ROLE OF THE NOVEL DNA SENSORS cGAS, IFI16, AND ZBP1, DURING VIRAL INFECTIONS IN GLIA

by

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ABSTRACT

AUSTIN MICHAEL JEFFRIES. Role of the novel DNS sensors cGAS, IFI16, and ZBP1, during viral infections in glia. (Under the direction of DR. IAN MARRIOTT)

Viral central nervous system (CNS) infections can lead to life threatening encephalitis and long-term neurological deficits in survivors. Resident CNS cell types, such as astrocytes and microglia, are known to produce key inflammatory and antiviral mediators following infection with neurotropic DNA viruses. However, the mechanisms by which glia mediate such responses remain poorly understood. Recently, a group of intracellular pattern recognition receptors (PRRs), collectively known as DNA sensors, have been identified in both leukocytic and non-leukocytic cell types. The ability of such DNA sensors to initiate immune mediator production and contribute to infection resolution in the periphery is increasingly recognized, but our understanding of their role in the CNS remains limited at best.

There is evidence for the expression and functionality of DNA sensors in resident brain cells, and these molecules likely play a role in neurotropic virus infections. The DNA sensors cyclic GMP-AMP synthase (cGAS) and interferon gamma inducible protein 16 (IFI16) are two such sensors that have been reported to be important for recognition of DNA pathogens such as herpes simplex virus 1 (HSV-1) in peripheral human cell types. In this dissertation, we provide the first demonstration that human microglia and astrocytes show robust levels of cGAS and IFI16 protein expression at rest

and following activation. Furthermore, we show that these cell types also constitutively express the critical downstream adaptor protein, stimulator of interferon genes (STING). Importantly, we demonstrate that, while cGAS serves as a significant component in interferon regulatory factor 3 (IRF3) activation and interferon beta (IFN-β) production by human microglial cell lines in response to foreign DNA, IFI16 is not required for such responses. Surprisingly, neither of these sensors mediate effective antiviral responses to HSV-1 in microglia, and this may be due, at least in part, to viral suppression of cGAS and/or IFI16 expression. As such, this ability may represent an important HSV immune evasion strategy in glial cells, and approaches that mitigate such suppression might represent a novel strategy to limit HSV-1 associated neuropathology.

Finally, our group has demonstrated the functional expression of Z DNA binding protein 1 (ZBP1) in murine glia. We report that this sensor serves as a restriction factor for HSV-1 in both murine microglia and astrocytes. Importantly, we have begun to determine the antiviral mechanisms initiated by ZBP1 responsible for such restriction and we show that ZBP1 contributes to the activation of cell death pathways in glia following HSV-1 infection. Interestingly, while ZBP1 is best known for its role in the activation of necroptotic pathways our data suggests it also can contribute to apoptosis. Together, these data indicate that ZBP1 represents an important restriction factor for HSV-1 in glia and may be an ideal target for therapeutic intervention during HSE.

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD domain
ATM	Ataxia-telangiectasia mutated
BDNA	B-form DNA
BMDCs	Bone marrow derived dendritic cells
BMDM	Bone marrow derived macrophages
BST2	Bone marrow stromal antigen 2
CARD	Caspase activation and recruitment domain
cccDNA	closed circular DNA
c-di-AMP	Cyclic dimeric adenosine monophosphate
c-di-GMP	Cyclic dimeric gunaosine monophosphate
cGAMP	cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cGAMP-synthase
CHIKV	Chikungunya virus
ChiP	Chromatin immunoprecipitation

CRISPR	Clustered regularly interspaced short palindromic repeats
DAMPs	Damage associated molecular patterns
DDX41	DEAD-Box Helicase 41
DENV	Dengue virus
DMXAA	dimethylxanthenone-4-acetic acid
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
dsDNA	double stranded DNA
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLP	G9a-like protein
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HFF	Human foreskin fibroblasts
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSE	HSV encephalitis

HSV-1	herpes simplex virus type 1
IAV	Influenza A virus
IFI16	Interferon gamma inducible protein 16
IFN-α	Interferon alpha
IFN-β	interferon-beta
IL-6	Interleukin 6
IRF3	Interferon regulatory factor 3
ISG	Interferon stimulated genes
KSHV	Kaposi sarcoma-associated herpesvirus
MCMV	Mouse cytomegalovirus
MEF	Mouse embryonic fibroblasts
MLKL	Mixed lineage kinase domain like pseudokinase
MLV	Murine leukemia virus
MOI	Multiplicity of infection
Nf-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP1	NLR family pyrin domain containing 1
NLRP3	NLR family pyrin domain containing 3
NLRs	NOD-like receptors

NOD	nucleotide-binding and oligomerization domain
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cell
PFU	Plaque forming units
PHYIN	pyrin and HIN domain
PRRs	Pattern recognition receptors
RHIM	RIP homotypic interaction motif
RIG-I	Retinoic acid-inducible gene-I
RIP	Receptor interacting protein
RIPK1	Receptor interacting protein kinase 1
RIPK3	Receptor interacting protein kinase 3
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
STING	stimulator of interferon genes
TBK1	Tank binding kinase 1
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TREX1	Three prime repair exonuclease 1

VSV	vesicular stomatitis virus
VZV	Varicella-zoster virus
WNV	West Nile virus
YDNA	Y-form DNA
ZBP1	Z-DNA binding protein 1
ZIKV	Zika virus

CHAPTER 1: INTRODUCTION

1.1 Introduction

Infection of the central nervous system (CNS) can result in encephalitis, a condition that is characterized by severe neuroinflammation resulting in fever, headaches, altered consciousness, seizures, and even death (Roos, 1999). Between 2000-2010 there were 7.3 encephalitis cases per 100,000, with most identified etiologies (48.2%) being attributable to viral infections (George et al., 2014). Since the mechanisms that lead to CNS inflammation following infection are poorly understood, current treatment strategies include general immune suppression and/or antiviral therapy (Chaudhuri and Kennedy, 2002; George et al., 2014; Venkatesan and Geocadin, 2014). Traditionally, it was thought that infiltrating peripheral monocytes and leukocytes were the major contributors of pro-inflammatory mediator production in encephalitis as most resident CNS cells were assumed to lack immune functions. However, it is now appreciated that glial cells, most notably microglia and astrocytes, play a critical role in immune surveillance in the CNS and are important contributors to both protective and detrimental host responses to infectious agents(Aloisi, 2000, 2001; M et al., 2002; Bowman et al., 2003; Bsibsi et al., 2006; Furr et al., 2008, 2011; Chauhan et al., 2009; Liu et al., 2010; Serramía et al., 2015). Glial cells can produce an array of proinflammatory and antiviral mediators following infection (Chauhan et al., 2009; Furr and Marriott, 2012) and it is now known that they accomplish this via members of multiple families of pattern recognition receptors (PRRs). These PRRs recognize numerous pathogen-associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) and trigger transcription factor activation that, in turn, elicits proinflammatory and antiviral mediator production. Of these, the most widely and best studied glial PRRs are the cell surface and endosomal Toll-like receptors (TLRs) and the cytosolic nucleotide-binding and

oligomerization domain (NOD)-like receptors (NLRs) (Sterka et al., 2006; Chauhan et al., 2009; Rebsamen et al., 2009; Liu et al., 2010; Dai et al., 2014; Reinert et al., 2016; Su and Zheng, 2017). More recently, multiple classes of cytosolic nucleic acid sensors have been discovered that are likely have an important function during active viral infections due to their intracellular location. These include RNA sensors, such as the retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) family, and the possible roles and importance of these molecules in glial immune responses have been discussed in depth elsewhere (Furr et al., 2008; Furr and Marriott, 2012; Carty et al., 2014; Nair and Diamond, 2015; Zohaib et al., 2016). However, the importance of DNA sensors, including cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS), in viral CNS infections and the initiation of glial immune functions remains more controversial, despite evidence for their antiviral function in peripheral lymphoid and myeloid cells (Unterholzner, 2013; Cai et al., 2014; Dhanwani et al., 2018; Lugrin and Martinon, 2018). Here, we will discuss the evidence for the expression and function of DNA sensors in resident CNS cells, their role during viral infections, and their potential as targets for therapeutic intervention.

1.2 cGAS/STING

Perhaps the most well-known and best studied cytosolic DNA sensor is cGAS. This molecule directly binds to double stranded DNA (dsDNA) and then catalyzes the production of the secondary messenger, 2'3'cyclic GMP-AMP (cGAMP) (Gao et al., 2013b; Sun et al., 2013; Zhang et al., 2013). This secondary messenger subsequently binds to the downstream adaptor protein stimulator of interferon genes (STING), which initiates the phosphorylation of tank binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3), and activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Such transcription factor activation precipitates the expression of interferon-beta (IFN-β) and other antiviral and/or pro-inflammatory cytokines (Ishikawa et al., 2009; Li et al., 2013; Abe and Barber, 2014; Fang et al., 2017; Sun et al., 2017; Aarreberg et al., 2019). Since its discovery in 2013, cGAS has been demonstrated to play a critical role in recognizing and eliminating a diverse array of pathogens, either through direct recognition of microbial DNA or indirect recognition of retroviral DNA intermediates or DAMPS, such as released mitochondrial DNA (Gao et al., 2013a; Abe and Barber, 2014; Dai et al., 2014; Herzner et al., 2015; Paijo et al., 2016; Fang et al., 2017; Sun et al., 2017; Cheng et al., 2018; Wong et al., 2019). As discussed elsewhere (Cai et al., 2014; Chen et al., 2016; Dhanwani et al., 2018; Ablasser and Chen, 2019), numerous studies have demonstrated the expression and function of cGAS in peripheral leukocytic and non-leukocytic cell types, such as human plasmacytoid dendritic cells, macrophages, monocytes, helper T-lymphocytes, and endothelial cells (as summarized in Table 1). However, less attention has been given to the role of this sensor in the CNS and the immune responses of glial cells (Lahaye et al., 2013; Li et al., 2013; Dai et al., 2014; West et al., 2015; Ma et al., 2015b; Bode et al., 2016;

Vermeire et al., 2016; Paijo et al., 2016; Luecke et al., 2017; Su and Zheng, 2017; Sun et al., 2017; Swanson et al., 2017).

The first description of cGAS expression in resident CNS cell types came from Cox and colleagues (Cox et al., 2015), who established the constitutive and inducible expression of mRNA encoding cGAS in murine microglia. Interestingly, while they observed neither constitutive nor IFN-β-inducible expression of cGAS in murine astrocytes, siRNA-mediated cGAS knockdown decreased IFN-β activity in both microglia and astrocytes following exposure to exogenous dsDNA (Cox et al., 2015). However, such expression has not been established in human microglia and astrocytes.

Circumstantial evidence for the functional importance of cGAS in the brain lays in the recognized ability of many important CNS pathogens to interfere with this sensor and/or it's signaling pathway (as summarized in Table 2). For example, herpes simplex virus 1 (HSV-1), the dsDNA virus that is the most common cause of fatal sporadic encephalitis, has multiple gene products that can interfere with the cGAS-STING signaling pathway (Bradshaw and Venkatesan, 2016). The HSV-1 encoded protein UL37 attenuates the enzymatic activity of cGAS and lowers cGAMP production in human monocytes and fibroblasts, thereby decreasing antiviral gene expression (Zhang et al., 2018). Mutations in UL37 that interfere with its deamidase activity prevent this protein from inhibiting cGAS and lead to lower HSV-1 titers in the brain following infection (Zhang et al., 2018). Interestingly, such an inhibitory activity appears to show species specificity as UL37 molecule does not appear to be important in infections in some non-human primate species (Zhang et al., 2018). Another HSV-1 product, UL41 (virion host shutoff protein), has been shown to decrease IFN-β production in a human epithelial cell line expression system by reducing cGAS protein expression (Su and Zheng, 2017), and its importance in

disrupting cGAS-mediated antiviral responses has been illustrated by the ability of cGAS knockdown to increase viral production of a UL41 null mutant HSV-1 strain, but not a wild type strain (Su and Zheng, 2017). Since all members of the family Herpesviridae appear to target cGAS (Table 2 and as reviewed in (Chan and Gack, 2016; Phelan et al., 2020), and such viruses can cause latent infections, it is tempting to speculate that the inhibition of this sensor may play a critical role in establishing viral latency. Such a mechanism could be of particular importance for HSV encephalitis (HSE) as the reactivation of a latent infection is thought to be a key contributor to the development of this condition (Menendez and Carr, 2017).

In addition to DNA viruses, other neurotropic viruses, such as positive stranded RNA viruses from the family Flaviviridae, can also hamper cGAS activity (Table 2). For instance, Dengue virus (DENV) that can cause encephalitis has been shown to target cGAS for degradation and prevent it from detecting released mitochondrial DNA in human monocyte-derived dendritic cells and monocytic and fibroblastic cell lines (Aguirre et al., 2017; Sun et al., 2017). Specifically, the DENV protein NS2B3 has been shown to directly interact with cGAS and target it for lysosomal degradation (Aguirre et al., 2017). In addition, this viral product can also cleave the downstream signaling molecule STING, further disrupting cGAS-mediated antiviral signaling. Interestingly, this viral product also shows species specificity similar to the HSV-1 product UL37 as NS2B3 targets human STING but does not cleave this molecule in mouse or non-human primate cells (Stabell et al., 2018). Similarly, Zika virus (ZIKV), which came to prominence for its role in CNS and peripheral nerve pathologies including microcephaly and Guillain-Barré syndrome, can cleave STING via its NS2B3 protein and this effect, again, is restricted to human rather than murine cells (Ding et al., 2018). ZIKV can further disrupt cGAS-STING signaling by stabilizing caspase-1 protein. This results in cGAS cleavage and reduced

expression of type I IFNs and interferon stimulated genes (ISGs), and the promotion of inflammasome activation and ZIKV production (Zheng et al., 2018). Importantly, Zheng and colleagues demonstrated that cGAS deficiency or caspase-1 inhibition leads to increased cellular levels of ZIKV genetic material (Zheng et al., 2018). Such findings therefore support the therapeutic potential of augmenting cGAS-STING mediated responses to combat debilitating neurotropic RNA virus infections.

More direct evidence of the importance of cGAS-STING signaling during viral CNS infections comes from the study of HSV-1 infection in STING deficient animals. Parker and coworkers (Parker et al., 2015) demonstrated that STING deficient animals show increased susceptibility to intracerebral HSV-1 infection, with all succumbing within 3-5 days of infection and little mortality in age-matched wildtype animals. Interestingly, it appears that this increased susceptibility is dependent on the route of infection, as STING deficient animals show survival rates comparable to wildtype mice following administration via the cornea, despite the presence of high viral titers in the cornea and trigeminal ganglion (Parker et al., 2015). This phenomenon was subsequently confirmed (Royer and Carr, 2016) and the apparent discrepancy in lethality is likely to be due to difference in the distribution of HSV-1 within the CNS. High viral titers are limited to the trigeminal ganglion following corneal HSV-1 infection, while intracerebral infection results in widely disseminated HSV-1 infection throughout the CNS (Parker et al., 2015). In addition, it should be noted that susceptibility to HSV-1 appears to be strain dependent as STING deficient animals succumb to neuroinvasive strains of HSV-1 following corneal infection more rapidly than wild type animals (Parker et al., 2015). Regardless, it is clear that STING plays a role in HSV-1 neuroinvasion and is critical for protective host responses once the virus has disseminated throughout the CNS (Parker et al., 2015; Royer and Carr, 2016). The

increased susceptibility of STING deficient animals to HSV-1 neuroinvasion may be due, at least in part, to a decreased expression of the ISG tetherin (also known as bone marrow stromal antigen 2 (BST2)), as these animals exhibit decreased expression of this ISG (amongst others) during infection (Royer and Carr, 2016), and tetherin depletion has been shown to increase HSV-1 titers in the trigeminal ganglion (Royer and Carr, 2016).

Consistent with these studies employing STING deficient animals, treatment with the STING agonist dimethylxanthenone-4-acetic acid (DMXAA) has been demonstrated to increase IFN-β expression by HSV-1 infected mouse fibroblasts and to lower the production of viral particles by these cells (Cerón et al., 2019). Importantly, in vivo DMXAA treatment can lower viral titers in the cornea, trigeminal ganglion, and brainstem, of mice infected with the neuroinvasive McKrae strain of HSV-1, and this is reflected by increased survival and improved neurological outcomes in these animals (Cerón et al., 2019). As such, these studies provide a tantalizing glimpse of the potential of targeting the cGAS-STING pathway to treat CNS infections.

While these studies illustrate the importance of cGAS-STING signaling in HSV-1 infections of the CNS, the specific role of this sensor system in glia was established by Reinert and colleagues (Reinert et al., 2016) in a mouse model of HSV-1 encephalitis. They demonstrated that cGAS deficiency resulted in a phenotype that matched that observed in STING deficient mice following ocular HSV-1 infection (Reinert et al., 2016). Furthermore, they established that microglia were the primary producers of IFN-β after HSV-1 challenge and showed that only this glial cell type produced higher viral titers in vitro following the loss of STING (Reinert et al., 2016). In vivo, however, neurons and astrocytes showed greater numbers of HSV-1 viral particles in STING deficient mice (Reinert et al., 2016). This discrepancy was

explained by the observation that astrocytes and neurons initiate antiviral programs in vivo in response to IFN-β produced by microglia in a TLR3-dependent manner (Reinert et al., 2016). This suggests that microglia represent the first responders to HSV-1 infection in the CNS.

1.3 IFI16

Interferon gamma inducible protein 16 (IFI16) is a member of the Pyrin and HIN domain (PHYIN) family of proteins that can serve as an intracellular DNA sensing molecule. PHYIN proteins are characterized by the presence of an N-terminal pyrin domain and one or two C-terminal HIN domains (Unterholzner et al., 2010). The HIN domains bind DNA while the pyrin domain is required for protein-protein interactions (Unterholzner et al., 2010). IFI16, and its mouse ortholog p204, was the first PHYIN family members demonstrated to induce IFN-β in response to transfected DNA (Unterholzner et al., 2010). Additionally, IFI16 has been shown to interact with STING and knockdown of either of these proteins leads to reduced IFN-β production by the human and mouse monocytic cell lines THP-1 and RAW 264.7, respectively (Cridland et al., 2012). Interestingly, similar findings were described in murine astrocytes and microglia where p204 knockdown was shown to reduce IFN-β expression following DNA transfection (Cox et al., 2015).

However, IFI16 does not appear to contribute to IFN-β expression by human foreskin fibroblasts (HFF) stimulated with exogenous DNA (Orzalli et al., 2015). It is possible this finding might be indicative of cell type-specific differences. However, Gray and colleagues (Gray et al., 2016) used a mouse model lacking all 13 PHYIN family members to demonstrate that these receptors were dispensable for IFN responses to DNA transfection in bone marrow derived macrophages and mouse embryonic fibroblasts. But despite an apparent lack of involvement in IFN production, this study did identify a requirement for PHYIN family members in inflammasome activation, as characterized by the maturation of the potent pro-inflammatory cytokines IL-1β and IL-18 (Gray et al., 2016). Such a role for IFI16 in linking DNA sensing and inflammasome activation is supported by multiple studies (Ansari et al., 2013, 2015; Johnson et

al., 2013; Dutta et al., 2015; Iqbal et al., 2016; Orzalli et al., 2016) and is discussed in depth elsewhere (Dell'Oste et al., 2015), but it is currently unknown whether IFI16-mediated inflammasome activation occurs in CNS cell types.

While there is conflicting evidence for the role of IFI16 in IFN-β responses to foreign DNA challenge, multiple lines of investigation indicate that this DNA sensor contributes to IFN-β and ISG expression following viral infection (Orzalli et al., 2012; Jakobsen et al., 2013; Ansari et al., 2015; Diner et al., 2015; Ma et al., 2015a; Orzalli et al., 2016; Zhang et al., 2016; Jønsson et al., 2017; Lum et al., 2019; Yang et al., 2019). For example, nuclear IRF3 translocation and subsequent IFN production in HSV-1 infected corneal epithelial cells has been shown to be dependent, at least in part, on p204 expression (Conrady et al., 2012), while TBK-1 phosphorylation and subsequent IFN-β expression by HSV-1 infected HFFs was found to require IFI16 and STING (Orzalli et al., 2012).

Interestingly, Orzalli and colleagues (Orzalli et al., 2012) used attenuated HSV-1 strains to determine that expression of the immediate early viral protein ICP0 leads to IFI16 degradation and reduced nuclear IRF3 translocation, and this degradation was subsequently shown to be dependent on proteasome activity (Cuchet-Lourenco et al., 2013). Such findings are supported in HFFs where IFI16 knockdown does not affect HSV-1 levels in cells infected with wild type HSV-1 but significantly increases viral titers following infection with an ICP0 null mutant virus (Diner et al., 2016). Similarly, IFI16 has been reported to be dispensable for IFN production in mice following human cytomegalovirus (HCMV) infection (Gray et al., 2016) and this apparent independence may also stem from the reported ability of HCMV to interfere with IFI16 signaling (Dell'Oste et al., 2014). However, it is should be noted that the ability of these viruses to reduce

IFI16 protein abundance and/or signaling may show cell type specificity as HSV-1 does not elicit such effects in either U2OS or HeLa cells (Orzalli et al., 2016).

Since the available evidence indicates that IFI16 has a role in virally-induced IFN signaling and that this is accomplished through via a STING-dependent pathway, it is possible that this molecule could work in concert with cGAS to stimulate antiviral responses. Evidence for this notion comes from the observation that knockdown of either STING, IFI16, or cGAS, in human fibroblasts leads to reduced HSV-1 infection-induced IFN-β expression (Orzalli et al., 2015). Interestingly, in the same study it was noted that cGAS knockdown reduces constitutive IFI16 protein expression and that this effect was dependent on proteasome activity (Orzalli et al., 2015). This suggests that cGAS may stabilize IFI16 protein levels to promote antiviral activity.

Additional support for cooperation between IFI16 and cGAS comes from the work of Jønsson and coworkers (Jønsson et al., 2017) who demonstrated that IFI16 knockdown reduces cGAMP production by THP-1 cells following foreign DNA challenge. Furthermore, they showed that HEK293t cells stably expressing IFI16 produce higher amounts of cGAMP following intracellular administration of a cGAS expression plasmid than IFI16 deficient cells (Jønsson et al., 2017). Similarly, another study showed that the co-transfection of increasing amounts of an IFI16 expression plasmid with constant levels of STING and cGAS increased IFN-β activity as assessed by IFN promoter driven luciferase activity, and demonstrated the ability of IFI16 and cGAS to interact directly (Almine et al., 2017). It should be noted that these investigators failed to detect significant changes in cGAMP levels in the absence or presence of IFI16 (Almine et al., 2017). Rather, they determined that IFI16 was required for cGAMP to fully activate STING as assessed by CCL5 expression, STING dimerization, and IRF3 nuclear translocation, in response to cGAMP transfection (Almine et al., 2017).

While cGAS and IFI16 might play redundant roles in STING activation, some evidence suggests that they promote similar responses through different mechanisms. For instance, it was found that IFI16 is not required for cGAS/STING/TBK-1 signaling in HFFs following HSV-1 or HCMV infection, but was for the transcription of IFN-β, ISG54, ISG56, and RANTES (Diner et al., 2016). Interestingly, the same investigators found that IFI16 also reduced the transcription of the HSV-1 genes icp27, icp8, and ul30 (Diner et al., 2016). This suggests that the antiviral functions previously attributed to IFI16 may occur through transcriptional regulation, rather than by direct activation of cGAS-STING signaling.

The ability of IFI16 to negatively regulate viral transcription has been reported for other herpesviruses, human papillomavirus (HPV), and hepatitis B virus (HBV) (Gariano et al., 2012; Lo Cigno et al., 2015; Roy et al., 2016, 2019; Pisano et al., 2017; Yang et al., 2019), and the modification of heterochromatin and euchromatin appears to be the primary mechanism by which this is accomplished. For example, U2OS cells or an immortalized human keratinocyte cell line (NIKS) overexpressing IFI16 exhibit elevations in heterochromatin markers, such as H3K9m2, and decreases in euchromatin markers, such as H3K4me2, in early and late HPV promoters as determined by chromatin immunoprecipitation (ChiP) analysis (Lo Cigno et al., 2015). In addition, IFI16 has been demonstrated to directly interact with the histone H3-K9 methyltransferases, SUV39h1 and G9a-like protein (GLP), and knockdown of these proteins in a B cell lymphoma latently infected with Kaposi sarcoma-associated herpesvirus (KSHV) (BCBL1 cells) led to increased viral transcription (Roy et al., 2019). Furthermore, in the same study, IFI16 knockdown reduced the recruitment of both methyltransferases to the KSHV genome (Roy et al., 2019).

