separate experiments.

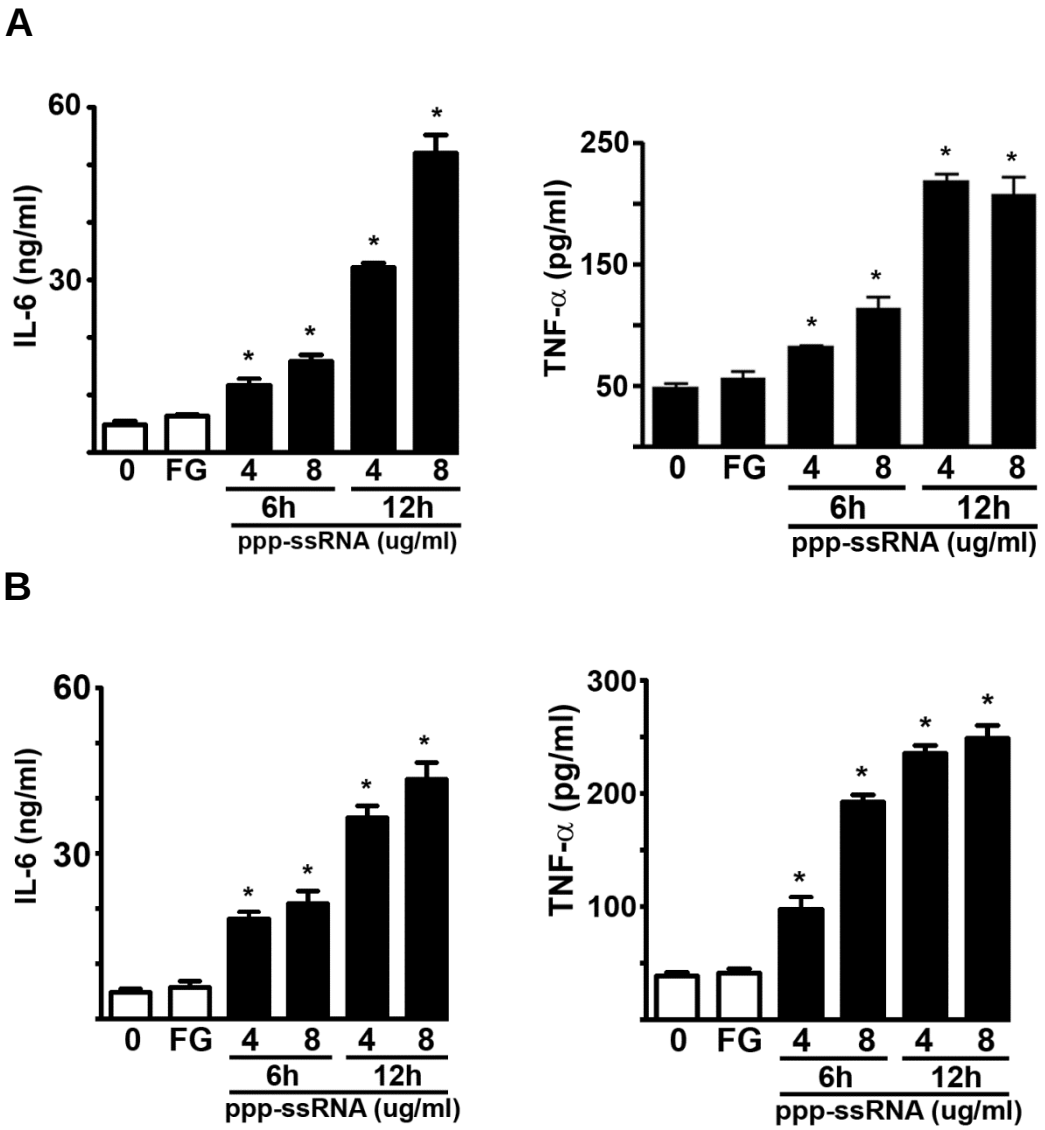

FIGURE 18: Single stranded RNA induces inflammatory mediator production by primary murine glial cells. Microglia (Panel A) and astrocytes (Panel B) were untreated (0) or treated with transfection reagent alone (FG), or exposed to 5' triphosphate ssRNA (5'ppp-ssRNA: 4 or 8 ug/ml) in the presence of the transfection reagent. At 6 hrs following transfection, supernatants were collected and IL-6 and TNF-α levels were measured by ELISA analyses. Data is expressed as mean +/- SEM (n = 3). An asterisk indicates a statistically significant difference from unstimulated cells ($p < 0.05$).

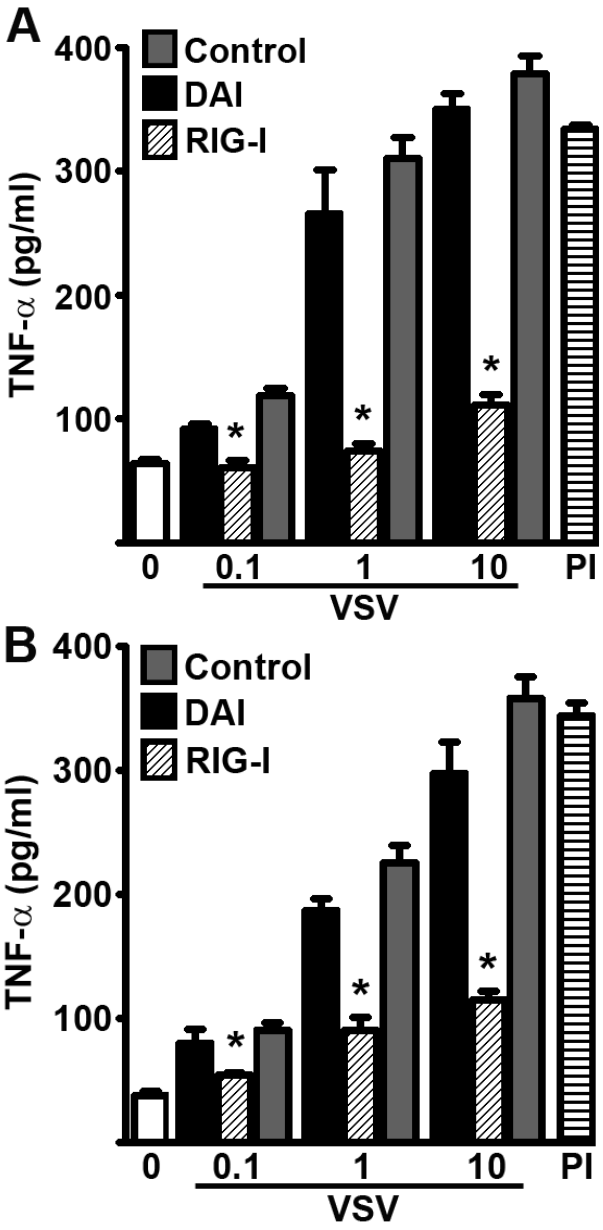

FIGURE 19: RIG-I knockdown abolishes VSV-induced inflammatory cytokine production by primary glia. Astrocytes (Panel A) and microglia (Panel B) were untreated (0), or transfected with siRNA targeting RIG-I, DAI, or scrambled siRNA (Control). At 72 hours following transfection, cells were infected with VSV (MOI of 0.1, 1, and 10) and the levels of TNF-α in the culture supernatants were assayed by specific capture ELISA at 12 hours p.i. Data is expressed as mean +/- SEM (n = 3). An asterisk indicates a statistically significant difference from unstimulated cells; a pound symbol indicates a statistically significant difference between cells transfected with siRNA directed against RIG-I or DAI versus scrambled siRNA ($p < 0.05$).