In a HBV covalently closed circular DNA (cccDNA) model of infection, overexpression of IFI16 has been shown to increase IFN-β and ISG expression along with decreased euchromatin and increased heterochromatin markers on cccDNA (Yang et al., 2019). Interestingly, knockdown of IFI16 in BCBL1 cells increased the transcription of immediate early, early, and late lytic KSHV genes, indicating reactivation of the lytic cycle, while the reintroduction of IFI16 reduced KSHV genome copy numbers (Roy et al., 2016). This was also found to be true for Akata and MUTU1 cell lines latently infected with Epstein-Barr virus (Pisano et al., 2017). As such, it will be important to determine whether IFI16 similarly contributes to HSV-1 latency in CNS cell types, since reactivation of latent infections is a key event in the onset of HSV-1 encephalitis (Menendez and Carr, 2017). If so, IFI16 could be a promising new therapeutic target, either as an intervention during CNS infection or to prevent reactivation of HSV-1 in at-risk populations.

1.4 ZBP1

Z-DNA binding protein 1 (ZBP1; also known as DNA-dependent activator of interferon regulatory factors (DAI) and DLM-1) was the first identified cytosolic DNA sensor, and was shown to directly bind dsDNA in murine L929 fibroblast-like cells (Takaoka et al., 2007). Importantly, this study demonstrated ZBP1 can interact with TBK and IRF3 and contribute to IFN-β mRNA expression following DNA transfection or infection with HSV-1 (Takaoka et al., 2007). However, it seems that this cytosolic DNA sensor may function in a cell type and ligand specific manner, as ZBP1 knockdown in mouse embryonic fibroblasts (MEFs) has little or no effect on exogenous DNA-induced IFN-β expression (Wang et al., 2008). Similarly, ZBP1 knockdown was found to significantly reduce IFN-β expression in L929 cells in response to B-form DNA (BDNA) transfection but had no effect in a similarly challenged human lung epithelial cell line (Lippmann et al., 2008).

In addition to the expression of antiviral cytokines, ZBP1 has also been shown to mediate the expression of the pro-inflammatory cytokine IL-6 (Takaoka et al., 2007), and Kaiser and colleagues (Kaiser et al., 2008) subsequently demonstrated the activation of a NF-κB-driven luciferase promoter in HEK293t cells overexpressing ZBP1. This group identified three RIP homotypic interaction motif (RHIM)-like repeats and hypothesized that such NF-κB activation occurs via a RHIM-dependent interaction with receptor interacting protein 1 (RIPK1) in a similar manner to that seen with TLR3 (Kaiser et al., 2008). This hypothesis was confirmed by the demonstration that ZBP1 can interact with both RIPK1 and receptor interacting protein 3 (RIPK3) through its first RHIM domain, and by the ability of RIPK1 knockdown or mutations in the RHIM domain in RIPK1 or ZBP1 to decrease NF-κB promoter activation. The ability of ZBP1 to interact with RIPK1 and RIPK3, and to activate NF-kB-mediated gene transcription was

subsequently confirmed in a similar HEK293 cell expression system (Rebsamen et al., 2009). In is interesting to note, however, that co-expression of ZBP1 and RIPK3 was also reported to elicit NF-kB activation in these studies, an observation that is in contrast to TLR3 signaling where RIPK3 blocks RIPK1-mediated NF-κB activation (Kaiser et al., 2008).

In agreement with these studies in non-CNS cell types, we have determined that murine microglia and astrocytes express ZBP1 in an inducible manner, and found that this sensor contributes to pro-inflammatory cytokine production by glia following HSV-1 infection (Furr et al., 2011). Furthermore, these studies also showed that HSV-1 infection induces the production of soluble neurotoxic mediators by astrocytes and microglia in a ZBP1-dependent manner (Furr et al., 2011). Surprisingly, combined knockdown of ZBP1 and RIG-I leads to greater reductions in tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) production by HSV-1 infected glia than either alone, suggesting that these dissimilar sensors can act in synergy (Crill et al., 2015). Together, these studies suggest a role for ZBP1 in inflammation and/or antiviral immunity both in the periphery and the CNS.

While the available evidence supports a role for ZBP1 as a DNA sensor capable of inducing cytokine production, some studies suggest that ZBP1 plays a broader role in antiviral immunity. For example, ZBP1 has been reported to work in concert with RIPK3 in murine fibroblasts and epithelial cells to induce necroptosis following infection with a mutant mouse cytomegalovirus (MCMV) strain (Upton et al., 2012) that lacks the expression of m45, a viral product that limits ZBP1/RIPK3 interactions due the presence of a RHIM domain (Table 1) (Rebsamen et al., 2009; Upton et al., 2012). Interestingly, a similar immune evasion mechanism has been observed for HSV-1 (Table 1), where the viral protein ICP6 also contains a RHIM domain that is capable of inhibiting necroptosis in peripheral human cells (Guo et al., 2015).

Necroptotic cell death initiated by simultaneous treatment with TNF-α and the caspase inhibitor zVAD-FMK was blocked following infection with wild type HSV-1, while an ICP6 deficient HSV-1 strain failed to prevent cell death (Guo et al., 2015; Sawai, 2016). This finding is in sharp contrast to studies in mouse cells, where infection with wild type HSV-1 elicits cell death in a RIPK3-dependent manner (Wang et al., 2014; Huang et al., 2015). Surprisingly, expression of ICP6 in MEFs was found to be enough to induce RIPK3-dependent cell death, while the presence of ICP6 containing mutations in the RHIM domain did not (Wang et al., 2014; Huang et al., 2015), suggesting that ICP6 may be able to directly induce necroptosis in this cell type (Wang et al., 2014). The reason for these apparently contradictory findings was discovered in more recent studies that show ICP6 has species-dependent effects, inducing necroptosis in cells from mice while inhibiting it in human cells, HSV's primary natural host (Huang et al., 2015; Guo et al., 2018).

Importantly, ZBP1 has been found to be a major contributor to necroptosis in both human and mouse fibroblasts following infection with either an ICP6-deficient or ICP6 RHIM mutant HSV-1 strain (Guo et al., 2018). The potential importance of this pathway in antiviral immunity within the CNS is underscored by the decreased survival and increased viral burden in the brain of RIPK3 deficient mice following HSV-1 infection (Wang et al., 2014; Huang et al., 2015). However, since necroptosis promotes inflammation, it will be important to determine whether this ZBP-mediated response also contributes to CNS pathology during HSV-1 encephalitis, especially in the human host (Dhuriya and Sharma, 2018).

While it is increasingly clear that ZBP1 is a PRR that is capable of initiating cell death pathways, it is less certain what ligands specifically initiate such as response. ZBP1 was initially shown to directly bind BDNA and it has recently been shown to recognize plasmid DNA (Wang

et al., 2008; Semenova et al., 2019). However, other studies have shown that ZBP1 is critical for the induction of necroptosis, pyroptosis, and apoptosis, in cells challenged with influenza virus, a segmented negative strand RNA virus (Kuriakose et al., 2016; Thapa et al., 2016), and this role is discussed in depth elsewhere (Dhuriya and Sharma, 2018). By pharmacologically inhibiting various stages of the MCMV life cycle, Sridharan and colleagues (Sridharan et al., 2017) were able to determine that active transcription was required for ZBP1-mediated cell death, suggesting that RNA serves as the activating ligand in this response. This notion was subsequently supported by two studies describing the ability of ZBP1 to bind endogenous RNA (Maelfait et al., 2017; Jiao et al., 2020).

An ability to sense both RNA and DNA accounts for the protective role of ZBP1 in influenza virus infection and following exposure to other RNA viruses including West Nile virus (WNV) and ZIKV (Daniels et al., 2019; Rothan et al., 2019). Interestingly, however, ZBP1-mediated protection against these neurotropic flaviviruses appears to be independent of cell death in neurons (Daniels et al., 2019; Rothan et al., 2019). Mice genetically deficient in ZBP1 show worse clinical scores, higher viral burdens in the brain, and increased mortality, following WNV infection than wild type mice (Rothan et al., 2019). Similarly higher viral burdens and mortality have been observed in WNV challenged RIPK3 deficient animals, and this effect was independent of cell death pathways (Daniels et al., 2017). Surprisingly, ZBP1 deficient animals demonstrate higher levels of antiviral and inflammatory cytokines/chemokines following WNV infection (Rothan et al., 2019), and this finding is in contrast to similarly infected RIPK3 deficient mice, which demonstrate decreased inflammatory cytokine production (Daniels et al., 2017). As such, it is possible these two molecules have independent roles during neuronal

infection, especially since peripheral cells undergo cell death in both a ZBP1 and a RIPK3-dependent manner (Daniels et al., 2017; Rothan et al., 2019).

A potential mechanism for the antiviral effects of ZBP1 and RIPK3 during neurotropic RNA virus infections comes from the studies of Daniels and coworkers (Daniels et al., 2019) that indicate a neuron-specific function for ZBP1. They found that ZBP1-induced antiviral gene expression in neurons following ZIKV infection occurs in a RIPK1 and RIPK3-dependent manner, and that loss of any of these signaling molecules results in increased viral burden and mortality (Daniels et al., 2019). Surprisingly, RIPK3 deficiency in primary microglial cultures did not result in increased ZIKV replication in these studies suggesting that such protection is intrinsic to neurons. Consistent with this notion, upregulation of the antiviral gene *IRG1* was required for protection against both ZIKV and WNV infection in neurons, but this gene was not upregulated in microglia (Daniels et al., 2019), indicating a cell type specific function for ZBP1. Since we have identified a role for this sensor in glia following HSV-1 infection, it will be interesting to see what role, if any, ZBP1 plays in glial responses to neurotropic RNA viruses (Furr et al., 2011; Crill et al., 2015). Regardless, it is apparent that ZBP1 is an important mediator of CNS innate immune responses to both RNA and DNA viruses.

1.5 AIM2

Absent in melanoma 2 (AIM2) is another member of the PHYIN family of interferon inducible proteins that has been found to act as a DNA sensor (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Adamczak et al., 2014). However, unlike IFI16, recognition of dsDNA by AIM2 has been shown to lead exclusively to inflammasome activation and the induction of pyroptosis, an inflammatory form of cell death (Adamczak et al., 2014). Upon binding to dsDNA, AIM2 associates with the downstream signaling molecule apoptosis-associated speck-like protein containing a CARD domain (ASC), which recruits and activates caspase-1 (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Caspase-1 then acts as the effector protein to cleave the immature form of IL-1β and IL-18, leading to the maturation and secretion of these potent inflammatory cytokines (Miao et al., 2011). Additionally, caspase-1 can cleave gasdermin D to initiate pyroptotic cell death, characterized by the formation of pores in the plasma membrane and the release of cellular contents into the extracellular environment (Kayagaki et al., 2015; Shi et al., 2015). The AIM2 inflammasome has been shown to form following infection with either DNA or RNA viruses in peripheral myeloid and lymphoid immune cell-types, such as bone marrow derived dendritic cells (BMDCs), bone marrow derived macrophages (BMDM), monocytes, and fibroblasts (Rathinam et al., 2010; Ekchariyawat et al., 2015; Schattgen et al., 2016; Huang et al., 2017a; Zhang et al., 2017), and the role of this and other inflammasomes during viral infection is discussed extensively elsewhere (Chen and Ichinohe, 2015; Lupfer et al., 2015; Man et al., 2016; Shrivastava et al., 2016; Lugrin and Martinon, 2018; Zhu et al., 2019).

While the AIM2 inflammasome is recognized to have an antiviral function in peripheral cell types, relatively little is known about its role in CNS infections despite having been shown

to be expressed in neurons and glia (Adamczak et al., 2014; Cox et al., 2015). Furthermore, AIM2 has been shown to function as a DNA sensor in neurons as the cytosolic administration of exogenous DNA induces the association of AIM2 with ASC and leads pyroptosis in these cells (Adamczak et al., 2014). Since neuronal cell death is typically detrimental to the host, it appears likely that the proinflammatory nature of the AIM2 inflammasome can be damaging in the context of viral CNS infections. Circumstantial evidence supporting this notion comes from the effect of deleting ataxia-telangiectasia mutated (ATM), a protein known for its role in activating DNA damage responses, in primary murine microglia (Song et al., 2019). Such a deletion results in cytoplasmic DNA accumulation and cellular activation as demonstrated by the retraction of processes (Song et al., 2019). Moreover, co-culture of microglia and neurons with an ATM inhibitor leads to neuronal cell damage, which is reversed with an IL-1 receptor antagonist consistent with a major role for the inflammasome in this effect (Song et al., 2019). Importantly, this study utilized co-immunoprecipitation approaches to demonstrate that inflammasome activation as a result of ASC association with AIM2 rather than other initiator molecules such as NLR family pyrin domain containing 3 (NLRP3) (Song et al., 2019). A detrimental role for AIM2 in CNS pathologies is further supported by the observation that AIM2 deficient mice show less brain atrophy and cognitive defects following stroke than their wild type counterparts (Kim et al., 2020). Furthermore, caspase-1 inhibition resulted in a similar phenotype in these studies indicating that the improved outcome was due to reduced AIM2 inflammasome activation (Kim et al., 2020).

Despite such evidence, some studies suggest that AIM2 can play a protective role in some infections. For example, the neurotropic RNA viruses, WNV and Chikungunya virus (CHIKV), have been shown to activate the AIM2 inflammasome in peripheral dermal fibroblasts

and AIM2 knockdown led to increased CHIKV genome copies in these cells (Ekchariyawat et al., 2015). Furthermore, the higher levels of AIM2, caspase 1, IL-1β, and IL-18, found in brain tissue from still births following ZIKV infection provides circumstantial evidence for a role for this sensor (de Sousa et al., 2018). However, it should be noted that the expression of two other inflammasome activators, NLRP3 and NLRP1, were also elevated in this tissue, and it is not clear whether the upregulation of inflammasome components reflect a protective host response or contribute to disease pathology. Similarly, AIM2 expression is upregulated in neurons following infection with enterovirus A71, the causative agent of hand foot and mouth disease and AIM2 knockdown in a neuronal cell line led to decreased IL-1β cleavage and increased viral copy numbers (Yogarajah et al., 2017). Yet, no mechanism has yet been defined for AIM2-mediated sensing of RNA viruses.

With regard to neurotropic DNA viruses, AIM2 was initially demonstrated to be dispensable for inflammasome activation following HSV-1 infection in peritoneal macrophages, but was for necessary for such responses to MCMV challenge (Rathinam et al., 2010). Conversely, another study indicated that IFI16 and NLRP3 were the initiators of inflammasome activation in HFFs following HSV-1 infection (Johnson et al., 2013). This apparent discrepancy may be due to the ability of the HSV-1 product VP22 to block AIM2-mediated inflammasome activation by preventing oligomerization (Table 2) (Maruzuru et al., 2018). Intracranial administration of an HSV-1 strain lacking VP22 leads to decreased viral burdens in wildtype mice but not those lacking AIM2, suggesting that this sensor can limit viral replication in the CNS (Maruzuru et al., 2018). Interestingly, the protective functions of AIM2 in the CNS may extend to bacterial pathogens as AIM2 has been shown to contribute to survival following CNS infection with *Staphylococcus aureus* (Hanamsagar et al., 2014).

It is clear from the available data that out current understanding of the role of AIM2 in the CNS is rudimentary. While some evidence suggests that AIM2 contributes to CNS disease pathology, some indicate protective functions. As such, it may be that AIM2 plays a context-dependent role where this molecule exacerbates sterile inflammation in neurodegenerative diseases when activation tends to be chronic, while acute activation assists in viral or bacterial clearance. Since our understanding of AIM2 in the CNS is based mostly on circumstantial evidence, further study is clearly required to determine the role of this molecule relative to other inflammasome activators, and to determine whether this pathway can be targeted for therapeutic intervention.

1.6 DDX41, Ku70/DNA-PK, and RNA polymerase III

Several other putative DNA sensors have been identified in peripheral cell types but their role as PRRs in the CNS remains more controversial. DEAD-Box Helicase 41 (DDX41) was first identified as a cytosolic DNA sensor in a murine dendritic cell-like line with the demonstration that this molecule can directly bind dsDNA and interact with the common DNA sensing and antiviral signaling components STING and TBK1 (Zhang et al., 2011b). Importantly, DDX41 knockdown was shown to decrease IFN-α production in these cells in response to dsDNA transfection or infection with either HSV-1 or adenovirus (Zhang et al., 2011b). Interestingly, DDX41 has also been shown to directly bind cyclic dinucleotides such as cyclic dimeric guanosine monophosphate (c-di-GMP) and cyclic dimeric adenosine monophosphate (c-di-AMP), and knockdown of DDX41 prevents STING association with TBK1 or IRF3 and reduces antiviral signaling in response to these molecules (Parvatiyar et al., 2012). As such, it is possible that DDX41 bolsters cGAS-STING signaling by promoting cGAMP-STING interactions. Evidence for such a suggestion comes from the demonstration that DDX41 knockdown further reduces murine leukemia virus (MLV)-induced IFN expression by cGAS deficient macrophages and dendritic cells (Stavrou et al., 2015). Furthermore, IFN-β expression could be rescued in macrophage-like cells following cGAS knockdown with the administration of exogenous cGAMP prior to MLV infection, but this procedure failed to rescue such responses in cells following DDX41 knockdown (Stavrou et al., 2018). Together, these studies suggest that DDX41 can act in a cooperative manner with cGAS to induce STING activation following viral challenge.

To date, it is not known whether DDX41 is expressed in the mammalian CNS. However, the drosophila DDX41 homolog, Abstrakt, has been shown to be involved in visual and CNS

system development (Irion and Leptin, 1999; Schmucker et al., 2000). Furthermore, DDX41 is highly expressed in the zebrafish brain and this gene product performs similar antiviral functions to mammalian DDX41 when expressed in a HEK293 expression system (Ma et al., 2018). Finally, circumstantial evidence of a role for DDX41 in the human CNS lays in the observation that HSV-1 has evolved an evasion mechanism targeting DDX41 (Table 2), suggesting that this molecule can act as a restriction factor for this neurotropic virus (Duan et al., 2019).

DNA protein kinase (DNA-PK) is a protein complex made up of a DNA protein kinase catalytic subunit (DNA-PKcs), ku70, and ku80, and is best known for its role in DNA double stranded break repair. However, several studies have shown that it can bind to transfected DNA and elicit the expression of IFN-β and other ISGS, independent of kinase activity (Ferguson et al., 2012; Harnor et al., 2017; Burleigh et al., 2020). In addition, the Ku70 subunit has also been identified as a possible DNA sensor in studies where plasmid transfected HEK293 cells produce the type three IFN, IFN-λ1, that can limit human immune deficiency virus (HIV) replication (Zhang et al., 2011a). In this work, Ku70 and Ku80 were both found to bind transfected DNA, but only the loss of Ku70 decreased IFN-λ1 expression in these cells (Zhang et al., 2011a). This finding was confirmed in splenocytes derived from ku70 deficient mice (Zhang et al., 2011a), and it was later determined that Ku70-mediated IFN-λ1 expression requires the expression of STING (Sui et al., 2017). However, it should be noted that these results are in contrast to a more recent report in which signaling through DNA-PK was found to be independent of the presence of STING (Burleigh et al., 2020). While it is presently unclear whether Ku70 functions alone or in concert with Ku80 and the DNA-PKcs to elicit antiviral activity, Ku70/DNA PK has been demonstrated to induce cytokine responses following infection of hepatocyte carcinoma and monocytic cell lines with HBV and human T-cell leukemia virus type 1, respectively (Li et al.,

2016; Wang et al., 2017a). Furthermore, vaccinia virus and adenovirus have both been shown to antagonize Ku70/DNA-PK signaling (Peters et al., 2013; Scutts et al., 2018; Burleigh et al., 2020). Together, these studies suggest that ku70/DNA-PK acts as a viral PRR in addition to its DNA repair functions.

Despite evidence for antiviral functions of ku70/DNA-PK in peripheral cell types such as monocytes and fibroblasts, little exists for such a role in the CNS (Li et al., 2016; Burleigh et al., 2020). Expression of DNA-PK in the CNS and its role in DNA repair has been established from the study of mutations in severe combined immunodeficiency (SCID) mice that result in a truncated kinase domain in DNA-PK (Chechlacz et al., 2001; Vemuri et al., 2001). This has been shown to cause increased neuronal cell death in vitro and in vivo, presumably as a consequence of accumulated dsDNA breaks (Chechlacz et al., 2001; Vemuri et al., 2001). Since the DNA repair functions of DNA-PK are found in CNS cell types, the DNA sensing abilities of this molecule seen in peripheral cells may also be retained in the brain and this possibility requires further investigation.

Finally, RNA polymerase III was simultaneously identified as a DNA sensor by two groups as they investigated the mechanisms responsible for RIG-I mediated DNA sensing (Ablasser et al., 2009; Chiu et al., 2009). They demonstrated that poly (dA:dT) was reverse transcribed to RNA that then served as a ligand to activate RIG-I and induce IFN-β production in monocytes, fibroblasts, and dendritic cells (Ablasser et al., 2009; Chiu et al., 2009). Our own work subsequently showed that RNA polymerase III is functionally expressed in murine glia and a human microglial cell line (Crill et al., 2015; Johnson et al., 2020), and that its inhibition reduces HSV-1-induced IRF3 activation and TNF-α production in murine microglia cells and astrocytes (Crill et al., 2015). In addition to HSV-1, evidence suggests that RNA polymerase III

also has a role in recognizing varicella-zoster virus (VZV) (Carter-Timofte et al., 2018, 2019). A RNA polymerase III mutation was identified in twins suffering from recurrent VZV CNS vasculitis and peripheral blood mononuclear cells (PBMCs) isolated from them showed reduced antiviral and/or inflammatory cytokine responses to poly(dA:dT) and VZV challenge (Carter-Timofte et al., 2018). Furthermore, additional RNA polymerase III mutations were identified in adult VZV encephalitis patients and PBMCs from these patients similarly showed reduced IFN-β and CXCL10 expression in response to poly (dA: dT). Interestingly, while PBMC cytokine response were unchanged following VZV challenge, patients with these RNA polymerase III mutations showed higher viral gene expression (Carter-Timofte et al., 2019). Together, these studies support a role for RNA polymerase III in combating viral CNS infections.

1.7 Concluding Remarks

It is now appreciated that resident CNS cells are important contributors to innate immunity and, due to their location, are likely the first responders to viral CNS infections. Resident CNS cells, especially astrocytes and microglia, are known to express an array of PRRs including TLRs, RLRs, NLRs, and now intracellular DNA sensors. In addition to their expression, multiple studies have demonstrated the functional nature of these sensors in various CNS cell types (as summarized in Figure 1 and Table 3). For example, Reinert and colleagues (Reinert et al., 2016) demonstrated that cGAS was required for the expression of IFN-β in microglia during infection with HSV-1 and that loss of this molecule promoted viral replication. However, our understanding of the role of DNA sensors in viral infections is limited and, in some cases, contradictory. It is currently unclear whether DNA sensors are beneficial or detrimental to the host during CNS infections, and it appears likely that outcomes following activation are pathogen, host cell-type, and even species, specific. This is exemplified by the finding that the HSV-1 product ICP6 blocks ZBP1-mediated responses in human cells but activates ZBP1 in murine cells (Guo et al., 2018). Because of this, future research on the role and therapeutic potential of DNA sensors must be cognizant of such variables.

Lastly, it is important to note that DNA sensors may contribute to other CNS pathologies, such as neurodegenerative diseases, which may be initiated or exacerbated by viral infection. For instance, three prime repair exonuclease 1 (TREX1) deficiency can cause accumulation of mis-localized DNA and lead to Aicardi Goutieres syndrome. This condition is characterized by permanent and often severe neurological damage due to IFN overproduction, and the loss of cGAS has been shown to rescue TREX1 deficient mice from disease pathology (Gray et al., 2016). Similarly, neurodegenerative diseases are associated with chronic overproduction of

proinflammatory mediators and neuroinflammation in diseases such as Alzheimer's disease could result from the chronic activation of DNA sensors by released DAMPS and/or viral infection. Regardless, it is clear that our understanding of these DNA sensors in the CNS remains rudimentary and further research is needed to define the cell type, species, and pathogen specificity of each. In doing so, it might be possible to target these molecules judiciously to limit damaging inflammation while allowing beneficial host responses to improve patient outcomes.

1.8 Hypothesis and goals of the present study

As shown in Figure 1, evidence for the expression and function of several DNA sensors is currently absent in human glia. In the present study, we have tested the hypothesis that human glial cells express the DNA sensors cGAS and IFI16, as well as the downstream adaptor molecule STING. Additionally, we have begun to investigate the functional nature of each sensor during exposure to foreign dsDNA. Furthermore, we have tested the hypothesis that these DNA sensors serve as critical restriction factors during HSV-1 infection in human glia.