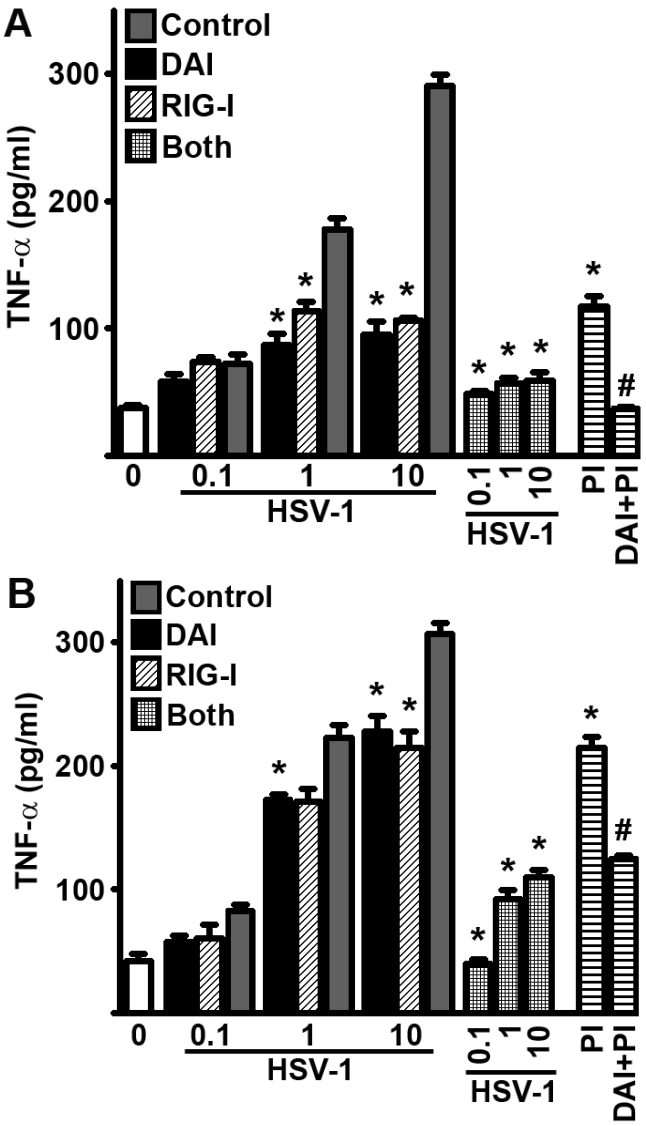

FIGURE 20: Both RIG-1 and DAI are involved in cellular responses to the DNA virus, HSV-1. Microglia (Panel A) and astrocytes (Panel B) were untreated (0), or transfected with siRNA targeting RIG-I, DAI, both RIG-I and DAI (Both) or scrambled siRNA (Control). At 72 hours following transfection, cells were infected with HSV-1 (MOI of 0.1, 1, and 10), and the levels of TNF-α in the culture supernatants were assayed by specific capture ELISA at 24 hours p.i. Additionally, cells were pretreated with RNA polymerase III inhibitor alone (PI) or in following DAI knockdown (DAI + PI). 8 hours following inhibitor treatment, cells were infected with HSV-1 at an MOI of 10, and the levels of TNF-α in the culture supernatants were assayed by specific capture ELISA at 24 hours p.i. Data is expressed as mean +/- SEM (n = 3). An asterisk indicates a statistically significant difference between cells transfected with siRNA directed against RIG-I or DAI versus scrambled siRNA, in cells infected with the same MOI of HSV-1 ($p < 0.05$). A pound symbol indicates a statistically significant difference between PI and DAI + PI. ($p < 0.05$).

CHAPTER SIX: SUMMARY AND CONCLUSIONS

6.1 DAI plays an important role in inflammatory and neurotoxic responses of glial cells to DNA viral pathogens

In the CNS, we have shown that microglia and astrocytes constitutively express DAI and its effector molecules RIP3 and STING, and show that such expression is upregulated following DNA virus challenge (Furr et al., 2011). In vivo HSV-1 infection elicited an upregulation in microglial DAI expression by up to 23.8 fold over that seen in resting cells, and despite strong constitutive expression, HSV-1 exposure was also able to further increase DAI expression in astrocytes with a maximal increase of 2.2 fold over that seen in unstimulated cells. Interestingly, the ability of viral challenge to augment DAI expression by primary glial cells was not limited to this neurotropic alphaherpesvirus, as the lymphotropic gammaherpesvirus, MHV-68, was also capable of eliciting robust increases in microglial DAI expression and caused modest increases in the expression of this molecule in astrocytes.

Importantly, we showed that transfection with the DAI specific ligand B-DNA elicits inflammatory cytokine production by isolated glial cells, with induced production of the inflammatory cytokines TNF-α and IL-6 as rapidly as 6 hours post-transfection at levels that matched or exceeded those elicited following a 24-hour HSV-1 challenge (Furr et al., 2011). To confirm the functional status of DAI in glial cells and to begin to determine the relative importance of this innate immune sensor

in their responses to neurotropic DNA viruses, we also assessed the effect of DAI knockdown on inflammatory cytokine production by HSV-1 challenged microglia and astrocytes (Furr et al., 2011). We found that siRNA directed against DAI significantly attenuated HSV-1 induced TNF-α and IL-6 production by murine microglia. Such an approach also markedly reduced IL-6 production by HSV-1 infected astrocytes but was not as effective in reducing TNF-α production by these cells, where a statistically significant reduction was only observed at the highest viral MOI used (Furr et al., 2011). Lastly, we demonstrated that HSV-1 challenged microglia and astrocytes release neurotoxic mediators and showed that such production is significantly attenuated following DAI knockdown (Furr et al., 2011). As such, we propose the model shown below in Figure 21.

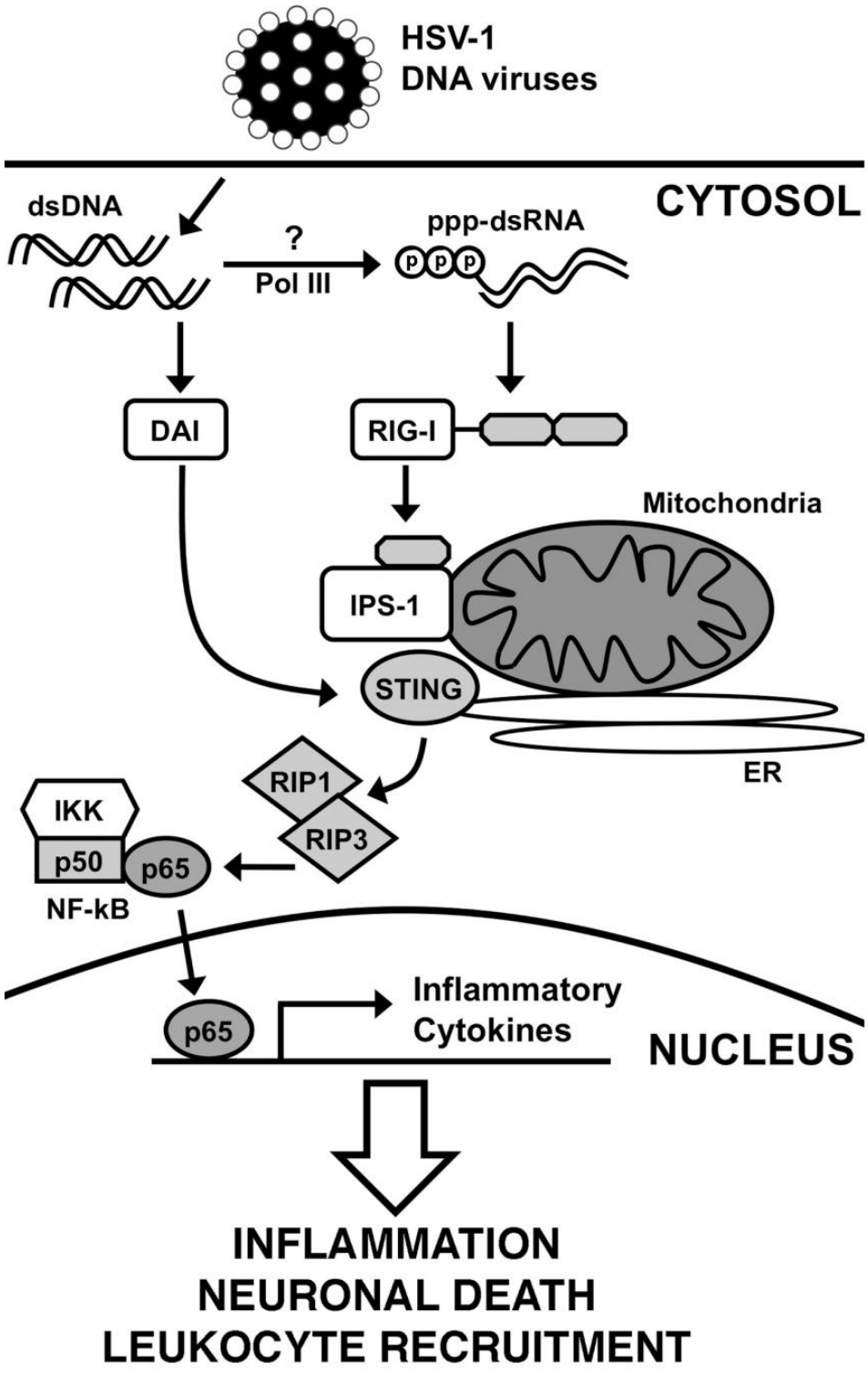

FIGURE 21. Proposed model by which glia recognize neurotropic DNA viruses such as HSV-1 and elicit inflammatory CNS damage.

We suggest that neurotropic double-stranded DNA viruses such as HSV-1 infect microglia and astrocytes and replicate within them, generating genomic DNA within the cytoplasm of each cell type. These viral DNA motifs are then recognized by DAI leading to the activation of downstream effector molecules including IPS-1, STING, and RIP3. Their activation ultimately liberates the RelA subunit of NF-κB allowing it to translocate to the nucleus and to initiate the *de novo* production of soluble inflammatory mediators. In addition, it is possible that RNA polymerase III in the cytosol could transcribe the viral DNA templates into double stranded RNA containing 5'-triphosphate which, in turn, could be recognized by RIG-I and similarly activate NF-κB via the adaptor molecules IPS-1 and STING. Once production is initiated, cytokines such as TNF-α and IL-6 would be anticipated to promote inflammation and to increase blood-brain barrier permeability, facilitating leukocyte recruitment into the CNS. In addition, other soluble inflammatory mediators such as NO can also initiate neuronal cell loss, either directly or via activation of resident/infiltrating myeloid cells. As such, the functional expression of DAI by glial cells may represent an important innate immune mechanism underlying the rapid and potentially lethal inflammation associated with neurotropic DNA virus infection (Figure 21).

6.2 RIG-I plays an important role in the initiation of inflammatory and neurotoxic responses to RNA pathogens in human astrocytes

Evidence for the functional nature of RIG-I expression in these cells comes from our observation that this molecule associates with IPS-1 following VSV infection and from the finding that the specific RIG-I ligand, 5’ppp-ssRNA, elicits human astrocyte immune responses (Furr et al., 2010). This work also demonstrated that RIG-I mediates

TNF-α production by human astrocytes (Furr et al., 2010). Importantly, we have shown that RIG-I knockdown significantly reduces inflammatory cytokine production by VSV-infected astrocytes and inhibits the production of soluble neurotoxic mediators by these cells. These findings directly implicate RIG-I in the initiation of glial inflammatory immune responses and suggest a potential mechanism underlying the neuronal cell death associated with acute viral CNS infections (Furr et al., 2010). Based upon the present study, we propose a model shown below in Figure 22.

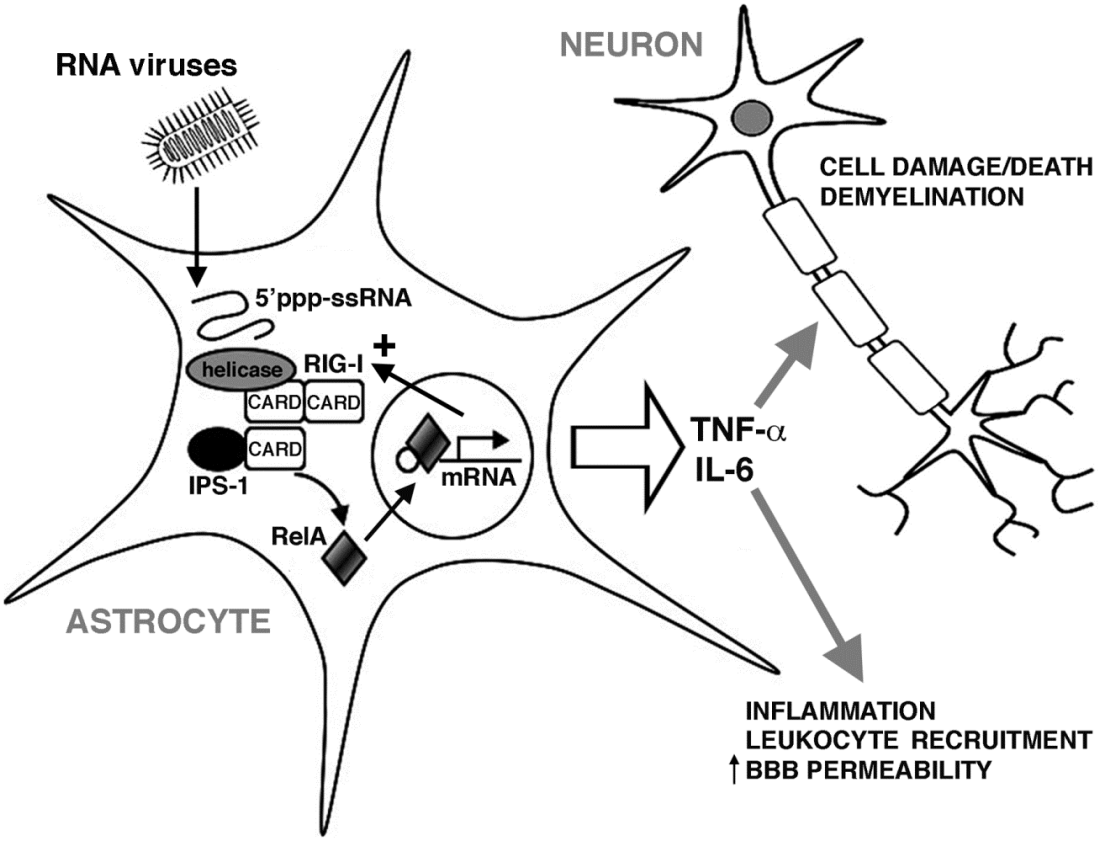

FIGURE 22. Proposed model by which human astrocytes recognize neurotropic RNA viruses and elicit inflammatory CNS damage.

We suggest that non-segmented, negative-sense RNA viruses infect astrocytes and replicate within them, generating uncapped genomic RNAs with phosphorylated 5'-ends. These motifs are recognized by RIG-I and such recognition leads to an association

of the CARD domains of this molecule with those of the downstream effector molecule, IPS-1. Activated IPS-1 ultimately liberates the RelA subunit of NF-κB allowing it to translocate to the nucleus and to initiate the de novo production of soluble inflammatory mediators. Mediators such as TNF-α and IL-6 are well recognized to initiate inflammation and to increase blood-brain barrier permeability, facilitating leukocyte recruitment into the CNS. In addition, such soluble mediators can also initiate neuronal cell loss, either directly or via activation of resident/infiltrating myeloid cells. The expression of this RLR by human astrocytes may therefore represent an important innate immune mechanism underlying the rapid and potentially lethal CNS inflammation associated with neurotropic RNA virus infections.

6.3 RIG-I is essential for the recognition of both RNA and DNA viral pathogens in the CNS

We have definitively shown important roles for both RIG-I and DAI in the recognition of RNA and DNA viral pathogens, respectively. Interestingly, the observation that both RIG-I and DAI are necessary to mount a maximal immune response to DNA pathogens is in line with our demonstration that DAI knockdown attenuates but does not abolish glial responses to HSV-1 (Furr et al., 2011). While the demonstrated ability of a specific inhibitor of RNA polymerase III to attenuate inflammatory cytokine production by infected glial cells provides strong support that RIG-I detects RNA moieties transcribed from cytosolic viral DNA, we cannot discount the possibility that other interactions between the DAI and RIG-I signaling pathways exist 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 the RIG-I and DAI signaling pathways converge at this component to facilitate cellular activation. Alternatively, it is also conceivable that RIG-I and DAI could interact either directly or via STING to promote inflammatory responses. In support of this possibility, previous immunoprecipitation studies have indicated 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 components associated with RIG-I signaling in glial cells. Collectively, these studies indicate that the transcription of cytosolic viral DNA by RNA polymerase III and subsequent RNA recognition by RIG-I may provide an additional mechanism for the perception of DNA viruses by CNS cells.

Taken as a whole, these studies provide evidence for the functional expression of the pathways shown below in solid lines following viral infection of glial cells (Figure 23).