Our previous research has shown that the DNA sensor ZBP1 contributes to pro-inflammatory mediator production during HSV-1 infection in murine astrocytes and microglia (Furr et al., 2011). In addition, we have demonstrated that this production of pro-inflammatory mediators contributed to neuronal cell death (Furr et al., 2011). Here, we have expanded on this work and tested the hypothesis that ZBP1 serves as an important restriction factor for HSV-1. Importantly, we have begun to describe the possible mechanisms by which ZBP1 restricts viral replication and address the hypothesis that this occurs through the activation of cell death pathways. In doing so, we begin to describe the mechanistic action of ZBP1 and investigate the therapeutic potential of this molecule.

1.9 TABLES AND FIGURES

Table 1: Expression and antiviral activity of intracellular DNA sensors in peripheral cell types

Sensor	Cell type	Ligand	Antiviral activity	Recognized pathogens	References
ZBP1	Mouse primary fibroblast, liver macrophages, BMDM Mouse cell lines, L929, SVEC4-10, NIH3T3, 3T3-SA, Human cell lines HT-29, A549, HepG2	dsDNA or RNA	IFN expression, NF-κB activation, cell death	HSV-1, IAV, CMV, vaccinia virus, ZIKV, WNV	Upton et al, 2012, Pham et al, 2013, Kuriakose et al, 2016, Lin et al, 2016, Maelfait et al, 2017, Guo et al, 2018, Yang et al, 2019, Ingram et al, 2019, Rothan et al, 2019, Daniels et al, 2019
cGAS	Mouse primary lung fibroblasts, GM-CSF DC, FLt3L DC, BMDM, Murine cell lines, L929, RAW 264.7 Hamster cell line BHK, Primary human PBMCs, MDM, MDDC, plasmacytoid dendritic cells, PBMCs, CD4+ T cells Human cell lines, THP1, HFFs, A549, HUVEC, EA.hy926	dsDNA	IFN expression, inflammasome priming	HIV, HSV, MLV, SIV, HCMV, DENV, ectromelia virus	Sun et al, 2013, Gao et al, 2013a, Li et al 2013, Herzner et al, 2015, Ma et al 2015a, Bode et al, 2016, Vermeire et al, 2016
IFI16	Mouse primary corneal epithelium Mouse cell line RAW 264.7 Primary human MDM, keratinocytes, PBMCs, CD4+ T cells, hepatocytes Human cell lines, THP1, hTCEpi, HFFs, BJAB, HMVEC, BCBL1, 184B5, HCC1937,	dsDNA	Inflammasome activation, IFN-β production, transcriptional regulation	HSV-1, KSHV, HIV, EBV, HBV	Unterholzner et al, 2010, Conrady et al, 2012, Orzalli et al, 2012, Cuchet-Lourenco et al, 2013, Jakobsen, 2013, Ansari et al, 2013, Dell'Oste et al, 2014, Diner et al, 2015, Dutta et al, 2015, Iqbal et al, 2016, Pisano et al, 2017, Roy et al, 2019, Lum et al, 2019, Yang et al, 2020

	TIME, HELF, U2OS, HUVEC, Akata cells, MUTU1, huh7, HepG2, HeLa, HepaRG cells, C666-1, Raji, LCL				
AIM2	Primary mouse BMDM, BMDC, MEF, alveolar macrophages, peritoneal macrophages Mouse cell lines NR9456, B6-MCL Primary human dermal fibroblasts, keratinocytes Human cell lines, THP1, ATII	dsDNA	inflammasome activation	HPV, HBV, HCMV	Hornung et al, 2009, Fernandes-Alnemri et al, 2009, Rathinam et al, 2010, Reinholz et al, 2013, Sagulenko et al, 2013, Ekchariyawat et al, 2015, Ekchariyawat et al, 2015, Corrales et al, 2016, Gray et al, 2016, Huang et al, 2017, Zhang et al, 2017, Nakaya et al 2017, Chen et al, 2018
DDX41	Primary mouse BMDC, peritoneal macrophages, MEF Mouse cell lines, D2SC, L929 Primary human PBMCs Human cell line THP1	dsDNA or DNA:RNA hybrid	IFN and ISG expression	HSV-1, adenovirus, MLV, IAV (mitochondrial DNA)	Zhang et al, 2011b, Zhang et al, 2013b, Lee et al, 2015, Stavrou et al, 2015, Moriyama et al, 2019
DNA-PK	Primary mouse MEF Primary human monocytes, MDDC Human cell lines, HEK293, HeLa, THP1, SK-hep-1, HepG2.2.15, U937s, HFFs	dsDNA	IFN expression	HSV-2, HTLV, HBV, vaccinia virus	Zhang et al, 2011a, Ferguson et al, 2012, Peters et al, 2013, Li et al, 2016, Wang et al, 2017, Scutts et al, 2018
RNA-pol III	Primary mouse MEF, dendritic cells Mouse cell line RAW264.7 Primary human PBMCs, MDDC Human cell line HEK293	RNA	IFN expression, NF-κB activation	adenovirus, HSV-1, EBV	Chiu et al, 2009, Ablasser et al, 2009

Table 2: Viral inhibitors of DNA sensing pathways

Sensor	Virus	Viral product	Inhibition mechanisms	References
STING	Coronavirus	Papain-like proteases	Blocks dimerization and signaling	Li Sun et al, 2012
	DENV	NS2B3	Cleavage (human only)	Aguirre et al, 2012
	HCMV	UL122 (IE86)	Facilitated degradation	Kim et al, 2017
		US9	Blocks dimerization and association with TBK-1	Choi et al, 2018
		UL82	Inhibits translocation and impairs TBK1 and IRF3 recruitment	Fu et al, 2017
	HCV	NS4B	Suppresses accumulation and activation	Yi et al, 2015
	HSV-1	ICP27	Inhibits TBK1/STING signaling	Christensen et al, 2016
		$\gamma_1$34.5	Disrupts translocation	Pan et al, 2018
cGAS	DENV	NS2B3	Autophagosomal cleavage/degradation	Aguirre et al, 2017
	EBV	KSHV ORF52 homolog	Inhibits activity	Wu et al, 2016
	HCMV	pUL83	Reduces cGAMP production	Biolatti et al, 2018
		UL31	Interferes with DNA binding	Huang et al, 2018
	HSV-1	UL37	Inhibits cGAMP production (not in NHP)	Zhang et al, 2018
		VP22	Inhibiting enzymatic activity	Huang et al, 2018
	KSHV	ORF52	Inhibiting enzymatic activity	Wu et al, 2016
	MHV68	KSHV ORF52 homolog		
	RRV	KSHV ORF52 homolog		
cGAS/STING	HIV-2/SIV	VPX	Blocks cGAS/STING mediated NF-κB activation	Su et al, 2019
	HSV-1	UL41	Unknown	Su and Zheng, 2017
		UL24	Prevents NF-κB translocation	Xu et al, 2017
		UL36	Prevents NF-κB activation by cleaving IkBa polyubiquiting chains	Ye et al, 2017

	KSHV	ORF36	Unknown	Ma et al, 2015
		ORF73		
		ORF57		
		ORF45		
		ORF55		
		vIRF1	Inhibits STING/TBK1 interactions	
	KSHV	cytoplasmic LANA	Binds cGAS and prevents TBK1 and IRF3 phosphorylation	Zhang et al, 2016b
cGAS/STING/RIG-I	HPV	SUV39H1	Represses transcription at their promoter regions	Cigno et al, 2020
IFI16	HCMV	pUL97	Mislocalization	Dell'Oste et al, 2014
	HSV-1	ICP0	Increases degradation	Orzalli et al 2012, 2016, Diner et al, 2015
	KSHV	Unknown	Increases degradation	Roy et al, 2016
IFI16/STING	HSV-1	UL46	Reduces protein expression of each and interferes with STING/TBK1 interaction	Deschamps et al, 2017
ZBP1	HSV-1	ICP6	Blocks human RIPK3/MLKL interactions (but activates RIPK3 in mice)	Wang et al, 2014, Huang, 2015, Guo et al, 2018
	MCMV	M45	Blocks ZBP1/RIPK3 interactions	Upton et al, 2012
AIM2	HCMV	UL83	Prevents IL-1β maturation and may increase IFI16 degradation	Huang et al 2017
		IE86	Blocks IL-1β secretion	Botto et al, 2019
		IE86	Inhibits NF-κB gene transcription and IL-1β release	
	HSV-1	VP22	Prevents inflammasome oligomerization	Maruzuru et al, 2018
DDX41	HSV-1	miR-H2-3p	Inhibits DDX41 transcription	Duan et al., 2019

Table 3: Expression and antiviral activity of intracellular DNA sensors in CNS cell types

Sensor	CNS cell type	Antiviral activity	References
ZBP1	Primary mouse whole brain tissue, cortical neurons, microglia, and astrocytes	Neuronal immunometabolism regulation, antiviral and proinflammatory cytokine production	Furr et. al, 2011, Daniels et. al, 2019, Rothan et. al, 2019
cGAS	Primary murine neurons, astrocytes, and microglia	IFN and ISG expression	Cox et. al, 2015, Reinert et. al, 2016,
IFI16	Primary mouse epithelial cells Human cell lines, corneal epithelial	Inflammasome activation	Conrady et. al, 2012, Coulon et. al, 2019,
AIM2	Primary mouse astrocytes and microglia Human cell line, SK-N-SH	Inflammasome activation	Cox et. al, 2015, Yogarajah et. al, 2017. Song et. al, 2019
DDX41	Zebrafish whole brain	IFN expression	Ma et. al, 2018
DNA PK	Primary mouse cerebral cortex and neurons	Unknown	Vemuri et. al, 2001, Chechlacz et. al, 2001
RNA pol III	Primary mouse astrocytes and microglia Mouse cell line, EOC13.31 Human cell line, hμglia	IFN expression and NF-κB activation via RIG-I	Crill et. al, 2015, Johnson et. al, 2020

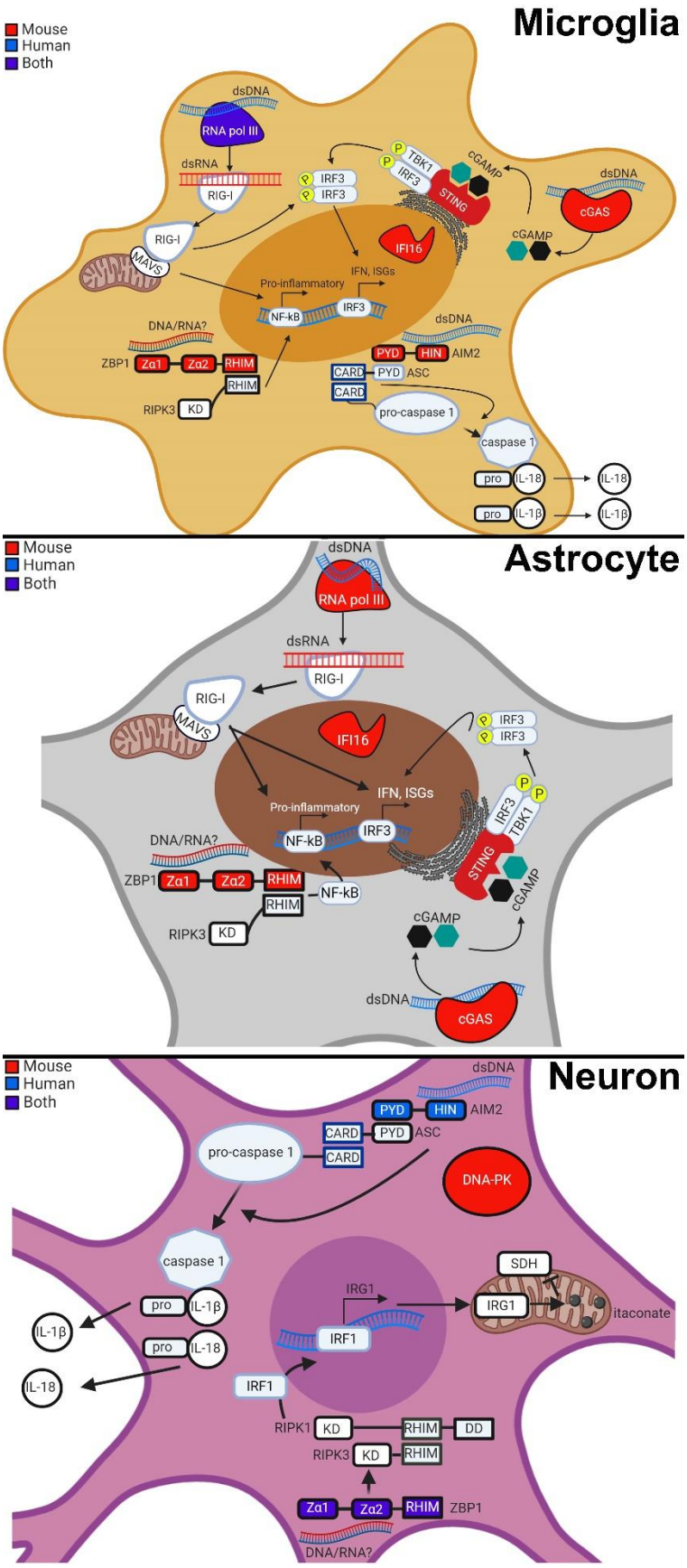

Figure. 1
Intracellular DNA sensors in resident CNS cell types.
Intracellular nucleic acid sensing by DNA sensors in microglia (A), astrocytes (B), and neurons (C). AIM2 sensing of dsDNA leads to the recruitment of apoptosis-associated speck-like protein containing a CARD (ASC) that then cleaves pro-caspase 1. Active caspase 1 then cleaves the precursor forms of IL-18 and IL-1β, causing their maturation and release from the cell. ZBP1 sensing of either dsDNA or RNA causes it to associate with RIPK3, activate the transcription factor NF-κB, leading to the production of pro-inflammatory mediators. In neurons, ZBP1 sensing results in the activation of IRF1, expression of IRG1, production of itaconate, and a reduction in succinate dehydrogenase (SDH) activity. Sensing of dsDNA by cGAS leads to the production of cGAMP, which binds to and activates STING causing the phosphorylation and translocation of interferon regulatory factor 3 (IRF3) to the nucleus. This results in the expression of IFN and ISGs in microglia and astrocytes. RNA pol III senses dsDNA and converts it into dsRNA that can then be sensed by RIG-I in microglia and astrocytes. RIG-I sensing of dsRNA causes it to associate with mitochondrial antiviral-signaling protein (MAVS) leading to activation and translocation of IRF3 and NF-κB, resulting in the expression of IFN, ISGs, and pro-inflammatory cytokines. DNA sensors in red indicate that studies were completed in mice, while blue indicates human origin, and purple indicates both species.

CHAPTER 2: Materials and Methods

2.1 Source and propagation of cell lines

U87 MG, an immortalized human astrocytic cell line, was obtained from the ATCC (HTB-14). Cells were maintained in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The human microglial cell line, hμglia, was a kind gift from Dr. Jonathan Karn (Case Western Reserve University)(Garcia-Mesa et al., 2017). These cells were derived from primary human cells transformed with lentiviral vectors expressing SV40 T antigen and hTERT, and have been classified as microglia due to their microglia-like morphology, migratory and phagocytic activity, presence of the microglial cell surface markers CD11b, TGFβR, and P2RY12, and characteristic microglial RNA expression profile (Garcia-Mesa et al, 2017). This cell line was maintained in Dulbecco's Modified Eagle Medium supplemented with 5% FBS and penicillin/streptomycin. Primary human astrocytes and microglia were purchased from ScienCell Research Laboratories (Carlsbad, CA) and were cultured in medium supplied by the vendor.

2.2 Murine glial cell isolation and culture

Primary murine glial cells were isolated as described previously by our laboratory (Bowman et al., 2003; Chauhan et al., 2008; Cooley et al., 2014; Crill et al., 2015). Briefly, six to eight neonatal C57BL/6J or ZBP1$^{-/-}$ (KO)(C57BL/6J background) 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 0.25% trypsin 1mM EDTA in serum-free RPMI 1640 medium for 5 min. The cell suspension

was then washed, and this mixed glial culture was maintained in RPMI 1640 containing 10% FBS and penicillin-streptomycin mix for two weeks.

Astrocytes were isolated from mixed glial cultures by trypsinization (0.25% trypsin-1 mM EDTA for 20-30 min) in the absence of FBS as previously described (Saura et al., 2003; Crill et al., 2015). The remaining intact layer of adherent cells was demonstrated to be >98% microglia by immunohistochemical staining for the microglial surface marker CD11b (Saura et al., 2003; Crill et al., 2015), and the isolated astrocytes were determined to be >96% pure based on morphological characteristics and the expression of the astrocyte marker glial fibrillary acidic protein (GFAP) as determined by immunofluorescence microscopy (Saura et al., 2003). Microglia were maintained for one week in RPMI 1640 with 10% FBS and 20% conditioned medium from LADMAC cells (ATCC number CRL-2420), a murine monocyte-like cell line that secretes colony stimulating factor-1 (CSF-1) (Cooley et al., 2014), while astrocytes were cultured in RPMI 1640 containing 10% FBS. All studies were performed in accordance with relevant federal guidelines and institutional polices regarding the use of animals for research purposes. ZBP1 deficient animals were a kind gift from Dr. Laura Knoll (University of Wisconsin-Madison, Madison, Wisconsin).

2.3 In vitro challenge of human microglia and astrocytes with nucleic acid ligands

Synthetic double-stranded B-form DNA analog poly(deoxyadenylic-deoxythymidylic) acid sodium salt (Poly(dA:dt)) and G3-ended Y-form short DNA, reported cGAS agonists (Herzner et al., 2015; Jeffries and Marriott, 2017), were purchased from InvivoGen (San Diego, CA). These ligands were directly introduced into microglial or astrocytic cells at concentrations of 0.01, 0.1, and/or 1.0 μg/ml using Lipofectamine 2000 transfection reagent (Thermo Fisher

Scientific, Waltham, MA) according to the manufacturer's instructions. At the indicated time points post-transfection, whole-cell protein isolates were collected and RNA was isolated for immunoblot analysis and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), respectively.

2.4 RNA extraction and semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated from cultured glial cells using Trizol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and quantified using a Nanodrop ND-1000 spectrophotometer. Prior to reverse transcription, RNA was treated with amplification grade DNase (Sigma Aldrich Cat. AMPD1) to remove genomic DNA. All RNA samples were diluted to the same concentration and reverse transcribed in the presence of random hexamers using 200 U of RNase H minus Moloney leukemia virus reverse transcriptase (Promega, Madison, WI) in the buffer supplied by the manufacturer. Semi-quantitative RT-PCR was performed on 16% of the reverse-transcribed cDNA product to assess the relative levels of expression of mRNA-encoding IFN-β, ICP-8, BST2, viperin, and the housekeeping gene product glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

TABLE 4. Primer sequences used in RT-PCR

Gene	Forward Primer	Reverse Primer	Size
IFN-β	GACGCCGCATTGACCATCTA	CCTTAGGATTTCCACTCTGACT	297 bp
ICP-8	GAGCTTCTGGCGTTACTGTC	TATGGTTACCTTGTCCGAGCC	465 bp
CXCL10	TGTACGCTGTACCTGCATCA	CTGTGTGGTCCATCCTTGGAA	268 bp
BST2 (Tetherin)	GATGGCCCTAATGGCTTCCC	TAACCGTGTTGCCCCATGAC	366 bp
IFITM1	TCAACATCCACAGCGAGACC	CAAAGGTTGCAGGCTATGGG	331 bp
RSAD2 (Viperin)	TGCTGGGAAGCTCTTGAGTG	CATTGCTCACGATGCTCACG	446 bp
GAPDH	CCATCACCATCTTCCAGGAGCGAG	CACAGTCTTCTGGGTGGCAGTGAT	347 bp

2.5 Immunoblot analysis

Whole-cell protein isolates were collected from microglial and astrocytic cells using Triton lysis buffer (10 mM TrisHCl pH 10.5, 5 mM MgCl2, and 1% (v/v) Triton X-100) and analyzed by immunoblot analysis. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to Immobilon-P transfer membranes (Millipore). Membranes were blocked with either 5% milk (cGAS, STING, IFI16, HSV-1 glycoprotein, ZBP1-1 or 5% BSA (phospho-IRF3, phospho-Mixed lineage kinase domain like pseudo kinase (MLKL)) for 1 h and then incubated overnight at 4 °C with primary antibodies directed against cGAS (Sigma Aldrich), STING (Abcam), IFI16 (Santa Cruz Biotechnology), pIRF3 (Cell Signaling Technology), HSV-1 glycoprotein g1 (GeneTex), ZBP1(AdipoGen), pMLKL (Abcam) and the housekeeping gene product β-actin (Abcam). Blots were then washed and incubated in the presence of a horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse IgG secondary antibody. Bound enzyme was detected with the Super Signal system (Thermo Fisher Scientific). A plasmid encoding full length human IFI16 was used to confirm the expression of this protein in human cells (Addgene plasmid cat #35064) (Liao et al., 2011). Immunoblots shown are representative of at least three separate experiments using the Bio-Rad ChemiDoc imaging system, and quantification analysis was performed using the ImageLab software (Bio-Rad).

2.6 Enzyme-linked immunosorbent assay

Specific capture enzyme-linked immunosorbent assays (ELISAs) were performed to quantify human and/or murine IL-6 and IFN-β release, as well as murine TNF-α. The human IL-6 ELISA was conducted using a rat anti-human IL-6 capture antibody (BD Pharmingen) and a biotinylated rat anti-human IL-6 detection antibody (BD Pharmingen). The human IFN-β ELISA

was carried out using a polyclonal rabbit anti-human IFN-β capture antibody (Abcam) and a biotinylated polyclonal rabbit antihuman IFN-β detection antibody (Abcam). The murine IL-6 ELISA was conducted using a rat anti-mouse IL-6 capture antibody (BD Pharmingen) and a biotinylated rat anti-mouse IL-6 (BD Pharmingen). The murine IFN-β ELISA was carried out using a polyclonal goat anti-mouse IFN-β capture antibody (Biolegend) and a biotinylated armenian hamster anti-mouse IFN-β detection antibody (Biolegend). The murine TNF-α ELISA was conducted using a commercially available kit (R and D Systems DuoSet ELISA). Bound antibody was detected using streptavidin-HRP (BD Biosciences) followed by the addition of tetramethylbenzidine (TMB) substrate. H2SO4 was used to stop the reaction and absorbance was measured at 450 nm. Dilutions of recombinant human IL-6 and IFN-β (BD Pharmingen, Abcam), or murine IL-6, IFN-β, and TNF-α (BD Biosciences, Biolegend, R and D systems respectively) were used to generate standard curves, and the concentration of each in study samples was determined by extrapolation to the standard curve.

2.7 Generation of heterozygous cGAS deletion microglial cell line

To investigate cGAS function, we created a heterozygous deletion microglial cell line that expresses cGAS at reduced levels, cGAS+/−, using CRISPR/Cas9 approaches. A sgRNA targeting cGAS was generated using the CRISPOR algorithm (http://crispor.tefor.net/), and a suitable sgRNA sequence was selected based on minimal off-targeting and proximity to the 5′ end of cGAS (5′ATCTTCTTAAGACAGGGGCACG-3′). The cGAS targeting sgRNA was cloned by BbsI digestion into the pX458 plasmid (Addgene plasmid cat #48138) (Ran et al., 2013) that promotes simultaneous expression of cGAS sgRNA, Cas9, and GFP. The hμglia human microglial cell line was transfected at 60% confluency with the cGAS sgRNA-pX458

plasmid (0.5 μg/ml) using Lipofectamine 2000 according to the manufacturer's instructions, and the cells were prepared for fluorescence-activated cell sorting (FACS) and clonal isolation at 72 h. GFP-positive cells were isolated by FACS and seeded at 100, 1000, and 10,000 cells per well in a six-well plate in complete growth media and maintained in culture until distinct colonies were visible. Individual cGAS+/− colonies were selected and propagated prior to analysis of cGAS expression by immunoblot analysis and subsequent experimental use.

2.8 siRNA transfection

ON-TARGETplus siRNA targeting human IFI16 and nontargeting pool siRNAs were purchased from Dharmacon (Lafayette, CO). Each was transfected into the hμglia human microglial cell line at a concentration of 5 nM using RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. At 48 h, IFI16 protein knockdown was confirmed by immunoblot analysis.