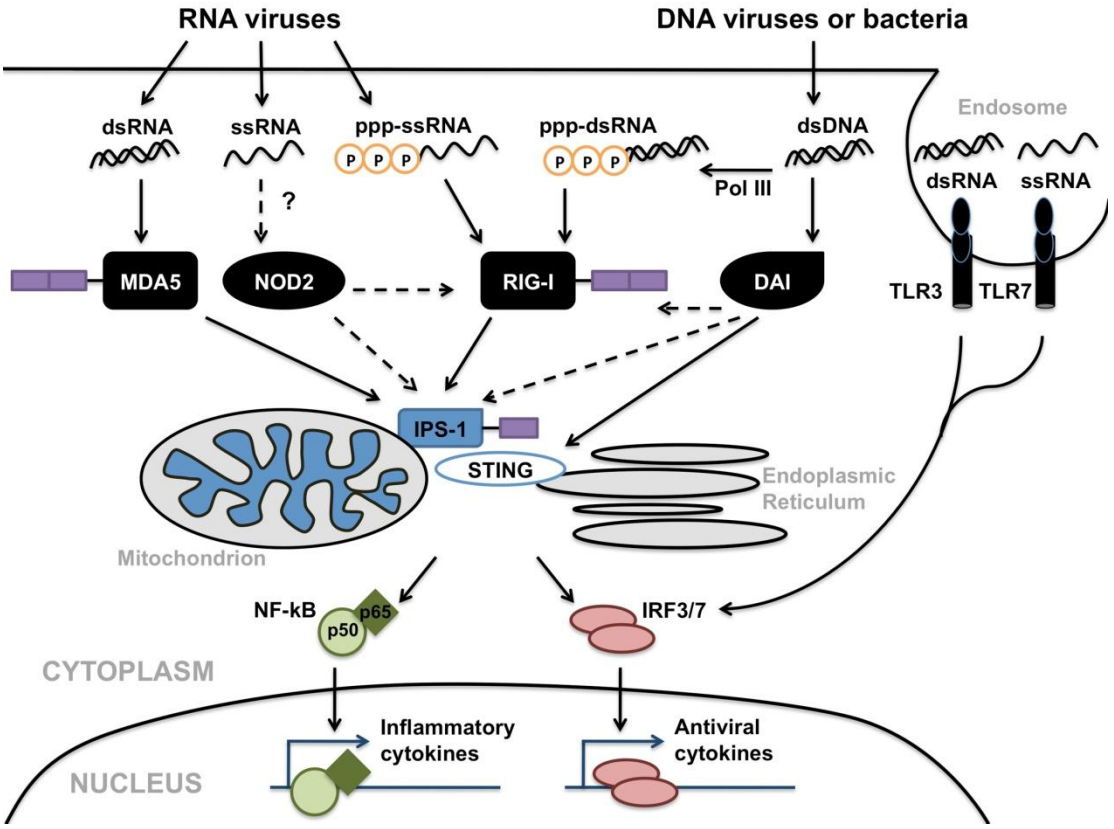

FIGURE 23. Proposed pathways involved in glial inflammatory responses to neurotropic viruses.

In this model, RNA viruses infect glial cells and generate disparate RNA motifs, which can be recognized by the RLRs, MDA5 and RIG-I. Following engagement with RIG-I, IPS-1 association results in translocation of NF-kB to the nucleus and the subsequent production of inflammatory cytokines. Similarly, DNA virus infection of glial cells results in detection of dsDNA moieties generated in the cytoplasm by DAI. DAI activation upregulates the expression of downstream adaptors, including STING and RIP3, and initiates the subsequent production of inflammatory cytokines. In addition, dsDNA from DNA virus infection can be transcribed into RNA that can be recognized by RIG-I through an RNA polymerase III-dependent pathway. However, it is highly likely that glial responses represent the sum of the inputs from multiple disparate cytosolic viral

sensors. In summary, the current study provides strong evidence that these cytosolic pattern recognition receptors serve an important role in the generation of potentially damaging neuroinflammation by glial cells in response to viral pathogens.

6.4 Potential for future studies

The importance of innate immunity in response to viral infection of the CNS is clear. Microglia and astrocytes express a diverse array of cell surface, endosomal and cytosolic molecules that can serve as sensors for RNA and DNA viruses, either constitutively or following activation. The diversity of ligands recognized, the exquisite control of the signaling pathways, and the complexity of the interactions between the innate and adaptive arms of the immune response are also apparent. However, it is also clear that we still do not fully understand the precise nature and structure of the ligands for many of these PRRs or their mode of generation during infection. Furthermore, the available data suggest that models that ascribe discrete PRRs to each particular pathogen are overly simplistic. Rather, it appears that TLRs, RLRs, NLRs, and DAI function as PRRs and/or adaptor molecules that act in a cooperative/synergistic manner to promote glial responses to viral pathogens.

Much work obviously remains to be done, as it is almost certain that additional PRRs await discovery in glial cells and the nature of the interactions between these viral sensors and their signaling pathways have not been determined (see Figure 23; dashed lines). In addition, it is presently unclear whether and to what degree viral CNS pathogens evade detection or manipulate the glial immune responses to their advantage. Answering these questions will lead to greater understanding of the fundamental mechanisms underlying resident CNS cell activation by neurotropic viruses and will

contribute significantly to our understanding of the events that underlie the development of either protective host responses or life threatening neuroinflammation.

REFERENCES

Ablasser A., Bauernfeind F., Hartmann G., Latz E., Fitzgerald K.A., Hornung V. (2009). RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol*. 10:1065-1072.

Aloisi F., Penna G., Cerase J., Menendez Iglesias B., Adorini L. (1997). IL-12 production by central nervous system microglia is inhibited by astrocytes. *J Immunol*. 159:1604-1612.

Anlar B., Söylemezoğlu F., Aysun S., Köse G., Belen D., Yalaz K. (2001). Tissue inflammatory response in subacute sclerosing panencephalitis (SSPE). *J Child Neurol*. 16:895-900.

Aravalli R.N., Hu S., Rowen T.N., Gekker G., Lokensgard J.R. (2006). Differential apoptotic signaling in primary glial cells infected with herpes simplex virus 1. *J Neurovirol.* 12:501-510.

Baloul L., Lafon M. (2003). Apoptosis and rabies virus neuroinvasion. *Biochimie*. 85:777-788.

Bauer J., Huitinga I., Zhao W., Lassmann H., Hickey W.F., Dijkstra C.D. (1995). The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis. *Glia.* 15:437-446.

Baringer JR. (2008). Herpes Simplex Infections of the Nervous System. *Neurol Clin*. 26:657-674.

Beers D.R., Henkel J.S., Kesner R.P., Stroop W.G. (1995). Spatial recognition memory deficits without notable CNS pathology in rats following herpes simplex encephalitis. *J Neurol Sci.* 131:119-127.

Bi Z., Barna M., Komatsu T., Reiss C.S. (1995). Vesicular stomatitis virus infection of the central nervous system activates both innate and acquired immunity. *J Virol.* 69:6466-6472.

Bingham A.C., Saiman L. (2000). Short Course Viral Encephalitis. *Office and Emerg Pediatr.* 13:184-189.

Bowman C.C., Rasley A., Tranguch S.L., Marriott I. (2003). Cultured astrocytes express Toll-like receptors for bacterial products. *Glia.* 43:281-291.

Bsibsi M., Persoon-Deen C., Verwer R.W., Meeuwsen S., Ravid R., Van Noort J.M. (2006). Toll-like receptor 3 on adult human astrocytes triggers production of neuroprotective mediators. *Glia*. 53:688-695.

Butchi N.B., Pourciau S., Du M., Morgan T.W., Peterson K.E. (2008). Analysis of the neuroinflammatory response to TLR7 stimulation in the brain: comparison of multiple TLR7 and/or TLR8 agonists. *J Immunol*. 11:7604-7612.

Camelo S., Lafage M., Lafon M. (2000). Absence of the p55 Kd TNF-alpha receptor promotes survival in rabies virus acute encephalitis. *J Neurovirol*. 6:507-518.

Campanella M., Sciorati C., Tarozzo G., Beltramo M. (2003). Flow cytometric analysis of inflammatory cells in ischemic rat brain. *Stroke*. 33:586-592.

Carpentier P.A., Duncan D.S., Miller S.D. (2008). Glial toll-like receptor signaling in central nervous system infection and autoimmunity. *Brain Behav Immun*. 22:140-147.

Chauhan V.S., Furr S.R., Sterka D.G. Jr, Nelson D.A., Moerdyk-Schauwecker M., Marriott I., Grdzelishvili V.Z. (2010). Vesicular stomatitis virus infects resident cells of the central nervous system and induces replication-dependent inflammatory responses. *Virology*. 10:187-196.

Chauhan V.S., Sterka D.G. Jr, Gray D.L., Bost K.L., Marriott I. (2008). Neurogenic exacerbation of microglial and astrocyte responses to Neisseria meningitidis and Borrelia burgdorferi. *J Immunol.* 180:8241-8249.

Cheeran M.C., Hu S., Sheng W.S., Rashid A., Peterson P.K., Lokensgard J.R. (2005). Differential responses of human brain cells to West Nile virus infection. *J Neurovirol.* 11:512-524.

Christian A.Y., Barna M., Bi Z., Reiss C.S. (1996). Host immune response to vesicular stomatitis virus infection of the central nervous system in C57BL/6 mice. *Viral Immunol.* 9:195-205.

Cliquet F., Picard-Meyer E. (2004). Rabies and rabies-related viruses: a modern perspective on an ancient disease. *Rev Sci Tech*. 23:625-642.

Conrady C.D., Drevets D.A., Carr D.J. (2010). Herpes simplex type I (HSV-1) infection of the nervous system: is an immune response a good thing? *J. Neuroimmunol.* 220:1-9.

Curran, J.A., Kolakofsky D. (1991). Rescue of a Sendai virus DI genome by other parainfluenza viruses: implications for genome replication. *Virology*. 182:168-176.

Daffis S., Samuel M.A., Suthar M.S., Gale M. Jr, Diamond M.S. (2008). Toll-like receptor 3 has a protective role against West Nile virus infection. *J Virol*. 82:10349-10358.

Das S.C., Nayak D., Zhou Y., Pattnaik A.K. (2006). Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells. *J Virol*. 80:6368-6377.

DeBiasi R.L., Tyler K.L. (2006). West Nile virus meningoencephalitis. *Nature Clin Practice Neurology*. 2:264-275.

Dix R.D., Hurst L.I., Keane R.W. (1992). Herpes simplex virus type 1 infection of mouse astrocytes treated with basic fibroblast growth factor. *Journal of General Virology*. 73:1845-1848.

Dixit E., Boulant S., Zhang Y., Lee A.S., Odendall C., Shum B., Hacohen N., Chen Z.J., Whelan S.P., Fransen M., Nibert M.L., Superti-Furga G., Kagan J.C. (2010). Peroxisomes are signaling platforms for antiviral innate immunity. *Cell.* 141:668-681.

Dong Y., Benveniste E.N. (2001). Immune function of astrocytes. *Glia*. 36:180-190.

Elsawa S.F., Taylor W., Petty C.C., Marriott I., Weinstock J.V., Bost K.L. (2003). Reduced CTL response and increased viral burden in substance P receptor-deficient mice infected with murine gamma-herpesvirus 68. *J Immunol.*170:2605-2612.

Esiri M.M., Drummond C.W.E., Morris C.S. (1995). Macrophages and microglia in HSV-1 infected mouse brain. *J Neuroimmunol*. 62:201-205.

Falsig J., van Beek J., Hermann C., Leist M. (2008). Molecular basis for detection of invading pathogens in the brain. *J Neurosci Res.* 86:1434-1447.

Fischer H.G., Reichmann G. (2001). Brain dendritic cells and macrophages/microglia in central nervous system inflammation. *J Immunol.* 166:2717-2726.

Jmor F., Emsley H.C., Fischer M., Solomon T., Lewthwaite P. (2008). The incidence of acute encephalitis syndrome in Western industrialised and tropical countries. *Virol J.* 5:134.

Fishbein D.B., Robinson L.E. (1993). Rabies. *N Engl J Med.* 329:1632-1638.

Flano E., Woodland D.L., Blackman M.A. (2002). A mouse model for infectious mononucleosis. *Immunol Res*. 25:201-217.

Frei K., Malipiero U.V., Leist T.P., Zinkernagel R.M., Schwab M.E, Fontana A. (1989). On the cellular source and function of interleukin 6 produced in the central nervous system in viral diseases. *Eur J Immunol*. 19:689-694.

Fu Y., Comella N., Tognazzi K., Brown L.F., Dvorak H.F., Kocher O. (1999). Cloning of DLM-1, a novel gene that is up-regulated in activated macrophages, using RNA differential display. *Gene*. 240:157-163.

Furr S.R., Chauhan V.S., Sterka D. Jr, Grdzelishvili V., Marriott I. (2008). Characterization of retinoic acid-inducible gene-I expression in primary murine glia following exposure to vesicular stomatitis virus. *J Neurovirol*. 14:503-513.

Furr S.R., Moerdyk-Schauwecker M., Grdzelishvili V.Z., Marriott I. (2010). RIG-I mediates nonsegmented negative-sense RNA virus-induced inflammatory immune responses of primary human astrocytes. *Glia*. 58:1620-1629.

Furr S.R., Chauhan V.S., Moerdyk-Schauwecker M.J., Marriott I. (2011). A role for DNA-dependent activator of interferon regulatory factor in the recognition of herpes simplex virus type 1 by glial cells. *J Neuroinflammation.*12;8:99.

Garg R.K. (2008). Subacute sclerosing panencephalitis. *J Neurol*. 255:1861-1871.

Ghosh D., Basu A. (2009). Japanese Encephalitis - A Pathological and Clinical Perspective. *PLoS Negl Trop Dis*. 3(9):e437.

Gitlin L., Barchet W., Gilfillan S., Cella M., Beutler B., Flavell R.A., Diamond M.S., Colonna M. (2006). Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci U S A.* 30:8459-8464.

Grdzelishvili V.Z., Smallwood S., Tower D., Hall R.L., Hunt D.M., Moyer S.A. (2005). A single amino acid change in the L-polymerase protein of vesicular stomatitis virus completely abolishes viral mRNA cap methylation. *J Virol.* 79:7327-7337.

Gubler D.J. (2008). The continuing spread of west Nile virus in the Western hemisphere. *Clin Infect Dis.* 45:1039-1046.

Ha S.C., Kim D., Hwang H.Y., Rich A., Kim Y.G., Kim K.K. (2008). The crystal structure of the second Z-DNA binding domain of human DAI (ZBP1) in complex with Z-DNA reveals an unusual binding mode to Z-DNA. *Proc Natl Acad Sci U S A.* 105:20671-20676.

Hashimoto C., Hudson K.L., Anderson K.V. (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell.* 52:269-279.

Hayashi T., Nishitsuji H., Takamori A., Hasegawa A., Masuda T., Kannagi M. (2010). DNA-dependent activator of IFN-regulatory factors enhances the transcription of HIV-1 through NF-κB. *Microbes Infect*. (12-13):937-947.

Hickey W.F., Kimura H. (1988). Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science.* 239:290-292.

Hiscott J. (2007). Convergence of the NF-kappaB and IRF pathways in the regulation of the innate antiviral response. *Cytokine Growth Factor Rev*. 18:483-490.

Hjalmarsson A., Blomqvist P., Sköldenberg B. (2007). Herpes simplex encephalitis in Sweden, 1990-2001: incidence, morbidity, and mortality. *Clin Infect Dis.* 45:875-880.

Hornung V., Ellegast J., Kim S., Brzozka K., Jung A., Kato H., Poeck H., Akira S., Conzelmann K.K., Schlee M., Endres S., Hartmann G. (2006). 5'-Triphosphate RNA is the ligand for RIG-I. *Science*. 314:994-997.

Huneycutt B.S., Bi Z., Aoki C.J., Reiss C.S. (1993). Central neuropathogenesis of vesicular stomatitis virus infection of immunodeficient mice. *J Virol*. 67:6698-6706.

Ikemoto K., Pollard R., Fukumoto T., Morimatsu M., Suzuki F. (1995). Small amounts of exogenous IL-4 increase the severity of encephalitis induced in mice by the intranasal infection of herpes simplex virus type 1. *J Immunol.* 155:1326-1333.

Isaacson E., Glaser C.A., Forghani B., Amad Z., Wallace M., Armstrong R.W., Exner M.M., Schmid, S. (2005). Evidence of human herpesvirus 6 infection in 4 immunocompetent patients with encephalitis. *Clin Infect Dis.* 40:890-893.

Ishii K.J., Coban C., Kato H., Takahashi K., Torii Y., Takeshita F. (2006). A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol*. 7:40-48.

Ishikawa H., Barber G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature*. 455:674-678. Erratum in: *Nature,* 2008 456:274.

Ishikawa H., Ma Z., Barber G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature*. 461:788-792.

Jack C.S., Arbour N., Manusow J., Montgrain V., Blain M., McCrea E., Shapiro A., Antel J.P. (2005). TLR signaling tailors innate immune responses in human microglia and astrocytes. *J Immunol.* 175:4320-4330.

Jogai S., Radotra B.D., Banerjee A.K. (2000). Immunohistochemical study of human rabies. *Neuropathology.* 20:197-203.

Juntrakul S., Ruangvejvorachai P., Shuangshoti S., Wacharapluesadee S., Hemachudha T. (2005). Mechanisms of escape phenomenon of spinal cord and brainstem in human rabies. *BMC Infect Dis.* 16:104.

Kaiser W.J., Upton J.W., Mocarski E.S. (2008). Receptor-interacting protein homotypic interaction motif-dependent control of NF-kappa B activation via the DNA-dependent activator of IFN regulatory factors. *J Immunol*. 1;181(9):6427-6434.

Kato H., Takeuchi O., Sato S., Yoneyama M., Yamamoto M., Matsui K., Uematsu S., Jung A., Kawai T., Ishii K.J., Yamaguchi O., Otsu K., Tsujimura T., Koh C.S., Reis e Sousa C., Matsuura Y., Fujita T., Akira S. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*. 441:101-105.