2.9 Preparation of viral stocks and in vitro infection of glial cells

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), ICP6 RHIM Mut strain (created from the HSV-1 F strain), or the parental F strain at a multiplicity of infection (MOI) of 0.01 and incubated for 48 to 72 h, at which time 100% of cells displayed cytopathic effects. Tissue culture flasks were then placed at − 80 °C for 15 min and subsequently warmed to room temperature inside a tissue culture hood. The cell suspension was removed and pulse sonicated (Vibra Cell; Sonics and Materials Inc., Newton, CT) to release intake virions. The sonicated material was centrifuged at 4000 RCF to remove unwanted cell debris and the

supernatant mixed with sterile milk for increased stability during freeze/thaw cycles. The stock was aliquoted and viral titers were quantified using a standard plaque assay of serial dilutions on Vero cells at 37 °C. The viral titer of the stock solutions was 1.2×10^7 PFU/ml for the MacIntyre and ICP6 RHIM Mut strains, and $1.5x10^7$ PFU/ml for the F strain. Human and murine astrocytes and microglia were infected with HSV-1 at MOIs of 0.02, 0.2, or 2.0 viral particles to glia, and the virus was allowed to adsorb for 1 h in DMEM in the absence of FBS or antibiotics. Cells were subsequently washed with PBS and cultures were maintained in appropriate growth medium for the indicated times prior to measuring cell viability or the collection of supernatants, whole-cell protein isolates, and/or total RNA. In experiments with inhibitors, following infection the RIPK1 inhibitor GSK 963 (Sigma), RIPK3 inhibitor GSK 872 (Sigma), and pan-caspase inhibitor zVAD (InvivoGen) were reconstituted in DMSO and added to the cultures at concentrations of 1μM, 5μM, and 20μM, respectively. The ICP6 RHIM Mut and parental F strains were a kind gift from Dr. Edward Mocarski (Emory University, Atlanta, Georgia).

2.10 Measuring cell viability and calculating percentage or kinetics of cell death.

Cell viability was measured for 24 hrs post-infection in isolated murine astrocytes and microglia using RealTime-Glo™ MT Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, the NanoLuc® Enzyme and MT Cell Viability Substrate was combined with the appropriate growth media containing the previously described inhibitors and added to glial cultures at one-hour post-infection. Starting at two hours, and continuing for 24 hrs, luciferase activity was measured using the SpectraMax® iD5 plate reader. Luciferase readings were normalized to uninfected controls and the resulting value was subtracted from one (our control) and multiplied by 100% to calculate the percent of cell death. Negative values were

recorded as zero. The slopes and standard deviations for each treatment were determined by linear regression using Microsoft Excel software.

2.11 Statistical analysis

Data is presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed by, two-way analysis of variance (ANOVA) or one-way analysis of variance ANOVA with Bonferroni's or Tukey 's post hoc tests, or Student's t test as appropriate using commercially available software (GraphPad Prism, GraphPad Software, La Jolla, CA). In all experiments, results were considered statistically significant when a P value of less than 0.05 was obtained.

Chapter 3: Human microglia and astrocytes express cGAS-STING viral sensing components

3.1 RATIONALE

Glia cells such as microglia and astrocytes are known to contribute to both damaging and protective immune responses during viral infections of the central nervous system (CNS). However, the mechanisms by which microglia and astrocytes respond to replicative viruses have been poorly understood, and it has only recently become apparent that cytosolic sensors for viral nucleic acids play a key role in glial cell responses (Furr and Marriott, 2012). For example, we have shown that the cytosolic RNA sensor retinoic acid-inducible gene I (RIG-I) is important for the recognition of productive infections with the RNA virus vesicular stomatitis virus (VSV) in murine glia and primary human astrocytes (Furr et al., 2010; Crill et al., 2015). Additionally, we have demonstrated that the DNA sensor, ZBP1 (Z-DNA binding protein 1), is expressed by murine glial cells and plays a significant role in the initiation of inflammatory cytokine production by these cells in response to HSV-1 infection (Furr et al., 2011). However, several other cytosolic DNA sensors have recently been identified in peripheral leukocytes that may also contribute to the innate immune responses of glia to replicative DNA viruses (Unterholzner, 2013; Dempsey and Bowie, 2015), some of which have been shown to be expressed in murine glial cells (Cox et al., 2015). However, far less is known about the expression and role of such sensors in human glial cells.

Of particular interest is the recently discovered cytosolic DNA sensor, cyclic GMP-AMP synthase (cGAS) (Sun et al., 2013). Upon binding to dsDNA, cGAS produces a unique secondary metabolite 2′3′ cyclic GMP-AMP that then binds to the critical adaptor protein, stimulator of interferon genes (STING). STING, in turn, recruits TANK binding kinase 1 (TBK1), which phosphorylates interferon regulatory factor 3 (IRF-3) that then dimerizes and

enters the nucleus to initiate transcription of interferon-stimulated genes including interferon-beta (IFN-β) (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013b; Zhang et al., 2013). The significance of cGAS is underscored by its apparent ability to recognize clinically important DNA viruses and retroviruses such as herpes simplex virus-1 (HSV-1) and human immunodeficiency virus-1 (HIV-1) that infect the human CNS (Gao et al., 2013a; Herzner et al., 2015). An antiviral role for cGAS in the CNS is supported by recent work in mice demonstrating that the microglial responses mediated by this sensor limit productive HSV-1 infection (Reinert et al., 2016). To date, the expression of cGAS and the critical adaptor protein STING has not been demonstrated in human glial cells. Here we show that human microglia and astrocytes functionally respond to cytosolic B form DNA and that these cell types constitutively express robust levels of cGAS and STING.

3.2 RESULTS

3.2.1 Human microglia and astrocytes functionally respond to B-form DNA

To determine if primary human astrocytes and microglia functionally respond to foreign cytosolic dsDNA, we transfected these cells with BDNA and assessed the expression of pIRF3 protein at 1, 2, and 3 hours post administration. The human microglial cell line, hμglia, and the astrocytic cell line, U87 MG, were transfected with BDNA (0.1 μg/mL) or left untreated, and whole cell lysates collected at the indicated time points. The presence of pIRF3 was determined by immunoblot analysis and was observed in hμglia and U87-MG cells as early as an hour following challenge (Fig. 2) with maximal increases seen at 3 hrs post transfection for hμglia (4.75 +/− 0.96 arbitrary densitometric units versus 0.10 +/− 0.40 in untransfected control cells: $p < 0.05$, $n = 3$) and at 2 hrs post transfection for U87-MG cells (1.40 +/− 0.15 arbitrary densitometric units versus 0.14 +/− 0.50 in untransfected control cells: $p < 0.05$, $n = 3$). In agreement with glial cell lines, primary human microglia and astrocytes similarly demonstrated rapid elevations in pIRF3 levels following challenge with BDNA (Fig. 2) with expression seen at 2 hrs post transfection in primary microglia from undetectable levels in untransfected control cells and significant increases in expression at 3 hrs post BDNA administration in primary astrocytes (0.79 +/− 0.16 arbitrary densitometric units versus 0.15 +/− 0.07 in untransfected control cells: $p < 0.05$, $n = 3$).

To determine the functional significance of changes in pIRF3 levels, we investigated the expression of IFN-β mRNA following BDNA challenge. Human astrocytes, microglia, and cell lines, were transfected with BDNA (0.1 μg/mL), or left untreated, prior to RNA isolation at 1, 2, and 3 hours post challenge. As shown in Figure 2, expression of IFN-β was significantly elevated

in hμglia microglia-like cells with a maximal increase seen at 3 hrs post transfection (1.69 +/− 0.06 arbitrary densitometric units versus 0.08 +/− 0.06 in untransfected control cells: $p < 0.05$, $n = 3$), and 24 hour exposure to BDNA elicited significant ($p < 0.05$, $n = 3$) IFN-β protein production by these cells at in a dose dependent manner (0.90 +/− 0.23 and 1.29 +/− 0.33 ng/ml with 1 and 5 ug/ml BDNA, respectively, from undetectable levels in untransfected cells and cells treated with transfection reagent alone). IFN-β mRNA was similarly detectable in primary human microglia and astrocytes within two hours of challenge. However, intracellular BDNA administration failed to induce demonstrable IFN-β mRNA expression in the U87 MG astrocytic cell line despite inducing IRF3 activation (Fig. 2).

3.2.2 Human microglia and astrocytes constitutively express robust levels of the viral DNA sensor cGAS

To begin to determine the mechanisms by which glial cells respond to BDNA, we investigated the expression of the recently identified cytosolic DNA sensor, cGAS, in human glia. Hμglia and U87 MG cell lines were transfected with BDNA (0.01 and 0.1 μg/mL), treated with transfection reagent alone (L2K), or left untreated (Con). After 24 hours, whole cell lysates were collected and the level of cGAS protein expression was determined by immunoblot analysis. Both hμglia and U87 MG cell lines at rest showed robust expression levels of a 50 kDa protein corresponding to the predicted size of one of the two cGAS isoforms, and in agreement with the size previously reported for cGAS in hepatic and tonsil tissue samples detected using this antibody as reported by the vendor (Sigma Aldrich). Such expression could not be elevated significantly by exposure to BDNA (Fig. 3). The expression of cGAS protein was confirmed in resting primary human microglia and astrocytes (Fig. 3). Interestingly, primary human microglia

showed a decrease in cGAS protein expression when challenged with the higher concentration of BDNA (Fig. 3) that could not be attributed to changes in cell viability as assessed by MTT assay (data not shown).

3.2.3 Human microglia and astrocytes constitutively express robust levels of the critical downstream adaptor protein STING

We next sought to determine the presence of the cGAS downstream adaptor protein STING in human glial cells. Hμglia and U87 MG cell lines were transfected with BDNA (0.01 and 0.1 μg/mL), treated with transfection reagent alone (L2K), or left untreated (Cells). At 24 hours following challenge, whole cell lysates were collected and the level of STING protein expression was determined by immunoblot. Both hμglia and U87 MG cell lines demonstrated robust constitutive expression of STING in unstimulated cells, and such expression was not elevated significantly by exposure to BDNA (Fig. 4). These findings were confirmed in primary human astrocytes and microglia, which also demonstrated robust constitutive expression of STING protein that was not elevated further with increasing concentrations of BDNA (Fig. 4).

3.3 Discussion

In the present study, we show that human microglia and astrocyte-like cell lines and primary human glial cells functionally respond to transfection with BDNA with the activation of the key transcription factor IRF3 and the expression of mRNA encoding the type I interferon IFN-β. This is in agreement with recent studies from our laboratory and others demonstrating the ability of primary murine glia to express IFN-β mRNA in response to BDNA (Cox et al., 2015; Crill et al., 2015). As such, both human glial cell types possess the means to perceive foreign cytosolic DNA. Importantly, we also provide the first demonstration that human microglia and astrocyte-like cell lines and authentic primary human glial cells express the novel cytosolic DNA sensor cGAS and its critical downstream adaptor molecule STING. We have determined that cultured human microglia and astrocytes constitutively show robust levels of cGAS and STING protein expression, levels that could not be elevated further following activation with foreign cytosolic DNA. Indeed, levels of cGAS protein expression actually showed a significant decrease in primary microglia at 24 hrs following transfection with the highest dose of BDNA, an effect that could not be attributed to a decrease in cell viability. The finding that human glial cells express components of the cGAS-STING DNA sensor pathway are in agreement with the recent study from Reinert and co-workers showing that murine microglia express cGAS (Reinert et al., 2016), and the demonstration by this group (Reinert et al., 2016) and our own (Furr et al., 2011) that murine glia possess STING.

It is interesting to note that while IFN-β mRNA expression was induced in primary human astrocytes following cytosolic BDNA administration, the human astrocytic cell line U87-MG failed to show such expression despite exhibiting BDNA-induced IRF3 activation. This observation perhaps reflects the limitations of the use of immortalized cell lines in such studies.

Finally, it is also important to note that BDNA could be recognized via other cytosolic DNA sensors in human glial cells, or may even be perceived using RNA sensors if the foreign DNA is transcribed by host cytosolic RNA polymerase III. As such, the present demonstration that human microglia and astrocytes express the principle components of the cGAS-STING pathway sets the stage for further studies to determine the relative importance of these molecules in human glial responses to clinically relevant viral pathogens.

3.4 FIGURES

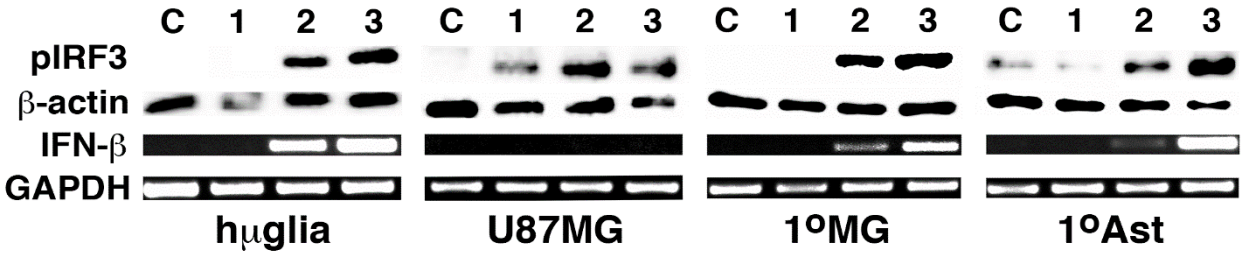

FIGURE 2: Human microglia and astrocyte cell lines and primary cells functionally respond to foreign intracellular dsDNA. Immortalized human microglial (hμglia) and an astrocytic cell line (U87MG) or primary human microglia (1°MG) and astrocytes (1°Ast) (3×10^5 cell per well) were treated with transfection reagent alone for 3 hrs (C) or exposed to intracellular B-form DNA (BDNA; 0.1 μg/mL) for 1, 2, or 3 hours (1, 2, 3). At these time points, whole cell lysates were collected and analyzed for the expression of phosphorylated IRF3 (pIRF3) by immunoblot analysis, and RNA was isolated and the expression of mRNA encoding IFN-β was determined by semi-quantitative RT-PCR. Expression of β-actin protein and GAPDH mRNA housekeeping gene products is shown. Results shown are representatives of at least three independent experiments and in some cases sections of the blots have been repositioned for presentation purposes.

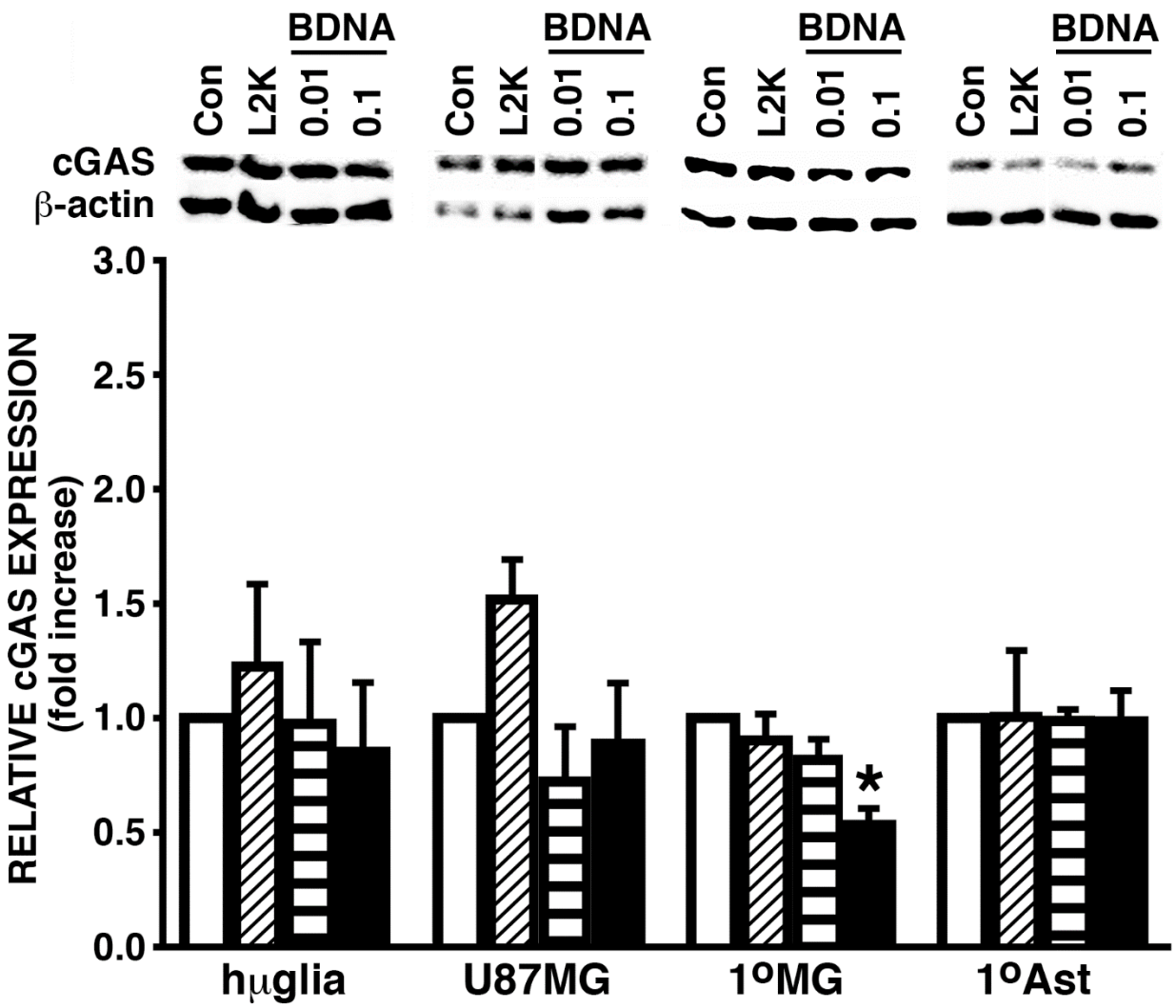

FIGURE 3: Human microglia and astrocyte cell lines and primary cells constitutively express robust levels of the viral DNA sensor cGAS. Immortalized human microglial (hμglia) and an astrocytic cell line (U87MG) or primary human microglia (1°MG) and astrocytes (1°Ast) (3×10^5 cell per well), were untreated (Con), treated with transfection reagent alone (L2K) or transfected with B-form DNA (BDNA; 0.01 or 0.1 μg/mL). At 24 hours post challenge, whole cell lysates were collected and analyzed for the expression of cGAS or the housekeeping gene product β-actin by immunoblot analysis. Blots shown are representatives of at least three independent experiments and in some cases sections of the blots have been repositioned for presentation purposes. The relative cGAS expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/− the standard error of the mean (SEM) of at least 3 independent experiments and an asterisk indicates a statistically significant difference from transfection reagent only treated cells.

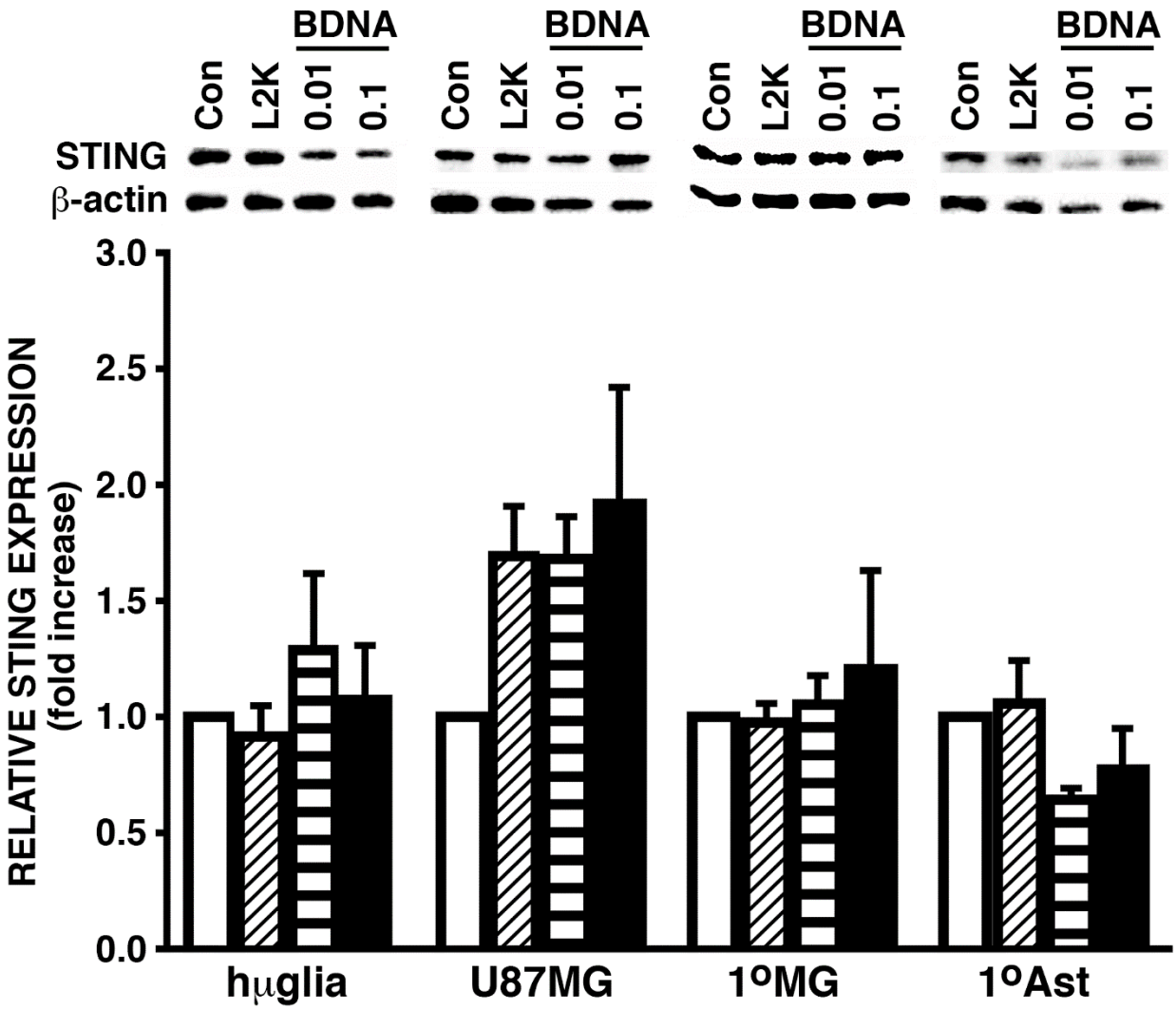

FIGURE 4: Human microglia and astrocyte cell lines and primary cells constitutively express robust levels of the cGAS downstream adaptor molecule STING. Immortalized human microglial (hμglia) and an astrocytic cell line (U87MG) or primary human microglia (1°MG) and astrocytes (1°Ast) (3×10^5 cell per well) were untreated (Cells), treated with transfection reagent alone (L2K) or transfected with B-form DNA (BDNA; 0.01 or 0.1 μg/mL). At 24 hours post challenge, whole cell lysates were collected and analyzed for the expression of STING or the housekeeping gene product β-actin by immunoblot analysis. Blots shown are representatives of at least three independent experiments and in some cases sections of the blots have been repositioned for presentation purposes. The relative STING expression was determined by densitometric analysis and normalized to untreated cells. Data is expressed as the mean +/− the standard error of the mean (SEM) of at least 3 independent experiments and no statistically significant differences were observed from transfection reagent only treated cells.

CHAPTER 4: The intracellular DNA sensors cGAS and IFI16 do not mediate effective antiviral immune responses to HSV-1 in human microglial cells

4.1 RATIONALE

It is now recognized that astrocytes and microglia play a critical role in the production of immune mediators that contribute to both protective host defense and disease pathology within the central nervous system (CNS) (Ghoshal et al., 2007; Swarup et al., 2007; Das et al., 2008; Marques et al., 2008; Furr et al., 2010, 2011; Furr and Marriott, 2012; Jiang et al., 2014; Crill et al., 2015; Reinert et al., 2016). The mechanisms by which glia recognize and respond to CNS pathogens are only now becoming apparent with the demonstration that microglia and astrocytes express a wide range of pattern recognition receptors (PRRs) capable of sensing pathogen and damage associated molecular patterns (PAMPs and DAMPs, respectively) (Sterka et al., 2006; Furr et al., 2010, 2011; Jeffries and Marriott, 2017). Similar to peripheral host cells, activation of glial PRRs initiate signaling cascades that lead to the production of soluble proinflammatory and/or antiviral mediators. Whether such production and release acts in a beneficial or detrimental manner in the CNS during infection is less well understood, and appears to be context dependent (Blank and Prinz, 2017).