Kawai T., Akira S. (2008). Toll-like receptor and RIG-I-like receptor signaling. *Ann NY Acad Sci.* 1143:1-20.

Kawai T., Akira S. (2007). Antiviral signaling through pattern recognition receptors. *J Biochem.* 141(2):137-45.

Huneycutt B.S., Bi Z., Aoki C.J., Reiss C.S. (1993). Central neuropathogenesis of vesicular stomatitis virus infection of immunodeficient mice. *J Viro.l* 67:6698-6706.

Keating S.E., Baran M., Bowie A.G. (2011). Cytosolic DNA sensors regulating type I interferon induction. *Trends Immunol.* 32:574-581.

Kiefer R., Lindholm D., Kreutzberg G.W. (1993). Interleukin-6 and transforming growth factor-beta 1 mRNAs are induced in rat facial nucleus following motoneuron axotomy. *Eur J Neurosci*. 5:775-781.

Kimberlin D.W. (2007). Management of HSV encephalitis in adults and neonates: diagnosis, prognosis and treatment. *Herpes.* 14:11-16.

Koh W.L., Ng M.L. (2005). Molecular mechanisms of West Nile virus pathogenesis in brain cell. *Emerg Infect Dis*.11(4):629-632.

Konat G.W., Kielian T., Marriott I. (2006). The role of Toll-like receptors in CNS response to microbial challenge. *J Neurochem*. 99:1-12.

Koskiniemi, M., Korppi, M., Mustonen, K., Rantala, H., Muttilainen, M., Herrgård, E., Ukkonen, P., and Vaheri, A. (1997). Epidemiology of encephalitis in children: a prospective multicentre study. *Eur J Pediatr.* 156:541-545.

Kowalinski E., Lunardi T., McCarthy A.A., Louber J., Brunel J., Grigorov B., Gerlier D., Cusack S. (2011). Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell*. 147(2):423-435.

Kurt-Jones E.A., Chan M., Zhou S., Wang J., Reed G., Bronson R., Arnold M.M., Knipe D.M., Finberg R.W. (2004). Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proc Natl Acad Sci U S A*. 101(5):1315-1320.

Kumar H., Kawai T., Akira S. (2011). Pathogen recognition by the innate immune system. Int *Rev Immunol.* 30:16-34.

Kumar H., Kawai T., Kato H., Sato S., Takahashi K., Coban C., Yamamoto M., Uematsu S., Ishii K.J., Takeuchi O., Akira S. (2006). Essential role of IPS-1 in innate immune responses against RNA viruses. *J Exp Med*. 203:1795-1803.

Lemaitre B., Nicolas E., Michaut L., Reichhart J.M., Hoffmann J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell.* 86(6):973-983.

Leung A.K., Davies H.D., Hon K.L. (2007). Rabies: epidemiology, pathogenesis, and prophylaxis. *Adv Ther*. 24:1340-1347.

Li J., Faber M., Dietzschold B., and Hooper D.C. (2011). The role of toll-like receptors in the induction of immune responses during rabies virus infection. *Adv Virus Res* 79:115-126.

Li X., Lu C., Stewart M., Xu H., Strong R.K., Igumenova T., Li P. (2009). Structural basis of double-stranded RNA recognition by the RIG-I like receptor MDA5. *Arch Biochem Biophys*. 488(1):23-33.

Liao C.L., Lin Y.L., Shen S.C., Shen J.Y., Su H.L., Huang Y.L., Ma S.H., Sun Y.C., Chen K.P., Chen L.K. (1998). Antiapoptotic but not antiviral function of human bcl-2 assists establishment of Japanese encephalitis virus persistence in cultured cells. *J Virol*. 72:9844-9854.

Licon Luna R.M., Lee E., Müllbacher A., Blanden R.V., Langman R., Lobigs M. (2002). Lack of both Fas ligand and perforin protects from flavivirus-mediated encephalitis in mice. *J. Virol.* 76:3202-3211.

Lima G.K., Zolini G.P., Mansur D.S., Freire Lima B.H., Wischhoff U., Astigarraga R.G., Dias M.F., das Graças Almeida Silva M., Béla S.R., do Valle Antonelli L.R., Arantes R.M., Gazzinelli R.T., Báfica A., Kroon E.G., Campos M.A. (2010). Toll-like receptor (TLR) 2 and TLR9 expressed in trigeminal ganglia are critical to viral control during herpes simplex virus 1 infection. *Am J Pathol*. 177(5):2433-2445

Liu P., Jamaluddin M., Li K., Garofalo R.P., Casola A., Brasier A.R. (2007). Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol*. 81(3):1401-1411.

Lokensgard J.R., Hu S., Sheng W., vanOijen M., Cox D., Cheeran M.C., Peterson P.K. (2001). Robust expression of TNF-alpha, IL-1beta, RANTES, and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus. *J Neurovirol.* 7(3):208.

Loo Y.M., Fornek J., Crochet N., Bajwa G., Perwitasari O., Martinez-Sobrido L., Akira S., Gill M.A., García-Sastre A., Katze M.G., Gale M. Jr. (2008). Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol*. 82(1):335-345.

Lui P., Li K., Garofalo R.P., Brasier A.R. (2008). Respiratory syncytial virus induces RelA release from cytoplasmic 100-kDa NF-kappa B2 complexes via a novel retinoic acid-inducible gene-I NF- kappa B-inducing kinase signaling pathway. *J Biol Chem*. 283:23169-23178.

Lund J., Sato A., Akira S., Medzhitov R., Iwasaki A. (2003). Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med*. 198:513-520.

Lundh B., Kristensson K., Norrby E. (1987). Selective infections of olfactory and respiratory epithelium by vesicular stomatitis and Sendai viruses. *Neuropathol Appl Neurobiol*. 13:111-122.

Lundh B. (1990). Spread of vesicular stomatitis virus along the visual pathways after retinal infection in the mouse. *Acta Neuropathol*. 79:395-401.

Mailles A., Vaillant V., Stahl J.P. (2007). Infectious encephalitis in France from 2000 to 2002: the hospital database is a valuable but limited source of information for epidemiological studies]. *Médecine et maladies infectieuses.* 37:95-102.

Mansur D.S., Kroon E.G., Nogueira M.L., Arantes R.M.E., Rodrigues S.C.O., Akira S., Gazzinelli R.T., Campos M.A. (2005). Lethal encephalitis in myeloid differentiation factor 88-deficient mice infected with herpes simplex virus 1. *Am J Pathol.* 166:1419-1426.

Mariani M.M., Kielian T. (2009). Microglia in infectious diseases of the central nervous system. *J Neuroimmune Pharmacol*. 4:448-461.

Marques C.P., Hu S., Sheng W., Cheeran M.C., Cox D., Lokensgard J.R. (2004). Interleukin-10 attenuates production of HSV-induced inflammatory mediators by human microglia. *Glia.* 47:358-366.

Marques C.P., Cheeran M.C., Palmquist J.M., Hu S., Urban S.L., Lokensgard J.R. (2008). Prolonged microglial cell activation and lymphocyte infiltration following experimental herpes encephalitis. *J Immunol*. 181:6417-6426.

Marquette C., Van Dam A.M., Ceccaldi P.E., Weber P., Haour F., Tsiang H. (1996). Induction of immunoreactive interleukin-1 beta and tumor necrosis factor-alpha in the brains of rabies virus infected rats. *J Neuroimmunol*. 68:45-51.

Martin F.C., Anton P.A., Gornbein J.A., Shanahan F., Merrill J.E. (1993). Production of interleukin-1 by microglia in response to substance P: role for a non-classical NK-1 receptor. *J Neuroimmunol.* 42:53-60.

McCormack J.G., Allworth A.M. (2002). Emerging viral infections in Australia. *Med J Aust.* 177:45-49.

McKimmie C.S., Johnson N., Fooks A.R., Fazakerley J.K. (2005). Viruses selectively upregulate Toll-like receptors in the central nervous system. *Biochem Biophys Res Commun.* 28:925-933.

McQuaid S., McMahon J., Herron B., Cosby S.L. (1997). Apoptosis in measles virus-infected human central nervous system tissues. *Neuropathol Appl Neurobiol*. 23:218-224.

Medzhitov R., Preston-Hurlburt P., Janeway C.A. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature.* 388:394-397.

Melchjorsen J., Rintahaka J., Søby S., Horan K.A., Poltajainen A., Østergaard L., Paludan S.R., Matikainen S. (2010). Early innate recognition of herpes simplex virus in human primary macrophages is mediated via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent pathways. *J Virol*. 84:11350-11358.

Meylan E., Tschopp J. (2006). Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell*. 22:561-569.