Of these PRRs, the expression of recently discovered cytosolic/nuclear RNA and DNA sensors by glial cells is of particular interest as their ability to interact with nucleic acids in the intracellular environment suggest an important role in the detection of viral pathogens. Consistent with this notion, we have demonstrated that the RNA sensor, retinoic acid inducible gene I (RIG-I), is important for the detection of vesicular stomatitis virus (VSV) in human glia, and we have shown that such recognition leads to the production of damaging proinflammatory mediators by these cells (Furr et al., 2010). In addition, we have demonstrated that murine glia

express Z-DNA binding protein 1 (ZBP1) and showed that this cytosolic DNA sensor contributes to glial inflammatory responses to the neurotropic DNA virus herpes simplex virus-1 (HSV-1) (Furr et al., 2011; Crill et al., 2015).

More recently, additional DNA sensors such as cyclic GMP-AMP synthase (cGAS), absent in melanoma 2 (AIM2), and interferon γ-inducible protein 16 (IFI16), been identified in peripheral cell types (Takaoka et al., 2007; Bürckstümmer et al., 2009; Unterholzner et al., 2010; Sun et al., 2013). Of these, cGAS has been the most widely studied and has been shown to recognize relevant CNS pathogens such as HSV-1 and HIV (Gao et al., 2013a; Li et al., 2013). This DNA sensor has been shown to directly bind dsDNA and to subsequently produce the secondary messenger 2'3'-cGAMP that then activates the critical downstream adaptor protein stimulator of interferon genes (STING). This activation leads to phosphorylation and nuclear translocation of the transcription factor, interferon regulatory factor 3 (IRF3), which induces the expression of type I interferons such as IFN-β (Sun et al., 2013). However, while we have recently demonstrated that human glia express cGAS and STING (Jeffries and Marriott, 2017), the functional significance of this DNA sensing pathway has not yet been established in these cells.

Here, we demonstrate that cGAS is functionally expressed by human microglial cells and underlies, at least in part, exogenous DNA-mediated cytokine production by this resident CNS cell type. Additionally, we have established that human glia also express the DNA sensor IFI16. However, while cGAS and IFI16 are thought to be important for DNA virus replication restriction in peripheral human cell types (Civril et al., 2013; Li et al., 2013; Johnson et al., 2014; Shu et al., 2014; Zhang et al., 2014; Diner et al., 2015; Ma et al., 2015a; Diner et al., 2016; Iqbal et al., 2016; Wang et al., 2017b; Merkl et al., 2018), our data indicates that neither of these

sensors mediate effective antiviral responses to HSV-1 in human microglia, perhaps due to an ability of HSV-1 to inhibit their expression in this cell type.

4.2 RESULTS

4.2.1 cGAS is functionally expressed by human microglia

We have previously demonstrated that primary human glia and cell lines can respond to the intracellular introduction of foreign DNA, and showed that these cells express cGAS and its downstream effector molecule STING (Jeffries and Marriott, 2017). Here, we have used CRISPR/Cas9 technology to generate a heterozygous deletion human microglial cell line that expresses cGAS at reduced levels, cGAS$^{+/-}$, to determine whether this cytosolic DNA sensor is functional in the hμglia human microglial cell line. As shown in Figures 5A and B, intracellular administration of microglia with B- and Y-form DNA, reported ligands for cGAS (Herzner et al., 2015; Jeffries and Marriott, 2017), elicited marked increases in levels of phosphorylated IRF3, and these responses were significantly reduced in cGAS$^{+/-}$ cells.

Consistent with their ability to induce IRF3 phosphorylation, intracellular administration of either BDNA or YDNA elicited significant increases in the secretion of the type I interferon, IFN-β, and was also able to induce the release of the potent inflammatory cytokine IL-6 by microglial cells (Figures 5C and D). As with phosphorylated IRF3 levels, decreased cGAS expression resulted in a significant reduction in IFN-β secretion following challenge with either BDNA or YDNA (Figures 5C and D). Interestingly, cGAS expression reduction failed to significantly decrease microglial IL-6 secretion, suggesting either that the markedly reduced cGAS levels are sufficient to initiate such expression, or that alternative DNA sensing molecules are responsible for the production of this inflammatory cytokine.

4.2.2 cGAS contributes to antiviral gene expression but does not restrict infectious HSV-1 particle release by infected human microglial cells

Since cGAS is functional in human microglia, we next investigated the role of this sensor in the antiviral microglial responses to the clinically relevant neurotropic DNA virus, HSV-1. Wild type and cGAS$^{+/-}$ microglia were exposed to HSV-1 at a MOI of 0.2 and the number of infectious HSV-1 particles released by these cells was determined by plaque assay at 24 hours following viral challenge. Surprisingly, reduced cGAS expression failed to elicit significant changes in the level of HSV-1 release (Figure 6A). Such a result could stem from an inability of these cells to secrete detectable levels of IFN-β at 8 hrs (Figure 6B) or 12 hrs (data not shown) following HSV-1 challenge, despite responding to this virus with significant levels of IL-6 release at 8 hrs (Figure 6C) and 12 hrs (data not shown) following infection. Again, cGAS expression reduction significantly reduced transfected BDNA-mediated IFN-β production by microglia, but did not significantly affect either BDNA or HSV-1-induced IL-6 release at either 8 hrs (Figures 6B and C) or 12 hrs (data not shown) following challenge.

Consistent with an absence of significant IFN-β production by HSV-1 challenged human microglial cells, wt cells did not show significant elevations in the expression of mRNA encoding the IFN-stimulated genes IFITM1, BST2, or viperin (Figures 6D-F). Interestingly, cGAS$^{+/-}$ microglia expressed significantly lower levels of IFITM1, BST2, and viperin, mRNA expression following HSV-1 infection than similarly challenged wt cells, and even showed a tendency for lower BST2 and viperin expression in uninfected cells (Figures 6D-F). Together, these data indicate that while cGAS is required, at least in part, for the maintenance of antiviral gene expression by human microglial cells, such cGAS-mediated responses are not sufficient to limit the release of infectious HSV-1 particles by these cells.

4.2.3 Human glia express IFI16 but this DNA sensor does not contribute to microglial responses to HSV-1

Another intracellular DNA sensor, IFI16, has previously been reported to function as an HSV-1 restriction factor in peripheral human cells (Conrady et al., 2012; Johnson et al., 2014; Liang et al., 2014; Diner et al., 2015, 2016; Merkl et al., 2018). Accordingly, we have determined if human glial cells express IFI16 and whether this molecule mediates, either constitutively or in the reduced expression of cGAS, microglial immune responses to HSV-1 challenge. As shown in Figure 7A, the hμglia human microglial cell line constitutively expresses IFI16 as determined by immunofluorescence microscopy, and this sensor is localized to the nucleus as previously described in other cell types (Roy et al., 2019). This expression was confirmed by immunoblot analysis with robust constitutive levels of a protein close to the predicted molecular weight of IFI16 (88 KDa), and at an identical size to that seen in whole cell protein isolates from HEK 293T cells transfected with a plasmid vector to express IFI16 (data not shown). Other fainter bands may correspond to the isotypic variants of IFI16 that have been reported to arise due to differential RNA splicing events (Johnstone et al., 1998). Such expression was also confirmed in a commercially available (Applied Biological Materials Inc.) immortalized human microglial cell line (data not shown). Levels of IFI16 protein expression were not increased further following stimulation with intracellular BDNA administration (Figure 7B). Interestingly, while the U87-MG human astrocytic cell line also constitutively expressed robust levels of IFI16 protein that were not increased following BDNA stimulation, primary human astrocytes only showed low levels of IFI16 at rest, but showed marked elevations in response to BDNA transfection (Figure 7B).

We then utilized siRNA approaches to assess the role of IFI16 in microglial immune responses to HSV1. We confirmed that siRNA targeting IFI16 markedly attenuated the expression of this protein and possible isotypic variants in both wt and cGAS$^{+/-}$ human microglia (Figure 7C) and determined the effect of IFI16 knockdown on microglial responses to intracellular BDNA administration and HSV-1 infection. As shown in Figure 7D, IFI16 knockdown had no demonstrable effect on BDNA-induced IFN-β production, expression of the HSV-1 gene product ICP8, or infectious HSV-1 particle release, by wt human microglial cells, and had no effect on IL-6 release (data not shown). Similarly, IFI16 knockdown had no demonstrable effect on BDNA-induced IFN-β production (Figure 7E), infectious HSV-1 particle release (Figure 7E), or IL-6 production (data not shown), by cGAS$^{+/-}$ microglial cells. Together, these data indicate that while human glia express IFI16 this DNA sensor does not contribute to BDNA or HSV-1 mediated antiviral mediator production by human microglia, or restrict the number of infectious HSV-1 particles released by infected cells.

4.2.4 HSV-1 infection down regulates cGAS and IFI16 expression by human glia

To begin to determine the mechanisms underlying the apparent resistance of HSV-1 to cGAS and/or IFI16 mediated antiviral microglial responses we have assessed the effect of this virus on the expression of each of these intracellular DNA sensors by human glia. As shown in Figure 8A, HSV-1 infection significantly decreased cGAS protein levels by human microglial cell and primary human astrocytes in a dose dependent manner, and showed a tendency to reduce cGAS expression in U87-MG astrocytic cells at 24 hours post-challenge. Similarly, HSV-1 infection elicited significant and dose-dependent decreases in IFI16 protein levels in the hμglia human microglial cell line, U87-MG astrocytic cells (Figure 8B), and a second immortalized

human microglial cell line (data not shown), and further decreased expression of this sensor in primary human astrocytes from low constitutive levels (Figure 8B). As such, the ability of HSV-1 to downregulate the expression of both of these intracellular DNA sensors may represent an important immune evasion strategy in human glia.

4.3 DISCUSSION

We have previously demonstrated that human glial cells express the DNA sensor cGAS and the downstream adaptor molecule STING (Jeffries and Marriott, 2017). In the present study, we expand upon this work by demonstrating that cGAS is functional in human microglial cells. We show that microglia respond to intracellular administration of either B or Y-form DNA, reported ligands for cGAS in other cell types (Herzner et al., 2015; Jeffries and Marriott, 2017), with increased levels of IRF3 activation and IFN-β secretion. Importantly, we show that these responses are due, in large part, to recognition via cGAS with the demonstration that reduced cGAS expression results in significantly reduced levels of IRF3 activation and IFN-β release in response to either of these ligands, while IL-6 responses are unchanged. These findings are in agreement with previous studies in peripheral cell types showing that cGAS is critical for cytoplasmic dsDNA recognition (Civril et al., 2013; Li et al., 2013; Sun et al., 2013; Zhang et al., 2013, 2014; Shu et al., 2014; Ma et al., 2015a).

Similar to cGAS, IFI16 has also been proposed to directly bind dsDNA and mediate the expression of antiviral mediators such as type I IFNs (Unterholzner et al., 2010). Interestingly, some studies have suggested that IFI16 works in concert with cGAS to initiate host immune responses to viral pathogens (Orzalli et al., 2015; Almine et al., 2017; Jønsson et al., 2017; Liu et al., 2017), while others point to separate and distinct antiviral functions for each (Diner et al., 2016). In the present study, we provide the first demonstration that human glial cells constitutively express the IFI16 protein. Surprisingly, and in contrast to peripheral human cell types that show IFI16-dependent IFN-β production in response to dsDNA ligands (Almine et al., 2017; Jønsson et al., 2017), our studies employing siRNA-mediated IFI16 knockdown indicate that this sensor does not play a significant role in human microglial IFN responses to exogenous

DNA administration. Such a finding cannot be explained on the basis of DNA sensing redundancy via cGAS as IFI16 knockdown similarly failed to affect BDNA-induced IFN-β expression in $cGAS^{+/-}$ microglia.

cGAS has previously been demonstrated to be an important PRR for combating numerous infections through direct detection of cytosolic microbial/viral DNA (Gao et al., 2013a; Li et al., 2013; Schoggins et al., 2014; Cox et al., 2015; Watson et al., 2015; Xia et al., 2016; Paijo et al., 2016; Vermeire et al., 2016; Ruangkiattikul et al., 2017; Sun et al., 2017; Cheng et al., 2018). Importantly, this sensor has been shown to be a critical component in the generation of protective immunity in a murine model of acute HSE (Reinert et al., 2016). This protection is thought to be mediated through the canonical cGAS-STING signaling pathway, which leads to the expression of type I IFNs that act in an autocrine and/or paracrine manner to promote an antiviral state (Reinert et al., 2016). Similarly, IFI16 exhibits antiviral capabilities in peripheral cell types (Conrady et al., 2012; Johnson et al., 2014; Merkl et al., 2018; Roy et al., 2019) and has been reported to be an important restriction factor for herpesviruses including HSV-1 in such cells (Ansari et al., 2015; Diner et al., 2015; Dutta et al., 2015; Iqbal et al., 2016). However, unlike cGAS, the mechanism of action of IFI16 and the signaling pathways that this intracellular sensor employs are poorly understood.

Given the documented importance of both cGAS and IFI16 as PRRs in the generation of host immune responses to HSV-1 in peripheral cell types, and the present description of constitutive expression of both sensors in glial cells, it is not unreasonable to assume that these sensors could serve a similar function in human microglia during HSV-1 infection. Surprisingly, our studies show that neither cGAS nor IFI16, alone or in concert, appear to significantly impact HSV-1 transcription or the production/release of infectious particles in human microglial cells.

While the lack of cGAS and IFI16-mediated antiviral responses to this neuroinvasive HSV-1 clinical isolate may be simply due to host cell type or species dependent differences in sensor function (Kalamvoki and Roizman, 2014; Orzalli et al., 2016), we have determined that HSV-1 infection elicits marked reductions in the expression level of both of these molecules in human microglial cells. Such HSV-1-mediated suppression could explain why siRNA directed against cGAS or IFI16 failed to elicit demonstrable effects in infected cells and is suggestive of a viral immune evasion mechanism. Indeed, these findings are consistent with the previously reported ability of HSV-1 to target the expression and/or the signaling pathways of cGAS and IFI16 in non-CNS cell types (Orzalli et al., 2012; Johnson et al., 2013; Kalamvoki and Roizman, 2014; Christensen et al., 2016; Su and Zheng, 2017; Huang et al., 2018; Zhang et al., 2018). Such a strategy could underlie, at least in part, the absence of IFN-β production by HSV-1 infected microglia with little or no induction in the expression of the antiviral interferon-stimulated genes BST2, viperin, and IFITM1, despite retaining an ability to release IL-6. However, it should be noted that IFI16 has been reported to restrict herpesvirus replication via transcriptional regulation rather than effects on IFN responses in other cell types (Johnson et al., 2014; Merkl et al., 2018; Merkl and Knipe, 2019; Roy et al., 2019), and so the downregulation of this molecule could impact microglial responses to HSV-1 via mechanisms other than by inhibiting IFN-β production.

Taken in concert, we have demonstrated that human microglial cells functionally express the cytosolic DNA sensor cGAS and exhibit robust constitutive expression of the nuclear DNA sensor IFI16. While cGAS serves as a significant component in IRF3 activation and IFN-β production by human microglial cells in response to intracellular administration of foreign DNA, IFI16 does not appear to be required for such responses, in contrast to previous reports in other

human cell types. Surprisingly, neither of these intracellular DNA sensors mediate effective antiviral responses by human microglial cells to the neurotropic DNA virus HSV-1, and this may be due, at least in part, to an ability of this virus to suppress the expression of cGAS and/or IFI16 in these cells. As such, this ability may represent an important HSV immune evasion strategy in glial cells, and approaches that mitigate such suppression might represent a novel strategy to limit HSV-1 associated neuropathology.

4.4 FIGURES

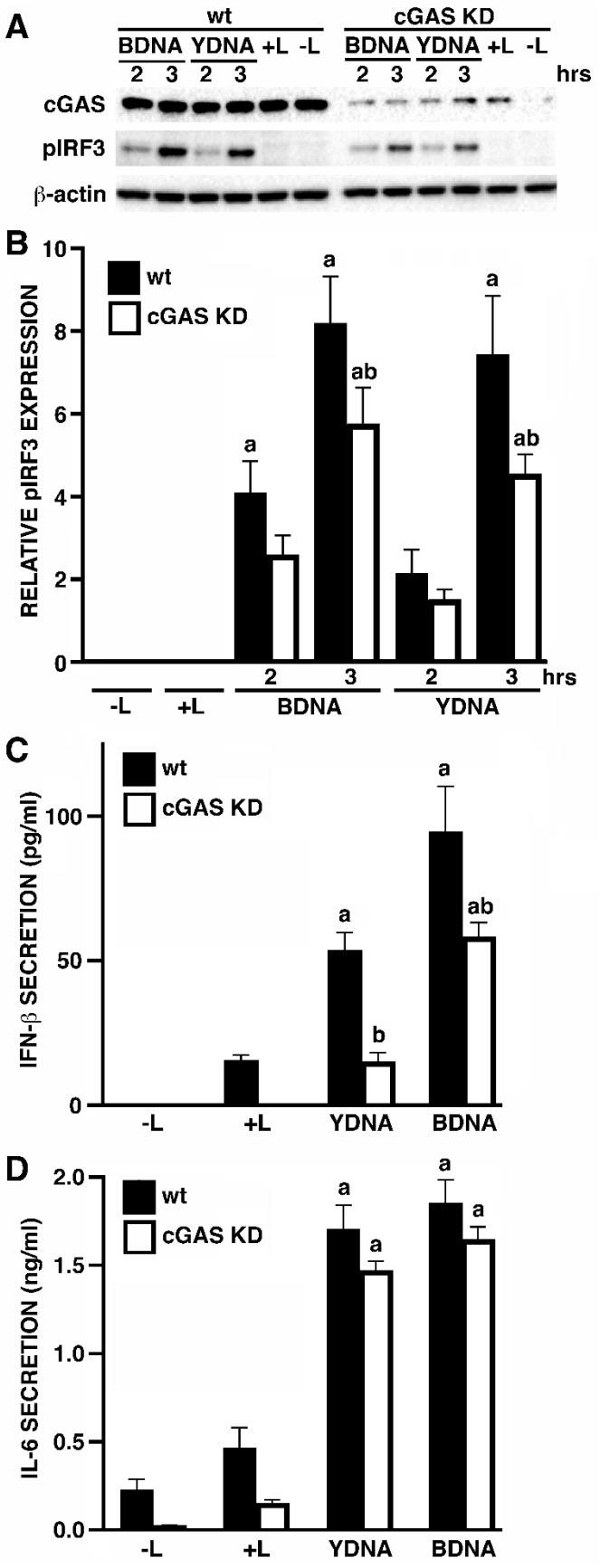

FIGURE 5: The DNA sensor cGAS is required for maximal antiviral mediator production by human microglia following intracellular administration of exogenous DNA. Panels A and B: Wildtype (wt) or heterozygous cGAS deletion (cGAS$^{+/-}$) immortalized human microglia (hμglia) were untreated (-L) or exposed to transfection reagent alone (+L) for 3 hrs, or were challenged with intracellular B-form DNA (BDNA; 0.1 μg/ml) or Y-form DNA (YDNA; 1 μg/ml) for 2 or 3 hours. Whole cell lysates were subsequently collected and analyzed for the expression of cGAS, phosphorylated IRF3 (pIRF3), or the housekeeping gene product β-actin by immunoblot analysis. Relative pIRF3 expression was determined by densitometric analysis and normalized to β-actin (n = 4-6). Panels C and D: wt or cGAS+/- hμglia were untreated (-L) or treated with transfection reagent alone (+L), or exposed to intracellular BDNA or YDNA (0.1 μg/ml and 1 μg/ml, respectively). After 24 hours, cell free supernatants were collected and the concentration of IFN-β (C) and IL-6 (D) was quantified by specific capture ELISA. Results are presented as the mean of four independent experiments +/- SEM (n = 4). The letter a indicates significant differences from cells exposed to transfection reagent alone while b indicates a significant difference from similarly treated wt cells.

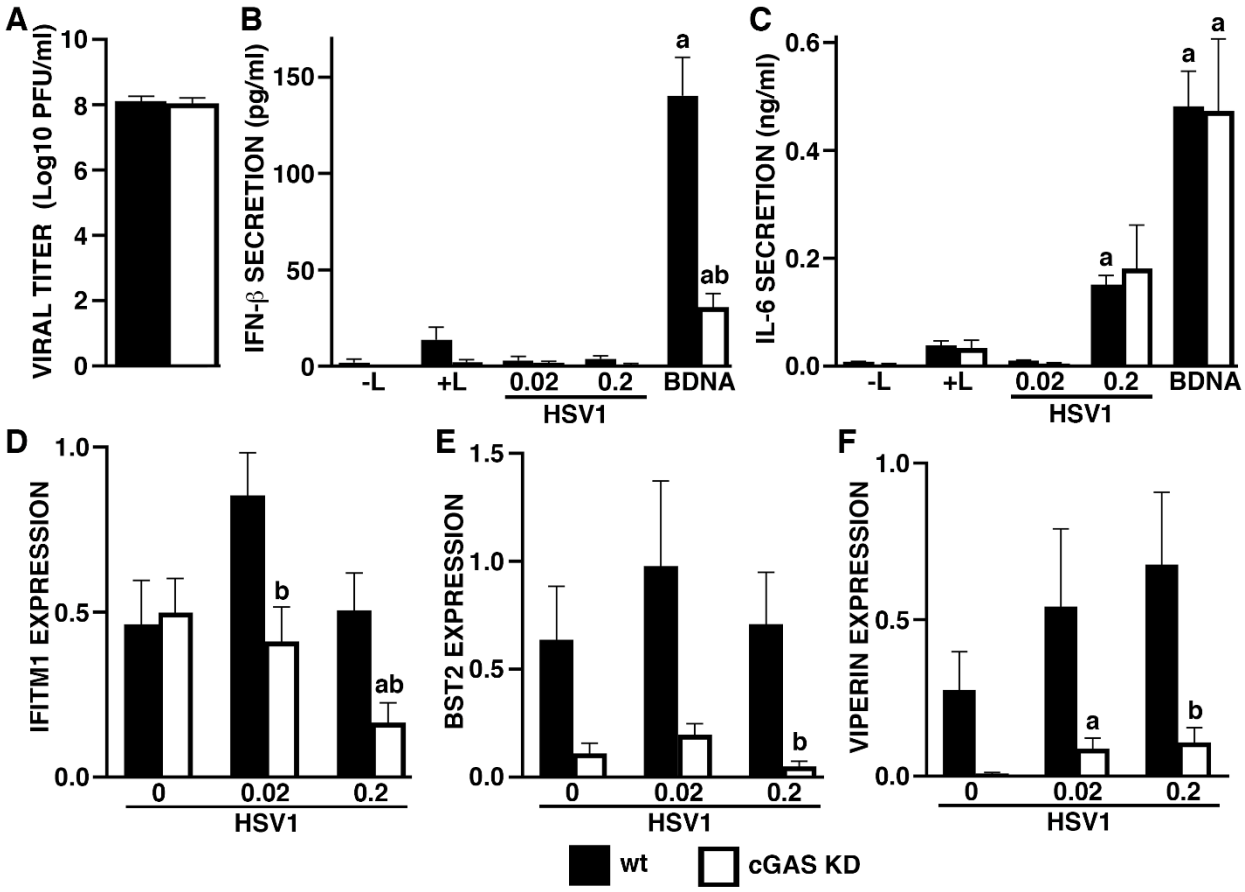

FIGURE 6: The DNA sensor cGAS contributes to antiviral gene expression, but does not restrict the number of infectious HSV-1 particles released by infected human microglia. Panel A: wt or cGAS+/- hμglia were infected with HSV-1 at an MOI of 0.2. At 24 hrs, cell free supernatants were collected and viral titers were determined by plaque assay (n = 7). Panels B and C: wt or cGAS+/- hμglia were transfected with 0.1 μg/mL BDNA or infected with HSV-1 (MOI of 0.02 and 0.2). At 8 hrs, cell free supernatants were collected and the concentrations of IFN-β (B) and IL-6 (C) were quantified by specific capture ELISAs (n = 7). Panels D-F: wt or cGAS+/- hμglia were infected with HSV-1 (MOI of 0.02 and 0.2). After 12 hrs, total RNA was isolated and the level of expression of mRNA encoding IFITM1 (D), BST2 (E), and Viperin (F), was determined by semi-quantitative RT-PCR and levels are reported relative to the expression of the housekeeping gene GAPDH (n = 4-7). In Panels B and C, the letter a indicates significant differences from cells exposed to transfection reagent alone while b indicates a significant difference from similarly treated wt cells. In Panels D-F, the letter a indicates significant differences from uninfected cells while b indicates a significant difference from similarly treated wt cells

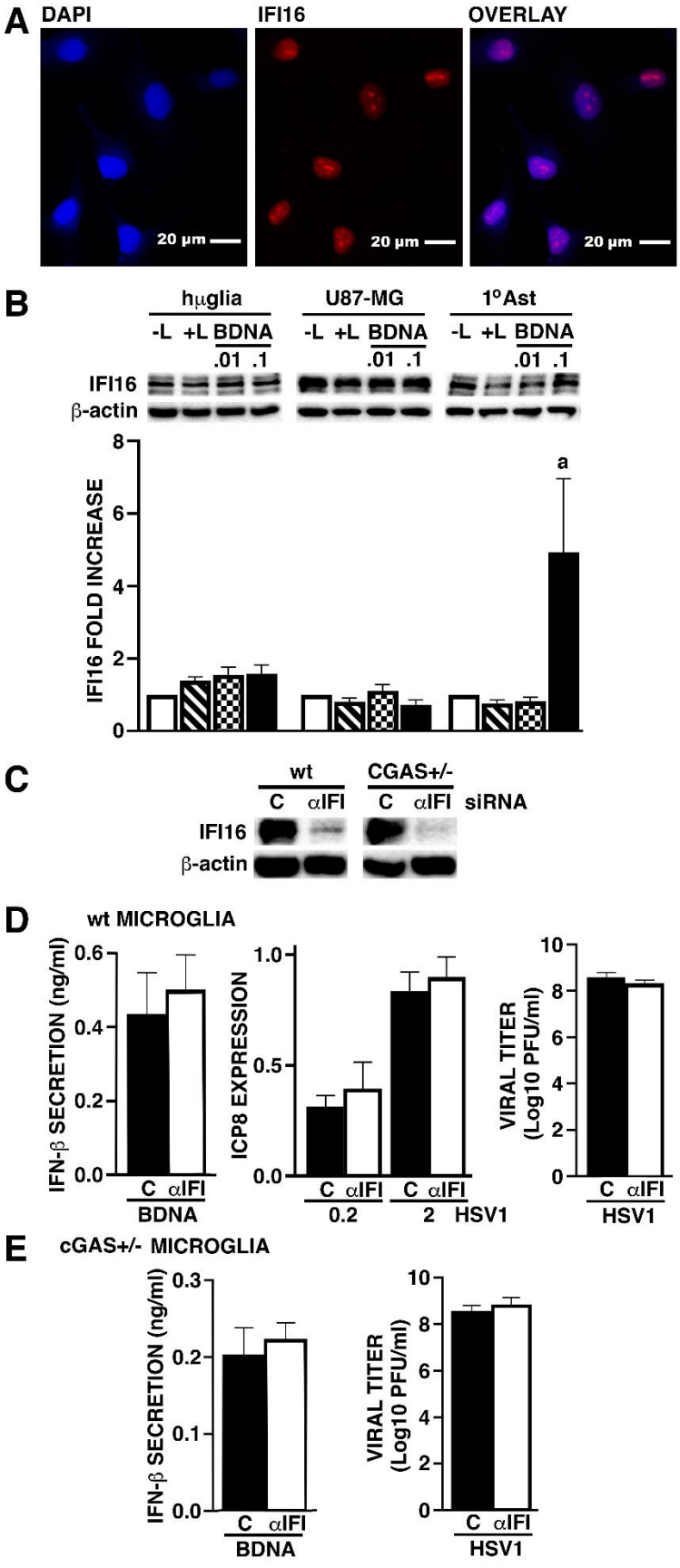

FIGURE 7: Human glia express IFI16, but this DNA sensor does not contribute to BDNA or HSV-1 mediated antiviral mediator production by human microglia, or restrict the number of infectious HSV-1 particles released by infected glia. Panel A: Micrographs show representative nuclear (DAPI), IFI16, and overlaid, immunofluorescence in hµglia human microglial cells (60X objective). Panel B: hµglia, U87-MG astrocytic cells, and primary human astrocytes (1°AST), were transfected with BDNA (0.01 and 0.1 ug/ml). At 24 hrs post-transfection, whole cell lysates were collected and tested for the presence of IFI16 by immunoblot analysis (n = 7-11). Panels C-E: wt or cGAS+/- hµglia were transfected with siRNA targeting IFI16 (αIFI; 5 nM) or scrambled RNA (C) for 48 hrs and IFI16 protein knockdown was confirmed by immunoblot analysis (Panel C; n = 2). After 48 hrs, control (C) or αIFI siRNA treated wt (Panel D) or cGAS+/- (Panel E) hµglia were either transfected with BDNA (0.1 ug/ml) or infected with HSV-1 (MOI of 0.2 and 2), and viral ICP8 mRNA levels relative to GAPDH expression were determined by RT-PCR at 8 hrs following challenge (n = 4), while supernatant IFN-β concentrations (n = 4-5) and viral titers (n = 8) were determined at 24 hrs post-challenge. Results are presented as the mean +/- SEM and no statistically significant differences were observed.

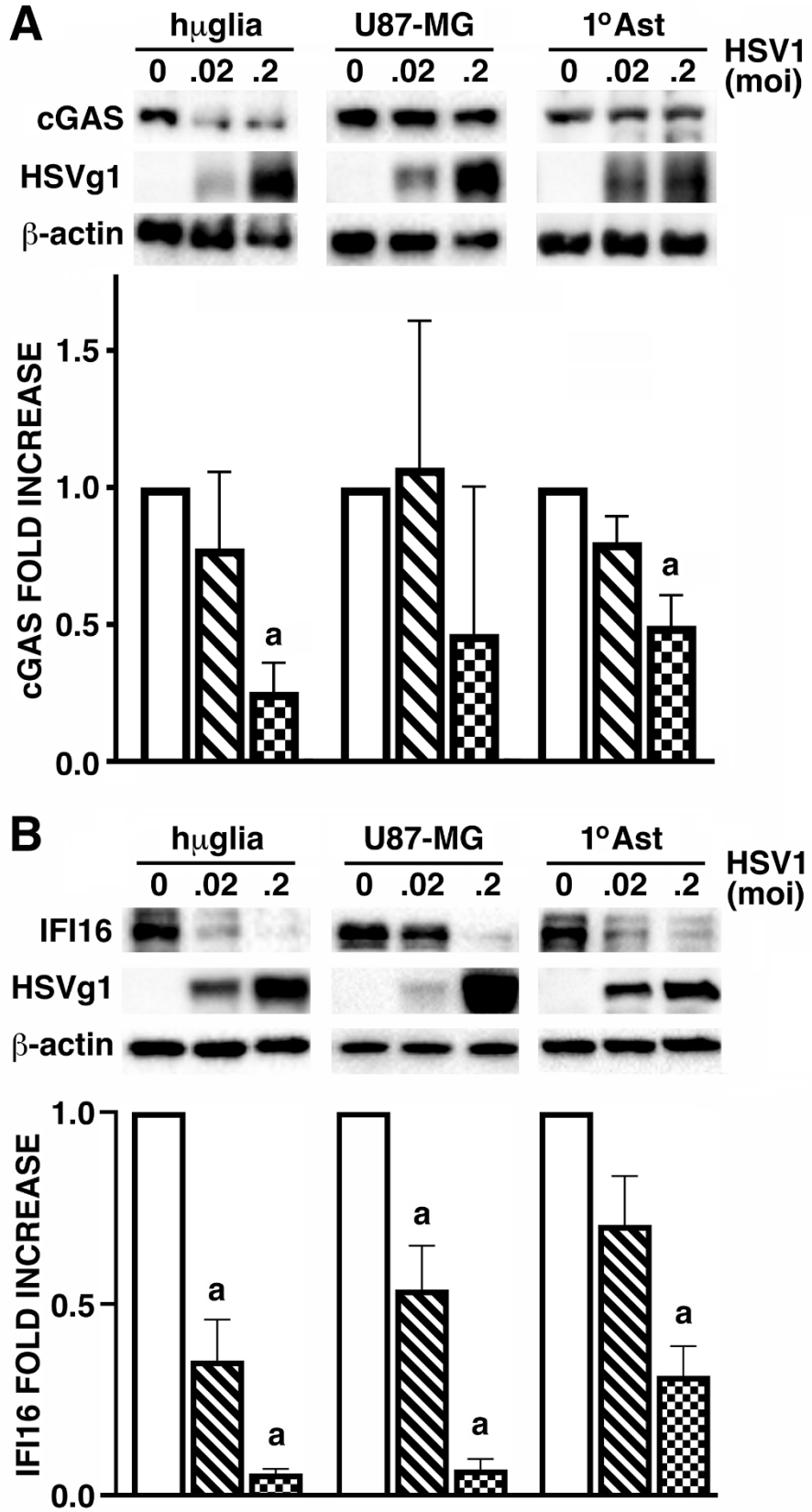

FIGURE 8: HSV-1 infection down regulates cGAS and IFI16 expression by human glia. hμglia, U87-MG, and primary human astrocytes (1°AST), were untreated or infected with HSV-1 (MOI of 0.02 and 0.2). At 24 hrs, whole cell lysates were collected and analyzed for the expression of cGAS (A; n = 3), IFI16 (B; n = 7-9), viral glycoprotein g1 (HSVg1), and the housekeeping product β-actin, by immunoblot analysis. An immunoblot with a longer exposure time is shown in Panel B for IFI16 expression in primary astrocytes due to the low levels seen in this cell type. Protein levels were determined by densitometric analysis relative to β-actin expression, and are shown as fold increases over untreated cells. Results are presented as the mean +/- SEM and the letter a indicates significant differences from uninfected cells.

CHAPTER 5: ZBP1 mediates necroptotic and apoptotic cell death pathways in glia

5.1 Rationale

Herpes simplex virus type 1 (HSV-1) is a highly successful human neurotropic DNA virus that currently infects nearly two thirds of the world's population (Xu et al., 2002). While most HSV-1 infections are asymptomatic, some result in life threatening brain inflammation and attendant swelling, known as encephalitis. HSV-1 is the most common cause of fatal sporadic encephalitis worldwide (Xu et al., 2006). If untreated, mortality rates are around 70% and even when patients receive appropriate care the infection still results in high morbidity (Baringer, 2008). Furthermore, of the patients who survive HSE, most will retain long-term neurological deficits (Xu et al., 2006). Much of the damage caused during HSE has been attributed to either a lack of control of HSV-1 propagation, mainly by a class of proteins known as type I IFNs, or excessive host inflammatory immune responses (Conrady et al., 2010). Therefore, understanding the initiation of early immune responses against HSV-1, and their relative contribution to protection or pathophysiology, is essential for the development of targeted therapeutics to reduce HSE associated morbidity and mortality.

It is now appreciated that resident non-neuronal brain cells, collectively known as glia, are capable of initiating innate immune signaling pathways following microbial infection (Chauhan et al., 2009; Furr and Marriott, 2012). Glia can initiate these responses through the recognition of conserved pathogen or damage associated molecular patterns (PAMPs or DAMPs, respectively) via the expression of pattern recognition receptors (PRRs). To date, several classes of PRRs have been identified with the best studied being the cell surface and endosomal toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Bowman et al., 2003; Sterka et al., 2006; Chauhan et al., 2009; Hanamsagar et al., 2012;

Jiang et al., 2014; Serramía et al., 2015; Huang et al., 2017b). However, the recent discovery of intracellular DNA sensors provides an additional mechanism by which glia may perceive viruses such as HSV-1. Of particular interest is the proposed DNA sensor Z-DNA binding protein 1 (ZBP1), as it has been demonstrated to be both protective and damaging to the host, depending on the context of the infection (Wang et al., 2014; Ingram et al., 2019; Momota et al., 2019).

ZBP1 was first identified as a DNA sensor capable of inducing type one IFNs in response to cytosolic DNA or viral infection (Takaoka et al., 2007). Additionally, ZBP1 was demonstrated to initiate nuclear factor kappa B (NF-κB) activation and pro-inflammatory mediator production following dsDNA stimulation (Takaoka et al., 2007; Kaiser et al., 2008; Rebsamen et al., 2009). In agreement with these studies, we have demonstrated that ZBP1 can contribute to pro-inflammatory mediator production during HSV-1 infection in murine astrocytes and microglia, and that these mediators cause neuronal cell death (Furr et al., 2011). Recently, ZBP1 has also been shown to initiate cell death pathways such as necroptosis in non-CNS cell types (Upton et al., 2012; Kuriakose et al., 2016; Thapa et al., 2016; Koehler et al., 2017; Maelfait et al., 2017; Sridharan et al., 2017; Guo et al., 2018; Jiao et al., 2020). Upon binding to nucleic acids, ZBP1 interacts with receptor interacting protein kinase 3 (RIPK3) via their RIP homotypic interaction motif (RHIM) domains. Following this interaction, RIPK3 phosphorylates mixed lineage kinase domain-like protein (MLKL) to induce necroptosis (Upton and Kaiser, 2017). While necroptosis can limit viral dissemination by killing the host cell prior to viral replication, it may also exacerbate damaging pro-inflammatory responses (Bonnet et al., 2011).

In the present study, we investigated the contribution of ZBP1 to HSV-1 mediated cell death in murine astrocytes and microglia. We confirm that HSV-1 can induce necroptosis and establish the ability of ZBP1 to initiate this cell death pathway in glia. Interestingly, while ZBP1

is best known for its role in necroptotic signaling, our findings indicate this sensor can also contribute to apoptosis in glia. Together, our findings indicate that ZBP1 may serve as a restriction factor for HSV-1 infection in these resident CNS cell types.

5.2 RESULTS

5.2.1 ZBP1 acts as an HSV-1 restriction factor in astrocytes

Since ZBP1 was shown to act as a restriction factor for HSV-1 in peripheral myeloid cells (Guo et al., 2018), we investigated the potential for this sensor to limit infection in glial cells. We challenged astrocytes and microglia derived from ZBP1+/+ (WT) and ZBP1-/- (ZBP1 KO) mice with HSV-1 and measured viral particle release. Lack of ZBP1 protein expression in astrocytes and microglia derived from ZBP1 KO mice was confirmed by Western blot analysis (Fig. 9). As shown in Fig. 10A, viral particle release was significantly increased in ZBP1 KO astrocytes and displayed a marked increase in microglia (Fig. 10B). To determine if this was due to a reduction in antiviral mediator production we measured the secretion of IFN-β. Both WT and ZBP1 KO astrocytes and microglia demonstrated similarly low levels of IFN-β induction (Fig. 10 C and D). We have previously demonstrated that siRNA mediated knockdown of ZBP1 led to a reduction in the secretion of inflammatory cytokines following HSV-1 infection in glia (Furr et al., 2011). To confirm these findings in a genetic ZBP1 knockout model we infected WT and ZBP1 KO glia and measured the production of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Fig. 10 C and D). Surprisingly, neither astrocytes or microglia demonstrated a significant difference in TNF-α production, and astrocytes displayed a significant increase in IL-6 secretion. This discrepancy from our previous findings may be due to unknown alterations resulting from a ZBP1 KO genetic background or the possible off target effects of ZBP1 targeting siRNA. In either case, the absence of IFN production and lack of decreased inflammatory mediator production suggests that another mechanism likely contributes to the reduction in HSV-1 particle release.

5.2.2 HSV-1 infection induces necroptosis in astrocytes

Several studies have now shown that ZBP1 can elicit the activation of necroptosis in non CNS cell types (Upton et al., 2012; Kuriakose et al., 2016; Thapa et al., 2016; Maelfait et al., 2017; Sridharan et al., 2017; Guo et al., 2018; Jiao et al., 2020). To determine if this was the case in glia, we infected astrocytes derived from WT and ZBP1 KO mice with HSV-1 and measured the change in cell death over 24 hours. However, because it was recently discovered that the HSV-1 protein ICP-6 also has a RHIM domain that is capable of directly interacting with receptor interacting protein kinase 1 (RIPK1) to initiate necroptosis in mice (Wang et al., 2014; Huang et al., 2015; Guo et al., 2018), we employed the use of an HSV-1 strain with mutations in the ICP6 RHIM domain (ICP6 RHIM Mut), as well as the parental F strain (ICP6 WT). Interestingly, not only was there a significant difference between the rate and percentage of cell death at 24 hrs in WT and ZBP1 KO astrocytes for the ICP6 RHIM Mut virus (Slope 3.078 ± 0.16 % death·hrs^{-1} vs. 1.30 ± 0.06 % death·hrs^{-1}, respectively, $p<0.0001$), but also for the parental ICP6 WT virus (2.72 ± 0.15 % death·hrs^{-1} vs. 1.30 ± 0.06 % death·hrs^{-1}, $p <0.0001$) (Fig. 11A). This is despite similar levels of phosphorylated MLKL during ICP6 WT infection (Fig. 11B), indicating necroptosis was not solely responsible for the differences in cell death. In agreement with this, we found that there were no significant differences in the kinetics, or percentage cell death at 24 hrs, during infection with either strain in the ZBP1 KO astrocytes (Fig. 11A), even though infection with ICP6 RHIM Mut resulted in significantly reduced P-MLKL (Fig. 11B).

To determine if RIPK1 was responsible for the phosphorylation of MLKL in ZBP1 KO astrocytes, we treated WT or ZBP1 KO derived astrocytes with the RIPK1 inhibitor, GSK963, during infection with either ICP6 WT and ICP6 RHIM Mut viruses. Again, there were

significant differences between the kinetics and percentage of cell death at 24 hrs for both the ICP6 RHIM Mut (2.25 ± 0.31 % death·hrs^{-1} vs 0.31 ± 0.12% death·hrs^{-1}, p<0.0001) and ICP6 WT virus (2.43 ± 0.24% death·hrs^{-1} vs. 0.50 ± 0.10% death·hrs^{-1}, p< 0.0001) (Fig. 11C), indicating the differences were not due to RIPK1 kinase activity. Importantly, treatment of ZBP1 KO derived astrocytes with a RIPK1 inhibitor significantly reduced the levels of phosphorylated MLKL following infection with ICP6 WT virus when compared to WT derived astrocytes (Fig. 11D), suggesting that phosphorylation of MLKL is dependent on RIPK1 in the absence of ZBP1. Together, these data indicate that the HSV-1 is capable of inducing both RIPK1 and ZBP1-mediated necroptosis.

5.2.3 Virally induced apoptosis and necroptosis requires ZBP1 in astrocytes

Since both RIPK1 and ZBP1 induced necroptosis require RIPK3 activity to phosphorylate MLKL, we employed the RIPK3 inhibitor, GSK872, during infection to ascertain whether necroptosis was the primary contributor to the differences observed in cell death. Surprisingly, both WT and ZBP1 KO derived astrocytes displayed significant differences in the kinetics of cell death regardless of viral strain (ICP6 WT: 1.80 ± 0.24% death·hrs^{-1} vs. 0.62 ± 0.06% death·hrs^{-1}, ICP6 RHIM Mut 2.11 ± 0.3% death·hrs^{-1} vs. 0.41 ± 0.08% death·hrs^{-1}; p <0.0001) (Fig. 12A), despite the reduction in levels of phosphorylated MLKL (Fig. 11B). Infection with the ICP6 RHIM Mut virus, but not ICP6 WT, resulted in a significant difference in the percentage of cell death at 24 hrs (Fig. 12A). These data indicate that necroptosis is not the only contributor to ZBP1 mediated cell death.

To see if cell death was also occurring via apoptosis, we treated WT and ZBP1 KO derived astrocytes with the pan caspase inhibitor zVAD following infection with either the ICP6

WT or ICP6 RHIM Mut HSV-1 virus. Interestingly, rather than reducing cell death in WT astrocytes, caspase inhibition appears to increase cell death in ZBP1 KO astrocytes (Fig. 12C). While the kinetics of cell death were still significantly different during caspase inhibition (ICP6 WT: 2.33 ± 0.11% death·hrs^{-1} vs. 1.30 ± 0.06% death·hrs^{-1}, ICP6 RHIM Mut: 2.42 ± 0.15% death·hrs^{-1} vs. 1.00 ± 0.09% death·hrs^{-1}; $p<0.0001$), the percentage of cell death at 24 hrs was not (Fig. 12C). Other studies have suggested that caspase inhibition may promote RIPK1 activation and, therefore, the induction of necroptosis (Degterev et al., 2005). To confirm this was occurring in astrocytes, we probed for phosphorylated MLKL protein following caspase inhibition and infection with ICP6 WT and ICP6 RHIM Mut viruses. Both WT and ZBP1 KO derived astrocytes displayed similar levels of phosphorylated MLKL regardless of viral strain (Fig. 12D), demonstrating that caspase inhibition permits ZBP1 independent necroptosis.

To determine if RIPK1 was responsible for the increased cell death in ZBP1 KO derived astrocytes following caspase inhibition, we simultaneously treated WT or ZBP1 KO astrocytes with RIPK1 and caspase inhibitors during infection with ICP6 WT or ICP6 RHIM Mut viruses. The kinetics of cell death remained significantly different between WT and ZBP1 KO astrocytes (ICP6 WT: 3.069 ± 0.31% death·hrs^{-1} vs. 1.64 ± 0.11% death·hrs^{-1}, ICP6 RHIM Mut: 2.91 ± 0.25% death·hrs^{-1} vs. 1.14 ± 0.08% death·hrs^{-1} $p<0.0001$) (Fig. 13A). Importantly, the percentage of cell death at 24 hrs was reduced in ZBP1 KO derived astrocytes and was significantly different from WT astrocytes (Fig. 13A), suggesting caspase inhibition permits RIPK1 mediated necroptosis during infection. Considering that neither caspase or RIPK3 inhibition alone reduced the percentage of cell death observed in WT derived astrocytes to that in ZBP1 KO astrocytes, we investigated whether simultaneous activation of both pathways could account for the differences in cell death. To test this, we treated WT and ZBP1 KO derived

astrocytes with both RIPK3 and caspase inhibitors during infection with ICP6 WT or ICP6 RHIM Mut virus. While the kinetics of cell death remained significantly different (ICP6 WT: 1.45 ± 0.10% death·hrs^{-1} vs. 0.83 ± 0.08% death·hrs^{-1}, ICP6 RHIM Mut 1.26 ± 0.07% death·hrs^{-1} vs. 0.67 ±0.09% death·hrs^{-1}, $p<0.0001$), the percentage of cell death at 24 hrs was reduced in WT derived astrocytes to levels similar to those observed in ZBP1 KO astrocytes (Fig 13B). These data suggest that ZBP1 mediates both apoptosis and necroptosis in astrocytes.

5.2.4 ZBP1 mediates apoptotic and necroptotic cell death in microglia

Thus far, we have focused on ZBP1's contribution to cell death in astrocytes, to establish that a similar phenotype occurs in microglia, we infected WT and ZBP1 KO derived microglia with ICP6 WT and ICP6 RHIM Mut viruses. Like astrocytes, WT derived microglia displayed a significantly higher rate of cell death compared to ZBP1 KO microglia for both ICP6 WT and ICP6 RHIM Mut viruses (2.03 ± 0.10% death·hrs^{-1} vs. 1.17 ± 0.07% death·hrs^{-1} and 2.06 ± 0.10% death·hrs^{-1} vs. 0.96 ± 0.07% death·hrs^{-1}, respectively, $p<0.0001$), as well as a trend for an increased percentage of cell death at 24 hrs. (Fig. 14A). As in astrocytes, treatment with the RIPK1 inhibitor had no effect on the rate or percentage of cell death at 24 hrs (Fig. 14B). Furthermore, WT and ZBP1 KO derived microglia treated with a RIPK3 inhibitor continued to have significant differences in the rate of cell death for both strains (ICP6 WT: 1.80 ± 0.096% death·hrs^{-1} vs. 1.11 ± 0.03% death·hrs^{-1}, ICP6 RHIM Mut 1.56 ± 0.1% death·hrs^{-1} vs. 0.854 ±0.04% death·hrs^{-1}, $p<0.0001$) (Fig. 14C), suggesting that necroptosis may not be the only pathway activated by ZBP1. Lastly, treatment with the pan caspase inhibitor zVAD appears to increase cell death in ZBP1 KO derived microglia to levels similar to those seen in WT

microglia, possibly due to RIPK1 activity as observed in astrocytes (Fig. 14D). As such, it is possible ZBP1 acts in microglia in an analogous manner to that in astrocytes.