Miyoshi K., Harter D.H., Hsu K.C. (1971). Neuropathological and immunofluorescence studies of experimental vesicular stomatitis virus encephalitis in mice. *J Neuropathol Exp Neurol.* 30:266-277.

Mori I., Goshima F., Ito H., Koide N., Yoshida T., Yokochi T., Kimura Y., Nishiyama Y. (2005). The vomeronasal chemosensory system as a route of neuroinvasion by herpes simplex virus. *Virology*. 334:51-58.

Nair A., Hunzeker J., Bonneau R.H. (2007). Modulation of microglia and CD8+ T cell activation during the development of stress-induced herpes simplex virus type-1 encephalitis. *Brain Behav Immun*. 21:791-806.

Nakajima H., Kobayashi M., Pollard R.B., Suzuki F. (2001). Monocyte chemoattractant protein-1 enhances HSV-induced encephalomyelitis by stimulating Th2 responses. *J Leukoc Biol.* 70:374-380.

Nuovo G.J., Defaria D.L., Chanona-Vilchi J.G., Zhang Y. (2005). Molecular detection of rabies encephalitis and correlation with cytokine expression. *Mod Pathol.* 18:62-67.

Parquet M.C., Kumatori A., Hasebe F., Morita K., Igarashi A. (2001). West Nile virus-induced bax-dependent apoptosis. *FEBS Lett*. 500(1-2):17-24.

Pérez de Diego R., Sancho-Shimizu V., Lorenzo L., Puel A., Plancoulaine S., Picard C., Herman M., Cardon A., Durandy A., Bustamante J., Vallabhapurapu S., Bravo J., Warnatz K., Chaix Y., Cascarrigny F., Lebon P., Rozenberg F., Karin M., Tardieu M., Al-Muhsen S., Jouanguy E., Zhang S.Y., Abel L., Casanova J.L. (2010). Human TRAF3 adaptor molecule deficiency leads to impaired Toll-like receptor 3 response and susceptibility to herpes simplex encephalitis. *Immunity*. 33:400-411.

Phares T.W., Kean R.B., Mikheeva T., Hooper D.C. (2006). Regional differences in blood-brain barrier permeability changes and inflammation in the apathogenic clearance of virus from the central nervous system. *J Immunol.* 176:7666-7675.

Pichlmair A., Schulz O., Tan C.P., Näslund T.I., Liljeström P., Weber F., Reis e Sousa C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science*. 10;314(5801):997-1001.

Pringle C.R. (1997). The order Mononegavirales-current status. *Arch Virol.* 142:2321-2326.

Raschilas F., Wolff M., Delatour F., Chaffaut C., De Broucker T., Chevret S., Lebon P., Canton P., Rozenberg F. (2002). Outcome of and prognostic factors for herpes simplex encephalitis in adult patients: results of a multicenter study. *Clin Infect Dis*. 35:254-260.

Rasley A., Bost K.L., Marriott I. (2004). Murine gammaherpesvirus-68 elicits robust levels of interleukin-12 p40, but not interleukin-12 p70 production, by murine microglia and astrocytes. *J Neurovirol*. 10:171-180

Ravi V., Parida S., Desai A., Chandramuki A., Gourie-Devi M., Grau G.E. (1997). Correlation of tumor necrosis factor levels in the serum and cerebrospinal fluid with clinical outcome in Japanese encephalitis patients. *J Med Virol*. 51:132-136.

Ray N.B., Power C., Lynch W.P., Ewalt L.C., Lodmell D.L. (1997). Rabies viruses infect primary cultures of murine, feline, and human microglia and astrocytes. *Arch Virol*. 142:1011-1019.

Rebsamen M., Heinz L.X., Meylan E., Michallet M.C., Schroder K., Hofmann K., Vazquez J., Benedict C.A., Tschopp J. (2009). DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. *EMBO Rep.* 10(8):916-922.

Rehwinkel J., Tan C.P., Goubau D., Schulz O., Pichlmair A., Bier K., Robb N., Vreede F., Barclay W., Fodor E., Reis e Sousa C. (2010). RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell.* 140:397-408.

Rehwinkel J., Reis e Sousa C. (2010). RIGorous detection: exposing virus through RNA sensing. *Science*. 327:284-286.

Ronnett G.V., Hester L.D., Nye J.S., Connors K., Snyder S.H. (1990). Human cortical neuronal cell line: establishment from a patient with unilateral megalencephaly. *Science*. 248: 603-605.

Roos, K.L. (1999). Central Nervous System Infections. *Neurol Clin* 17:813-833.

Rosler A., Pohl M., Braune H.J., Oertel W.H., Gemsa D., Sprenger H. (1998). Time course of chemokines in the cerebrospinal fluid and serum during herpes simplex type 1 encephalitis. *J Neurol Sci*. 157:82-89.

Rothenburg S., Schwartz T., Koch-Nolte F., Haag F. (2002). Complex regulation of the human gene for the Z-DNA binding protein DLM-1. *Nucleic Acids Res.* 30(4):993-1000.

Sabbah A., Chang T.H., Harnack R., Frohlich V., Tominaga K., Dube P.H., Xiang Y., Bose S. (2009). Activation of innate immune antiviral responses by Nod2. *Nat Immunol*. 10(10):1073-1080.

Saito T., Hirai R., Loo Y.M., Owen D., Johnson C.L., Sinha S.C., Akira S., Fujita T., Gale M. Jr. (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A.* 104(2):582-587.

Saito T., Gale M. Jr. (2008). Differential recognition of double-stranded RNA by RIG-I-like receptors in antiviral immunity. *J Exp Med*. 205:1523-1527.

Samaratunga H., Searle J.W., Hudson N. (1998). Non-rabies lyssavirus human encephalitis from fruit bats: Australian bat lyssavirus (pteropid lyssavirus) infection. *Neuropathol Appl Neurobiol*. 24:331-335.

Samuel, C.E. (2001). Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14:778-809.

Savarin C., Bergmann C.C. (2008). Neuroimmunology of central nervous system viral infections: the cells, molecules and mechanisms involved. *Curr Opinion in Pharmacology*. 8:472-479.

Schachtele S.J., Hu S., Little M.R., Lokensgard J.R. (2010). Herpes simplex virus induces neural oxidative damage via microglial cell Toll-like receptor-2. *J Neuroinflammation*. 28;7-35

Schroder K., Tschopp J. (2010). The inflammasomes. *Cell* .140(6):821-832.

Seal B.S., King D.J., Sellers H.S.. (2000). The avian response to Newcastle disease virus. *Dev Comp Immunol*. 24:257-268.

Sejvar, J.J. (2006). The evolving epidemiology of viral encephalitis. *Curr Opin Neurol*. 19:350-357.

Shrestha B., Gottlieb D., Diamond M.S. (2003). Infection and injury of neurons by West Nile encephalitis virus. *J Virol*. 77(24):13203-13213.

Solanki A., Radotra B.D., Vasishta R.K. (2009). Correlation of cytokine expression with rabies virus distribution in rabies encephalitis. *J Neuroimmunol*. 217:85-89.

Solomon T., Ni H., Beasley D.W., Ekkelenkamp M., Cardosa M.J., Barrett A.D. (2003). Origin and evolution of Japanese encephalitis virus in southeast Asia. *J Virol.* 77:3091-3098.

Stoll G., Jander S. (1999). The role of microglia and macrophages in the pathophysiology of the CNS. *Prog Neurobiol.* 58:233-247.

Stahl J.P., Mailles A., Vaillant V., Floret D., Encephalitis Protocol Group. (2008). Acute infectious encephalitis and pathogens coming from animals. *Medicina (Kaunas).* 44:821-826

Stetson D.B., Medzhitov R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity*. 24:93-103.

Streit W.J., Semple-Rowland S.L., Hurley S.D., Miller R.C., Popovich P.G., Stokes B.T. (1998). Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. *Exp Neurol.* 152:74-87.

Sumpter R. Jr, Loo Y.M., Foy E., Li K., Yoneyama M., Fujita T., Lemon S.M., Gale M. Jr. (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol.* 79(5):2689-2699.

Suthar M.S., Ma D.Y., Thomas S., Lund J.M., Zhang N., Daffis S., Rudensky A.Y., Bevan M.J., Clark E.A., Kaja M.K., Diamond M.S., Gale M. Jr. (2010). IPS-1 is essential for the control of West Nile virus infection and immunity. *PLoS Pathog.* 6(2):e1000757.

Suzumura A., Sawada M., Takayanagi T. (1998). Production of interleukin-12 and expression of its receptors by murine microglia. *Brain Res.* 787:139-142.

Takahasi K,, Yoneyama M,, Nishihori T,, Hirai R,, Kumeta H,, Narita R,, Gale M, Jr, Inagaki F,, Fujita T. (2008). Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Mol Cell*. 29:428-440.