5.2.5 Infection with a neuroinvasive strain of HSV-1 initiates ZBP1 mediated cell death pathways in astrocytes

Because the strains employed so far are attenuated when compared to clinical isolates, we wanted to confirm that a neuroinvasive strain of HSV-1 initiated similar ZBP1 dependent responses. To do this, we infected WT and ZBP1 KO derived astrocytes with the MacIntyre strain of HSV-1 (HSV-1 (Mac)) and measured cell viability over 24 hrs. HSV-1 (Mac) infection resulted in a significantly lower rate ($2.93 \pm 0.24\%$ death$\cdot$hrs^{-1} vs. $1.27 \pm 0.09\%$ death$\cdot$hrs^{-1}, $p<0.0001$) and percentage of cell death at 24 hrs in ZBP1 KO astrocytes (Fig. 15A), and this trend was unaltered by treatment with a RIPK1 inhibitor (Fig. 15B) ($2.34 \pm 0.28\%$ death$\cdot$hrs^{-1} vs. $0.63 \pm 0.113\%$ death$\cdot$hrs^{-1}, $p<0.0001$). Like the attenuated HSV-1 strains, when WT and ZBP1 KO derived astrocytes were treated with a RIPK3 inhibitor during infection they continued to display significant differences in the rate ($2.29 \pm 0.25\%$ death$\cdot$hrs^{-1} vs. $0.60 \pm 0.07\%$ death$\cdot$hrs^{-1}, $p<0.0001$) and percentage of cell death at 24 hrs (Fig. 15C). Furthermore, ZBP1 KO derived astrocytes treated with a pan caspase inhibitor demonstrated a tendency for increased cell death during infection, as observed previously (Fig. 15D). WT derived astrocytes treated with a caspase inhibitor continued to have a higher rate of cell death then similarly treated ZBP1 KO astrocytes (2.33 ± 0.10 vs. 1.23 ± 0.04. $p<0.0001$) (Fig. 15D). To determine if caspase inhibition permitted virally induced necroptosis via RIPK1, we simultaneously treated WT and ZBP1 KO astrocytes with RIPK1 and caspase inhibitors during infection with HSV-1 (Mac). Surprisingly, this combination treatment did not significantly reduce the percentage of cell death at 24hrs to

the same degree as that observed with attenuated HSV-1 strains, and the rates of cell death did not differ significantly (2.7 ± 0.24% death·hrs^{-1} vs. 2.13 ± 0.13% death·hrs^{-1}, p=0.0522) (Fig. 15E). This suggests that caspase inhibition may permit cell death independent of RIPK1 and ZBP1 during infection with a neuroinvasive strain of HSV-1. However, treatment with both RIPK3 and caspase inhibitors during infection with HSV-1 (Mac) resulted in similar levels of cell death at 24 hrs (Fig. 15F), suggesting that ZBP1 is responsible for the induction of both apoptotic and necroptotic cell death pathways. Together, these data indicate that ZBP1 mediates similar cell death responses to a neuroinvasive HSV-1 clinical isolate.

5.2.6 Infection with a neuroinvasive strain of HSV-1 initiates ZBP1 mediated cell death pathways in microglia

Next, we wanted to confirm that ZBP1 contributes to cell death in microglia following infection with the neuroinvasive strain of HSV-1. To accomplish this, WT and ZBP1 KO derived microglia were infected with HSV-1 (Mac) and cell viability was measured over 24 hrs. As with the attenuated strains of HSV-1, WT microglia had a higher rate of cell death when compared to ZBP1 KO microglia (1.85 ± 0.17% death·hrs^{-1} vs. 1.22 ± 0.14% death·hrs^{-1}, p=0.0003), but did not differ significantly in the percentage of cell death at 24 hrs (Fig. 16A). Surprisingly, ZBP1 KO derived microglia treated with a RIPK1 inhibitor and infected with HSV-1 (Mac) displayed lower initial cell viability than WT microglia. However, the rate of cell death was significantly higher for WT derived microglia (1.53 ± 0.2% death·hrs^{-1} vs. 0.20 ± 0.08% death·hrs^{-1}, p<0.0001) and both WT and ZBP1 KO microglia had similar levels of cell death at endpoint (Fig. 16B). Additionally, treatment with a RIPK3 inhibitor did not affect these trends, as WT microglia demonstrated a significantly higher rate of cell death compared to ZBP1 KO microglia

(1.36 ± 0.11% death·hrs^{-1} vs. 0.77 ± 0.09% death·hrs^{-1}, $p<0.0001$), although there were no significant differences in the percentage of cell death at 24 hrs (Fig. 16C). Lastly, treatment with a caspase inhibitor during infection resulted in similar rates of cell death between WT and ZBP1 KO derived microglia (1.36 ± 0.07% death·hrs^{-1} vs. 1.27 ± 0.06% death·hrs^{-1}, $p=0.67$), and ZBP1 KO microglia displayed a trend for increased cell death at 24 hrs (Fig. 16D). Together, these data suggest that ZBP1 contributes to an increased rate of cell death during infection with a neuroinvasive strain of HSV-1.

5.3 DISCUSSION

HSV-1 is a highly successful neurotropic DNA virus and the most common cause of fatal sporadic encephalitis worldwide (Xu et al., 2006). Damage caused during HSE is attributable either to the lack of control of HSV-1 replication or the over-production of inflammatory mediators (Conrady et al., 2010). As such, it is critical to determine the mechanisms leading to the initiation of early immune responses and their relative contribution to protection or pathophysiology. We have previously demonstrated that the DNA sensor ZBP1 contributes to the production of inflammatory mediators, and such mediators are damaging to neuronal cells (Furr et al., 2011). In the present study, we expand on this work by demonstrating the contribution of this sensor to HSV-1 restriction and the initiation of cell death pathways in astrocytes and microglia. We show that the loss of ZBP1 in astrocytes results in a significant increase in the number of released viral particles with similar trends seen in microglia. Furthermore, we demonstrate that this increase in viral particle release may result from a mechanism unrelated to IFN or inflammatory cytokine production.

Recently, ZBP1 has been identified as an important mediator of necroptosis during infection with DNA and RNA viruses (Kuriakose et al., 2016; Sridharan et al., 2017; Guo et al., 2018; Jiao et al., 2020). In agreement with these studies, we have demonstrated that HSV-1 initiates necroptosis in a ZBP1 dependent manner in astrocytes. Additionally, we have confirmed that the HSV-1 protein ICP6 can activate necroptosis independently of ZBP1 through RIPK1. Surprisingly, blocking necroptosis with a RIPK3 inhibitor did not reduce cell death in WT derived astrocytes to the same level as that observed in ZBP1 KO astrocytes. Interestingly, inhibition of apoptotic pathways also did not reduce cell death in WT derived astrocytes and, instead, increased cell death in ZBP1 KO astrocytes in a RIPK1-dependent manner. This is in

line with other studies, which suggests caspase-8 acts as a negative regulator of RIPK1-mediated necroptosis (Pasparakis and Vandenabeele, 2015; Silke et al., 2015; Grootjans et al., 2017; Weinlich et al., 2017). Furthermore, we show that simultaneous inhibition of caspases and RIPK1 reduced the percentage of cell death in ZBP1 KO derived astrocytes, supporting the role of caspases as negative regulators of RIPK1 induced necroptosis. Additional evidence indicates that RIPK3 can initiate apoptosis independent of its kinase activity (Mandal et al., 2014). In the presence of RIPK3 kinase inhibitors RIPK3 associates with RIPK1 via their respective RHIM domains. This interaction leads to the recruitment and activation of caspase 8 and, therefore, the induction of apoptosis (Mandal et al., 2014). Our data suggest this may be the case in astrocytes, as simultaneous inhibition of RIPK3 and caspases resulted in a reduction of cell death in ZBP1 WT derived astrocytes to levels similar to those found in ZBP1 KO astrocytes. These findings are summarized in graphical form in Fig. 15. Importantly, we have confirmed these findings during infection with a neuroinvasive strain of HSV-1 (Krinke and Dietrich, 1990). Together, we have demonstrated that ZBP1 functions as an intracellular sensor for HSV-1 and induces both apoptotic and necroptotic signaling pathways in astrocytes.

Similar to astrocytes, we show that ZBP1 KO derived microglia demonstrate a reduction in the rate of cell death during infection with HSV-1, highlighting the importance of this sensor in the activation of these pathways. Furthermore, treatment with a pan caspase inhibitor appears to increase cell death in ZBP1 KO derived microglia, likely in a similar manner to that seen astrocytes. This was also true during infection with a neuroinvasive strain of HSV-1 (Krinke and Dietrich, 1990). However, because we did not observe a significant reduction in the percentage of cell death at 24 hrs in the absence of inhibitors, it remains to be determined if ZBP1 mediates the same responses as those found in astrocytes. Regardless, it is clear that ZBP1 has some effect

on the initiation of cell death pathways, as the rate of death in ZBP1 KO derived microglia is significantly lower than that seen in WT microglia.

Taken in concert, we have shown that ZBP1 serves as an important restriction factor for HSV-1 in glia. We demonstrate that the reduction in viral particle release is likely due to the induction of apoptotic and necroptotic cell death pathways. Next, we will need to address what affect these pathways may have on neighboring cells, especially neurons. In doing so, we will be able to start identifying whether the activation of ZBP1 and downstream death pathways represent protective host responses or, rather, contribute to the pathology of HSE. These studies will allow researchers to identify potential targets for intervention to reduce HSE associated morbidity and mortality.

5.4 FIGURES

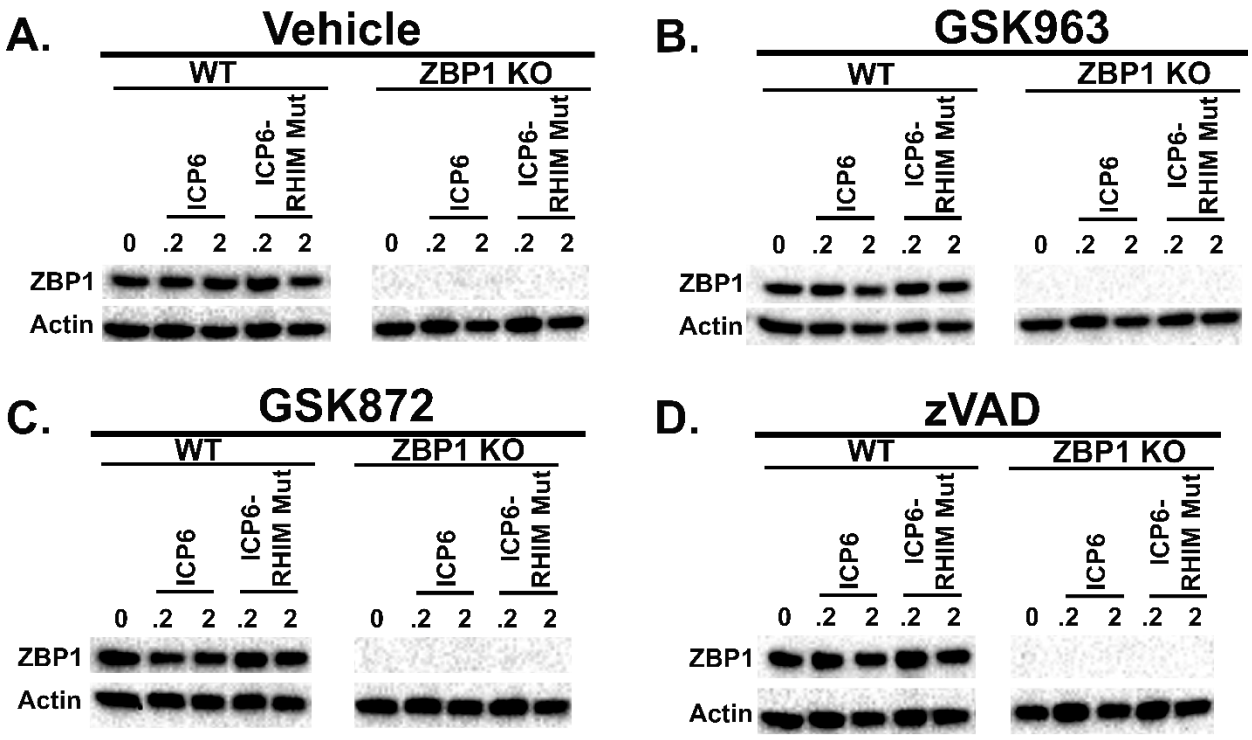

Fig. 9 Confirmation of ZBP1 deficiency in ZBP1$^{-/-}$ astrocytes.
WT or ZBP1 KO murine astrocytes were infected with ICP6 or ICP6 RHIM Mut HSV-1 and one hour following infection were treated with (A) DMSO (vehicle), (B) GSK963, (C) GSK872, (D) zVAD. After 24hrs total cell lysates were collected and analyzed for the presence of ZBP1 or the house keeping gene β-actin via Western Blot analysis.

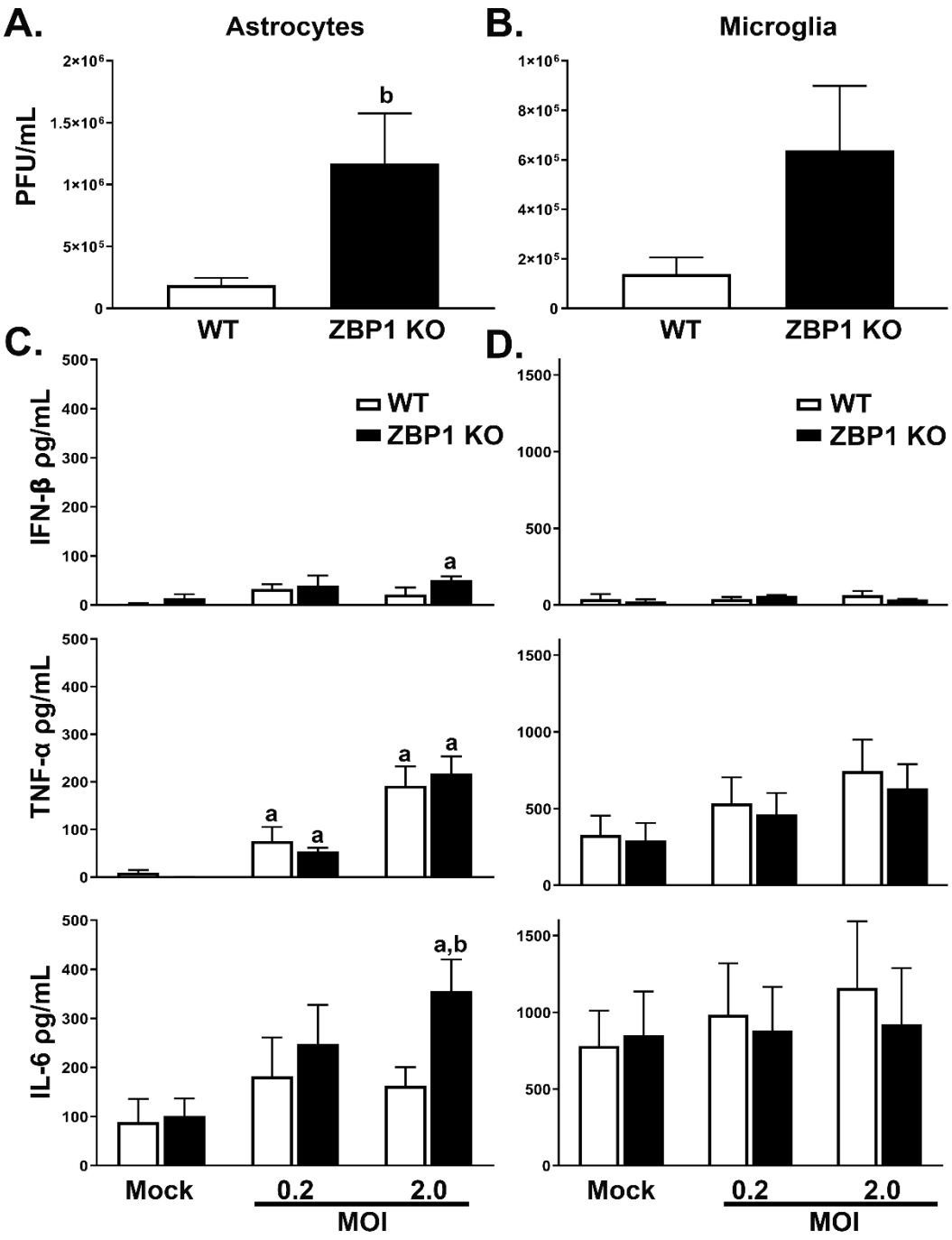

Fig. 10 ZBP1 restricts HSV-1 replication in astrocytes independent of interferon production. WT or ZBP1 KO murine astrocytes (A.) and microglia (B.) where infected with HSV-1 (MacIntyre strain) at a MOI of 0.2 for 24hrs. Cell free supernatants were then collected and analyzed for viral titer by plaque assay. WT or ZBP1 KO murine astrocytes (C.) and microglia (D.) where infected with HSV-1 at a MOI of 0.2 or 2.0 for 24hrs. After 24hrs, cell free supernatants were collected and the concentration of IFN-β, IL-6, and TNF-α was quantified by specific capture ELISA. The letter a indicates significant difference from mock infected cell while b indicates a significant difference from similarly treated WT cells. Data is presented as +/- the SEM of at least three independent replicates.

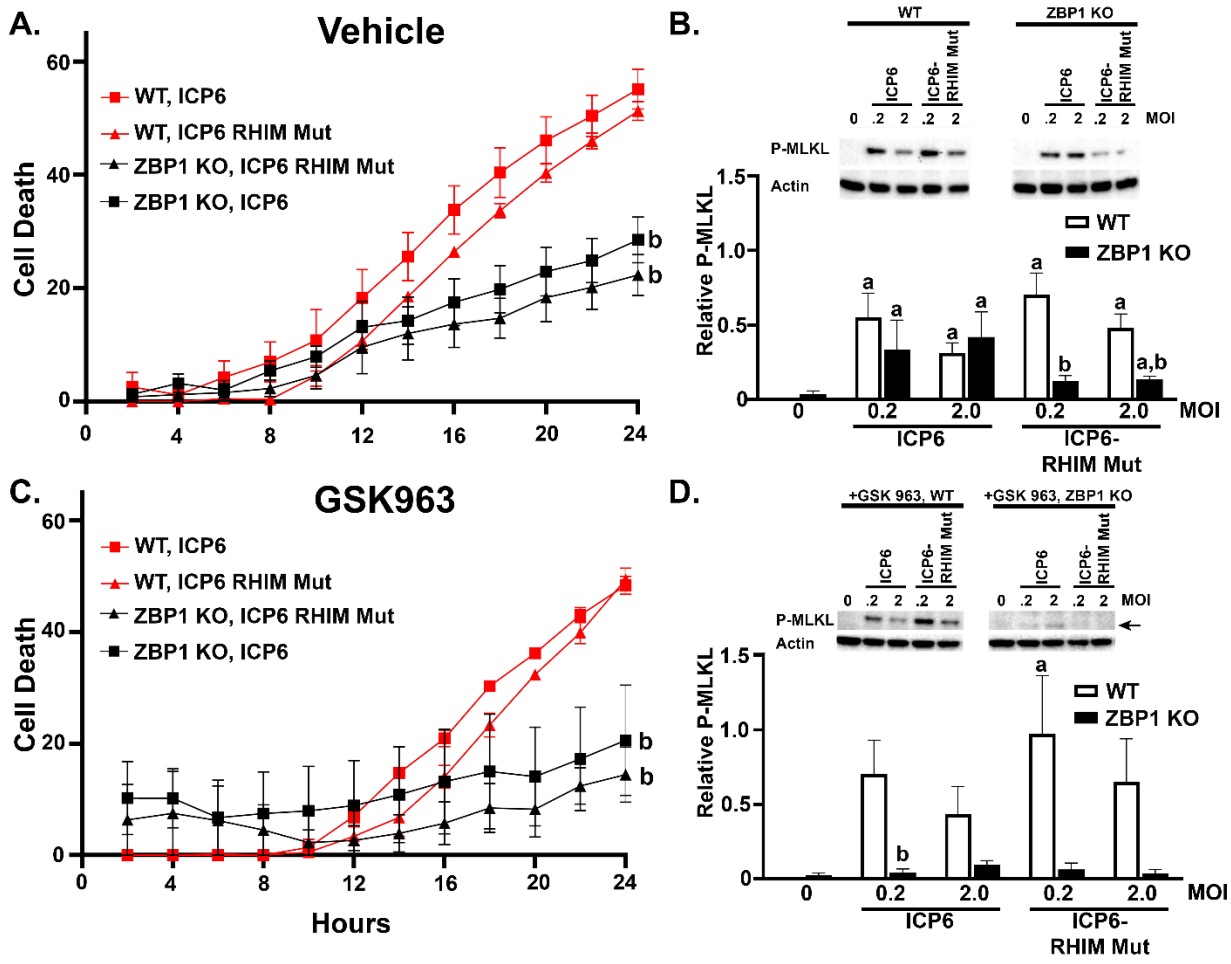

Fig. 11 HSV-1 induces both RIPK1 and ZBP1 mediated necroptosis in murine astrocytes. WT or ZBP1 KO murine astrocytes were infected with HSV-1 ICP6 WT (ICP6) or HSV-1 ICP6 RHIM mutant virus. One hour following infection cells were treated with either DMSO (vehicle) (A) or GSK963 (RIPK1 inhibitor) (C). Cell viability was measured with RealTime-Glo™ MT Cell Viability Assay starting two hours after infection and was continually measured every two hours for a 24-hour period. WT or ZBP1 KO murine astrocytes were treated with either DMSO (vehicle) (B) or GSK963 (RIPK1 inhibitor) (D) and infected with HSV-1 ICP6 or ICP6 RHIM mutant for 24hrs. After 24hrs, total cell lysates were collected and analyzed for the presence of phosphorylated MLKL (P-MLKL) or the housekeeping gene product β-actin via Western blot analysis. Relative P-MLKL expression was determined by densitometric analysis and normalized to β-actin. The black arrow in panel D corresponds to the predicted size of P-MLKL. The letter a indicates significant difference from mock infected cells while b indicates a significant difference from similarly treated WT cells. Data is shown as +/- SEM for three independent experiments.

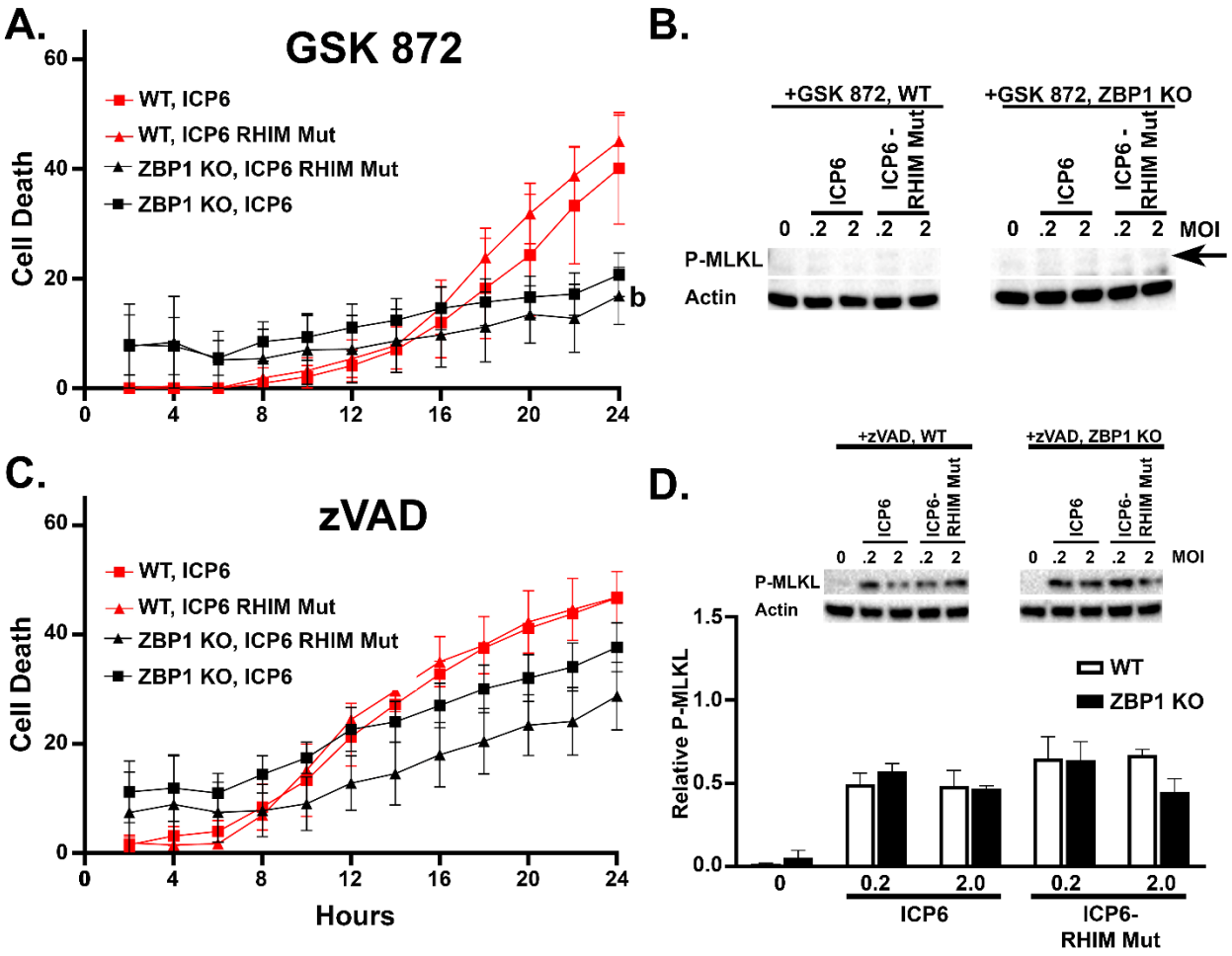

Fig. 12 Caspase inhibition permits virally induced necroptosis in astrocytes independent of ZBP1 expression. WT or ZBP1 KO murine astrocytes were infected with HSV-1 ICP6 WT (ICP6) or HSV-1 ICP6 RHIM mutant virus. One hour following infection cells were treated with either GSK 872 (RIPK3 inhibitor) (A) or zVAD (pan caspase inhibitor) (C). Cell viability was measured with RealTime-Glo^{TM} MT Cell Viability Assay starting two hours after infection and was continually measured every two hours for a 24-hour period. WT or ZBP1 KO murine astrocytes were treated with either GSK872 (RIPK3 inhibitor) (B) or zVAD (pan caspase inhibitor) (D) and infected with HSV-1 ICP6 WT (ICP6) or ICP6 RHIM mutant for 24hrs. After 24hrs, total cell lysates were collected and analyzed for the presence of phosphorylated MLKL (P-MLKL) or the housekeeping gene product β-actin via Western blot analysis. The black arrow in panel B. corresponds to the predicted size of P-MLKL. Relative P-MLKL expression was determined by densitometric analysis and normalized to β-actin. Data is shown as +/- SEM for three independent experiments. The letter b indicates a significant difference from similarly treated ZBP1$^{+/+}$ cells.