Takaoka A., Wang Z., Choi M., Yanai H., Negishi H., Ban T., Lu Y., Miyagishi M., Kodama T., Hondra K., Ohba Y., Taniguchi T. (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature*. 448:501-506.

Takeuchi O., Akira S. (2007). Recognition of viruses by innate immunity. *Immunol Rev*. 220:214-224.

Takeuchi O., Akira S. (2008). MDA5/RIG-I and virus recognition. *Curr Opin Immunol*. 20(1):17-22.

Takeuchi O., Akira S. (2010). Pattern recognition receptors and inflammation. *Cell*. 19;140(6):805-820.

Takahasi K., Kumeta H., Tsuduki N., Narita R., Shigemoto T., Hirai R., Yoneyama M., Horiuchi M., Ogura K., Fujita T., Inagaki F. (2009). Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. *J Biol Chem*. 284(26):17465-17474.

Theerasurakarn S., Ubol S. (1998). Apoptosis induction in brain during the fixed strain of rabies virus infection correlates with onset and severity of illness. *J. Neurovirol.* 4:407-414.

Town T., Bai F., Wang T., Kaplan A.T., Qian F., Montgomery R.R., Anderson J.F., Flavell R.A., Fikrig E. (2009). Toll-like receptor 7 mitigates lethal West Nile encephalitis via interleukin 23-dependent immune cell infiltration and homing. *Immunity.* 30:242-253.

Trifilo M.J., Lane T.E. (2003). Adenovirus-mediated expression of CXCL10 in the central nervous system results in T-cell recruitment and limited neuropathology. *J Neurovirol*. 9:315-324.

Tunkel A.R., Glaser C.A., Bloch K.C., Sejvar J.J., Marra C.M., Roos K.L., Hartman B.J., Kaplan S.L., Scheld W.M., Whitley R.J. (2008). The management of encephalitis: clinical practice guidelines by the Infectious Disease Society of America. *Clin Infect Dis.* 47:303-327.

Tyler K.L. (2009). Emerging viral infections of the central nervous system: part 2. *Arch Neurol*. 66(9):1065-1074.

van Marle G., Antony J., Ostermann H., Dunham C., Hunt T., Halliday W., Maingat F, Urbanowski M.D., Hobman T., Peeling J., Power C. (2007). West Nile virus-induced neuroinflammation: glial infection and capsid protein-mediated neurovirulence. *J Virol.* 81:10933-10949.

Wang T., Fikrig E. (2004). Immunity to West Nile virus. *Curr Opin Immunol*. 16:519-523.

Wang T., Town T., Alexopoulou L., Anderson J.F., Fikrig E., Flavell R.A. (2004). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med.* 10:1366–1373.

Wang J.P., Bowen G.N., Zhou S., Cerny A., Zacharia A., Knipe D.M., Finberg R.W., Kurt-Jones E.A. (2012). Role of specific innate immune responses in herpes simplex virus infection of the central nervous system. *J Virol*. 86(4):2273-81.

Wang Z., Choi M.K., Ban T., Yanai H., Negishi H., Lu Y., Tamura T., Takaoka A., Nishikura K., Taniguchi T. (2008). Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules. *Proc Natl Acad Sci U S A.* 105(14):5477-5482.

Wang J.J., Liao C.L., Chiou Y.W., Chiou C.T., Huang Y.L., Chen L.K. (1997). Ultrastructure and localization of E proteins in cultured neuron cells infected with Japanese encephalitis virus. *Virology*. 10;238(1):30-39.

Whitley R.J., Kimberlin D.W. (1999) Viral Encephalitis. *Ped Rev*. 20(6):192-198.

Whitley R.J., Alford C.A., Hirsch M.S., Schooley R.T., Luby J.P., Aoki F.Y., Hanley D., Nahmias A.J., Soong S.J. (1986). Vidarabine versus acyclovir therapy in herpes simplex encephalitis. *N Engl J Med.* 314:144-149.

Wildemann B., Ehrhart K., Storch-Hagenlocher B., Meyding-Lamade U., Steinvorth S., Hacke W., Haas J. (1997). Quantitation of herpes simplex virus type 1 DNA in cells of cerebrospinal fluid of patients with herpes simplex virus encephalitis. *Neurology.* 48:1341-1346.

Wilkins C., Gale M. Jr. (2010). Recognition of viruses by cytoplasmic sensors. *Curr Opin Immunol*. 22:41-47.

Wong K.T., Robertson T., Ong B.B., Chong J.W., Yaiw K.C., Wang L.F., Ansford A.J., Tannenberg A. (2009). Human Hendra virus infection causes acute and relapsing encephalitis. *Neuropathol Appl Neurobiol*. 35:296-305.

Xu F., Sternberg M.R., Kottiri B.J., McQuillan G.M., Lee F.K., Nahmias A.J., Berman S.M., Markowitz, L.E. (2006). Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA.* 296:964-973.

Yoneyama M., Fujita T. (2007). RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. *Cytokine Growth Factor Rev.* 18:545-551.

Yoneyama M., Fujita T. (2010). Recognition of viral nucleic acids in innate immunity. *Rev Med Virol.* 20(1):4-22.

Yoneyama M., Kikuchi M., Matsumoto K., Imaizumi T., Miyagishi M,. Taira K., Foy E., Loo Y.M., Gale M. Jr, Akira S., Yonehara S., Kato A., Fujita T. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol.* 175(5):2851-2858

Yoneyama M., Kikuchi M., Natsukawa T., Shinobu N., Imaizumi T., Miyagishi M., Taira K., Akira S., Fujita T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*. 7:730-737.

Yoshida H., Imaizumi T., Lee S.J., Tanji K., Sakaki H., Matsumiya T., Ishikawa A., Taima K., Yuzawa E., Mori F., Wakabayashi K., Kimura H., Satoh K. (2007). Retinoic acid-inducible gene-I mediates RANTES/CCL5 expression in U373MG human astrocytoma cells stimulated with double-stranded RNA. *Neurosci Res.* 58:199-206.

Zhang S.Y., Jouanguy E., Ugolini S., Smahi A., Elain G., Romero P., Segal D., Sancho-Shimizu V., Lorenzo L., Puel A., Picard C., Chapgier A., Plancoulaine S., Titeux M., Cognet C., von Bernuth H., Ku C.L., Casrouge A., Zhang X.X., Barreiro L., Leonard J., Hamilton C., Lebon P., Héron B., Vallée L., Quintana-Murci L., Hovnanian A., Rozenberg F., Vivier E., Geissmann F., Tardieu M., Abel L., Casanova J.L. (2007). TLR3 deficiency in patients with herpes simplex encephalitis. *Science*. 317:1522-1527.

Zhou Y., Ye L., Wan Q., Zhou L., Wang X., Li J., Hu S., Zhou D., Ho W. (2009). Activation of Toll-like receptors inhibits herpes simplex virus-1 infection of human neuronal cells. *J. Neurosci. Res.* 87:2916-2925.

APPENDIX: PUBLICATIONS

Furr SR and Marriott I. (2012). Initiation of glial immune responses during neurotropic viral infections. *Frontiers in Microbial Immunology*. Submitted for publication.

Furr SR and Marriott I. (2012). RIG-I is an essential component in glial responses to neurotropic RNA and DNA viruses. Manuscript in preparation.

Furr SR, Chauhan VS, Moerdyk-Schauwecker MJ, Marriott I. (2011). A role for DNA-dependent activator of interferon regulatory factor in the recognition of herpes simplex virus type 1 by glial cells. *J Neuroinflammation*. 12;8:99.

Furr SR, Moerdyk-Schauwecker M, Grdzelishvili VZ, Marriott I. (2010). RIG-I mediates nonsegmented negative-sense RNA virus-induced inflammatory immune responses of primary human astrocytes. *Glia.* 58(13):1620-1629.

Chauhan VS, Furr SR, Sterka DG Jr, Nelson DA, Moerdyk-Schauwecker M, Marriott I, Grdzelishvili VZ. (2010). Vesicular stomatitis virus infects resident cells of the central nervous system and induces replication-dependent inflammatory responses. *Virology*. 10;400(2):187-196

Chauhan VS, Sterka DG Jr, Furr SR, Young AB, Marriott I. (2009). NOD2 plays an important role in the inflammatory responses of microglia and astrocytes to bacterial CNS pathogens. *Glia*. 57(4):414-423.

Furr SR, Chauhan VS, Sterka D Jr, Grdzelishvili V, Marriott I. (2008). Characterization of retinoic acid-inducible gene-I expression in primary murine glia following exposure to vesicular stomatitis virus. *J Neurovirol*. 14(6):503-513.