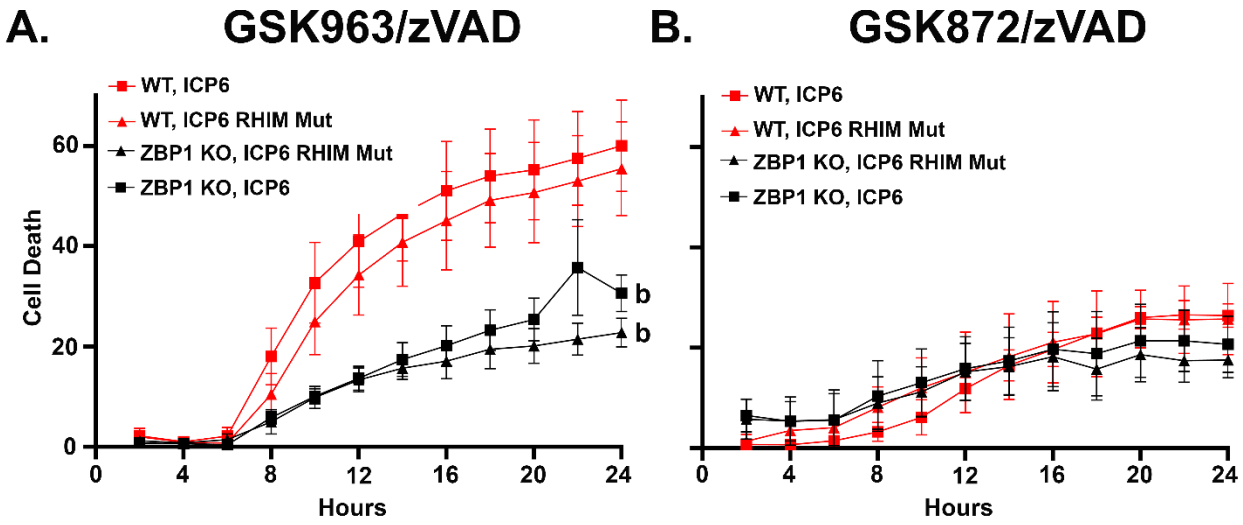

Fig. 13 ZBP1 induces both apoptotic and necroptotic cell death pathways in astrocytes. WT or ZBP1 KO murine astrocytes were infected with HSV-1 ICP6 WT (ICP6) or HSV-1 ICP6 RHIM mutant virus. One hour following infection cells were treated with either (A) GSK963 and zVAD (RIPK1/pan caspase inhibitor) or (B) GSK872 and zVAD (RIKP/pan caspase inhibitor). Cell viability was measured with RealTime-Glo™ MT Cell Viability Assay starting two hours after infection and was continually measured every two hours for a 24-hour period. The letter b indicates a significant difference from similarly treated WT cells. Data is shown as +/- SEM for three independent experiments.

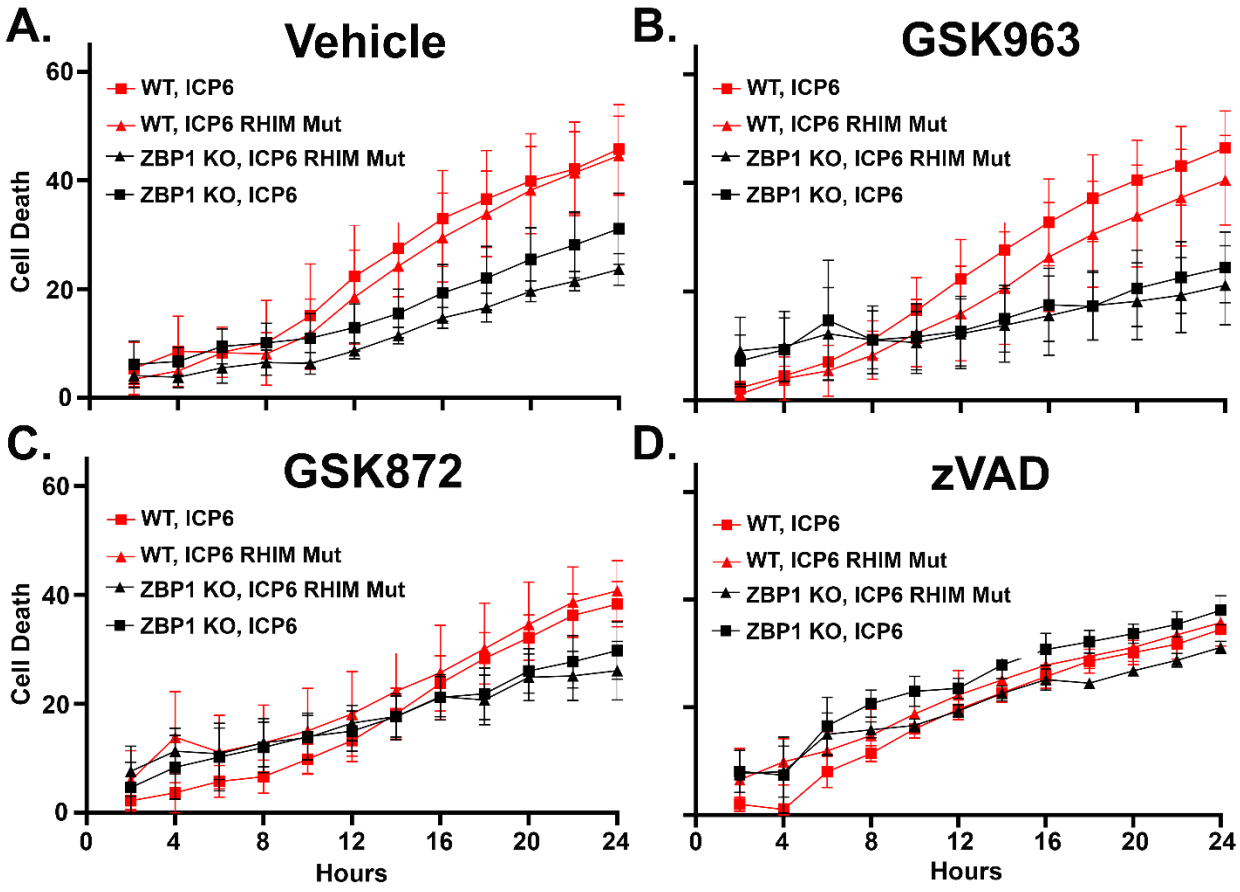

Fig. 14 ZBP1 induces apoptotic but not necroptotic cell death in microglia. WT or ZBP1 KO murine microglia were infected with HSV-1 ICP6 WT (ICP6) or HSV-1 ICP6 RHIM mutant virus. One hour following infection cells were treated with either (A) DMSO (vehicle), (B) GSK963 (RIPK1 inhibitor), (C) GSK 872 (RIPK3 inhibitor), or (D) zVAD (pan caspase inhibitor). Cell viability was measured with RealTime-Glo™ MT Cell Viability Assay starting two hours after infection and was continually measured every two hours for a 24-hour period. Data is shown as +/- SEM for three independent experiments.

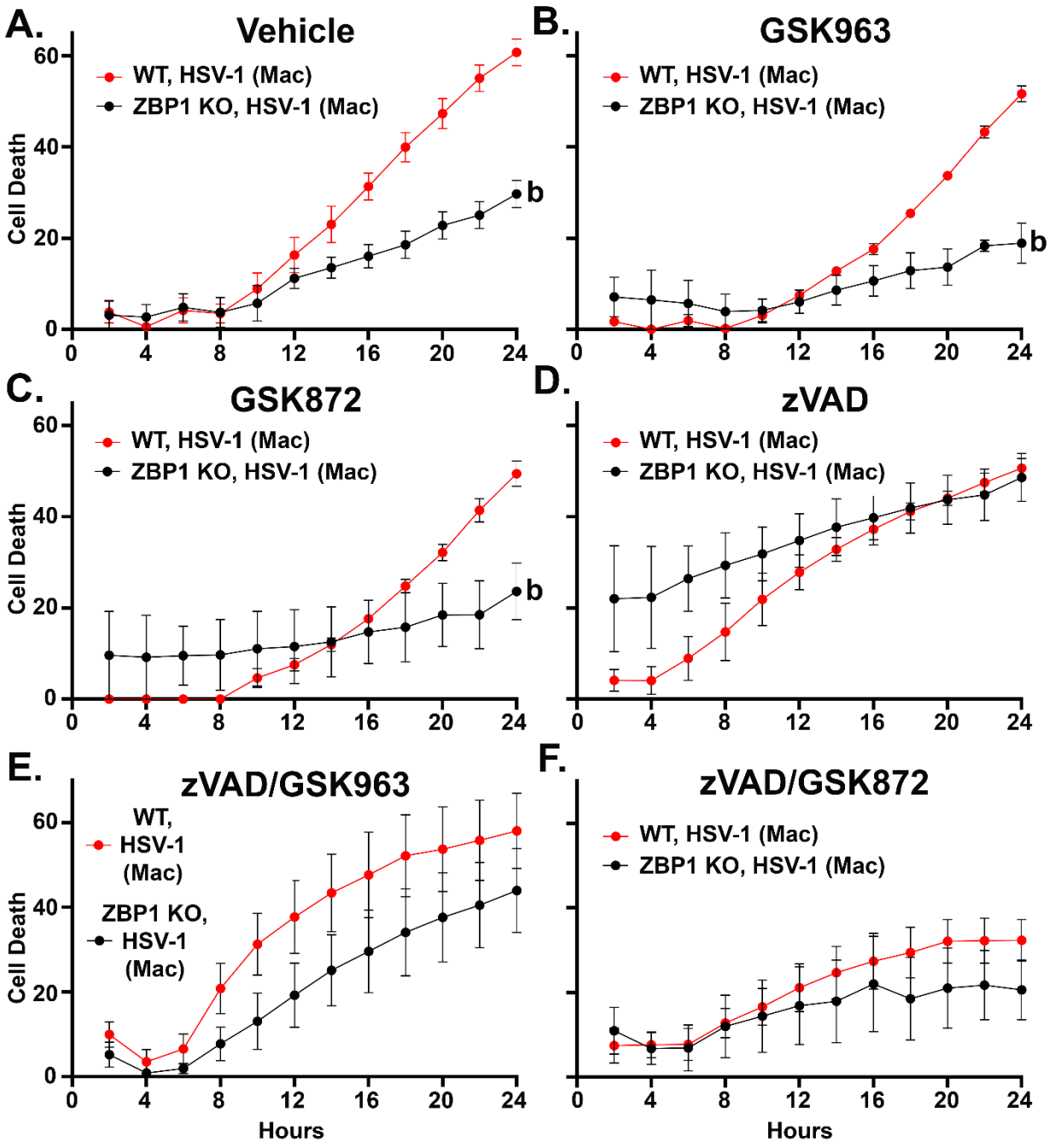

Fig. 15 A neuroinvasive strain of HSV-1 induces ZBP1 mediated necroptosis and apoptosis in astrocytes. WT or ZBP1 KO murine astrocytes were infected with HSV-1 (MacIntyre strain) and one hour following infection cells were treated with (A) DMSO (vehicle), (B) GSK963 (RIPK1 inhibitor), (C) GSK872 (RIPK3 inhibitor), (D) zVAD (pan caspase inhibitor), (E) zVAD and GSK963, or (F) zVAD and GSK872. Cell viability was measured with RealTime-Glo™ MT Cell Viability Assay starting two hours after infection and was continually measured every two hours for a 24-hour period. The letter b indicates a significant difference from similarly treated WT cells. Data is shown as +/- SEM for three independent experiments.

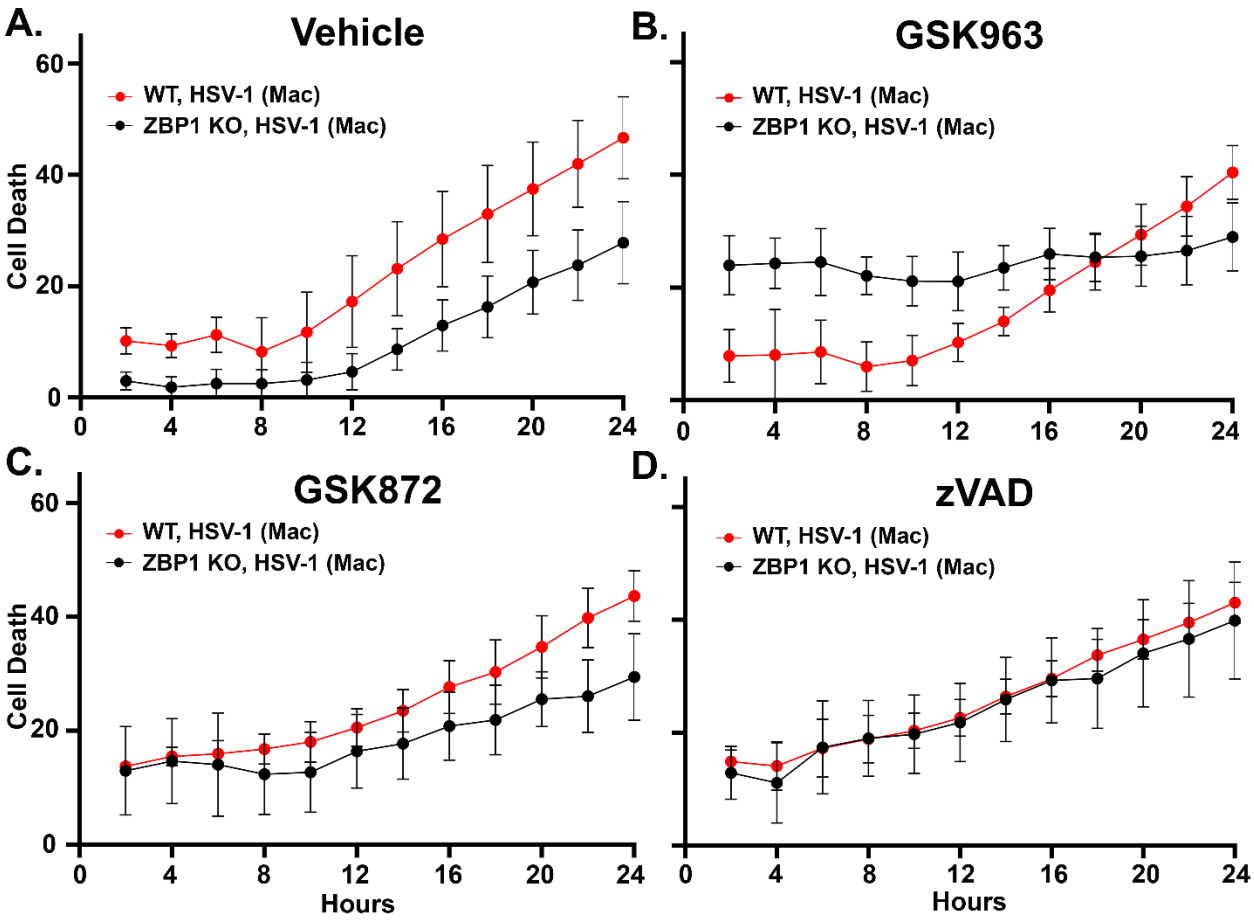

Fig. 16 A neuroinvasive strain of HSV-1 induces ZBP1 mediated apoptotic but not necroptotic cell death in microglia. WT or ZBP1 KO murine microglia were infected with HSV-1 (MacIntyre strain) and one hour following infection cells were treated with either (A) DMSO (vehicle), (B) GSK963 (RIPK1 inhibitor), (C) GSK 872 (RIPK3 inhibitor), or (D) zVAD (pan caspase inhibitor). Cell viability was measured with RealTime-Glo^{TM} MT Cell Viability Assay starting two hours after infection and was continually measured every two hours for a 24-hour period. Data is shown as +/- SEM for three independent experiments.

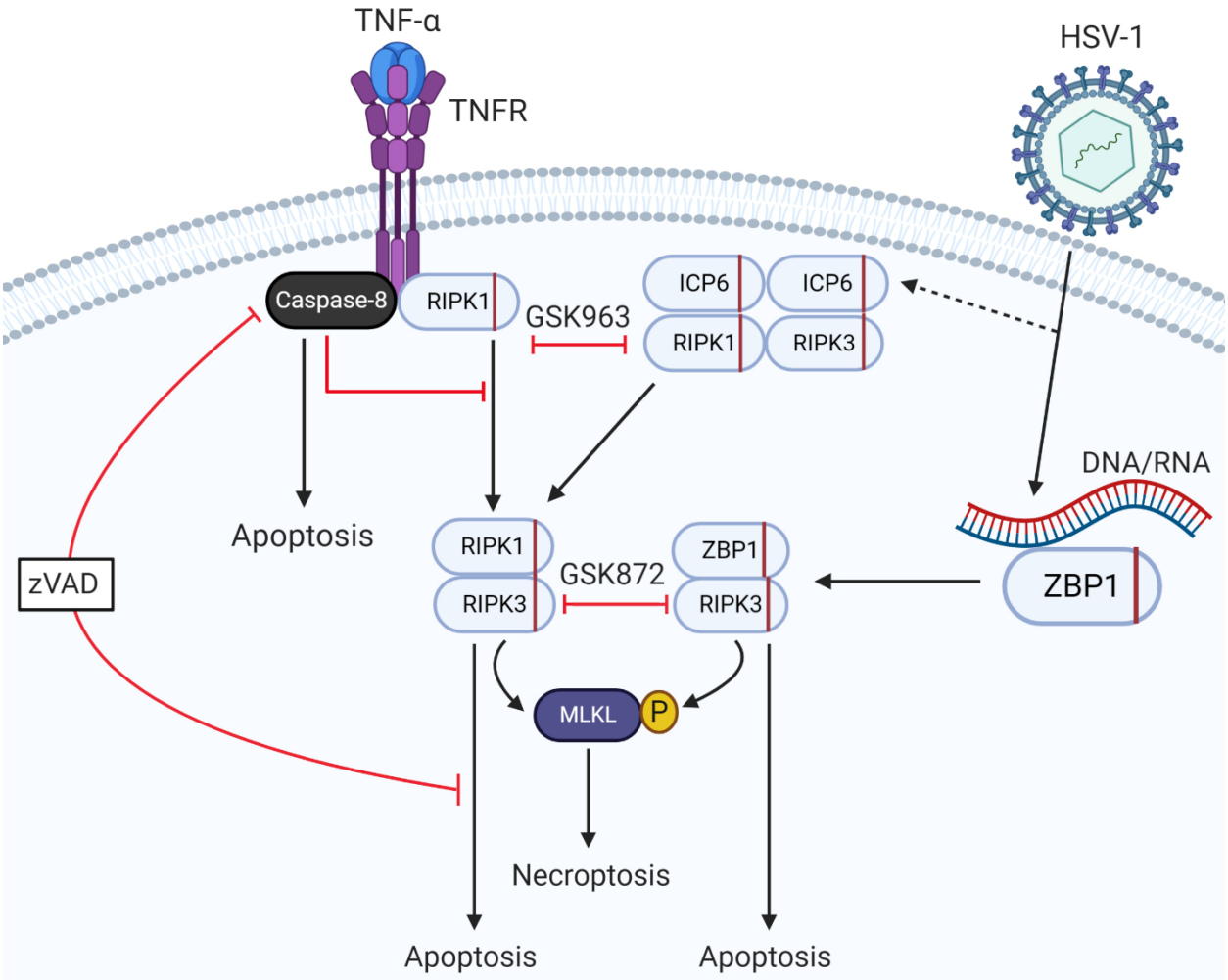

Fig. 17 HSV-1 infection induces cell death pathways in murine astrocytes and microglia. HSV-1 infection results in the activation of apoptosis and necroptosis in murine astrocytes and microglia via several mechanisms. First, the release of genetic material or transcription of viral genes leads to nucleic acid recognition by ZBP1 and its association with RIPK3 through their respective RHIM domains (shown as a red line). Association of ZBP1 with RIPK3 causes the phosphorylation of MLKL and the execution of necroptosis. In addition, ZBP1 association with RIPK3 induces apoptosis through a currently unknown mechanism. Alternatively, the HSV-1 viral protein ICP6 can directly interact with RIPK1 and RIPK3 through their respective RHIM domains. This causes RIPK1 and RIPK3 to associate and leads to the induction of necroptosis and apoptosis in a manner similar to ZBP1/RIPK3. Lastly, viral infection elicits the production of TNF-α that binds to its receptor to initiate cell death pathways. If caspase-8 is present and functional then TNF-α binding results in apoptotic signaling. However, if caspases are inhibited or absent then TNF-α binding to its receptor will result in the association of RIPK1 with RIPK3 and subsequent induction of necroptosis.

CHAPTER 6 SUMMARY AND CONCLUSIONS

6.1 Human astrocytes and microglia express cGAS-STING

It is now appreciated that glial cells are important contributors to innate immune responses during CNS infections. This is accomplished through the expression of a wide range of PRRs capable of recognizing numerous conserved PAMPs. Recently, a class of intracellular PRRs have been identified that are capable of recognizing foreign or mis-localized DNA. Because of their unique location, it is likely these PRRs, collectively known as DNA sensors, are capable of recognizing viral motifs. In agreement with this hypothesis, we have shown that human glial cells can functionally respond to exogenous DNA with the activation of IRF3 and expression of mRNA encoding IFN- β. Importantly, we have provided the first demonstration that the DNA sensor cGAS and its critical downstream adaptor molecule STING are constitutively expressed in human astrocyte and microglial-like cells, as well as in authentic primary astrocytes and microglia. Furthermore, we have determined that such robust expression could not be further elevated by BDNA transfection.

Interestingly, while we observed an increase in IFN-β expression in primary human astrocytes following BDNA transfection, we did not see an induction of IFN-β mRNA expression in the astrocytic cell line U87-MG. This is in spite of the ability of U87-MG cells to phosphorylate the upstream transcription factor IRF3 in response to exogenous DNA administration. It is possible that this observed difference is due to the inherent limitations of an immortalized cell line, such as variations in gene expression when compared to primary cells. This is why, when able, we confirm such findings in primary cells. It is also important to note, that while glial cells can respond to foreign DNA with the induction of IFN-β expression, it is possible that this ligand was perceived via the expression of additional DNA sensors. As such,

further study is required to determine whether these responses are initiated via the expression of cGAS-STING in human glia.

6.2 The intracellular DNA sensors cGAS and IFI16 do not mediate effective antiviral responses against HSV-1 in human microglial cells

In these studies, we confirmed that our previous observations regarding the activation of IRF3 and induction of IFN-β expression following BDNA transfection were due, at least in part, to cGAS expression. Using CRISPR/Cas9 techniques we generated heterozygous knockouts of cGAS (cGAS+/-) in the microglial cell line hμglia. Importantly, when challenged with either BDNA or YDNA, these cGAS+/- microglia demonstrated a significant reduction in the levels of phosphorylated IRF3 and a corresponding decrease in the production of IFN-β. While this demonstrates that cGAS is functionally expressed, a reduction in cGAS protein expression had little effect on the number of released viral particles from HSV-1 infected cells. In agreement with this observation, we found that HSV-1 infection resulted in minimal production of IFN-β protein and did not significantly increase the expression of ISGs. Interestingly, despite the lack of increased ISG expression, cGAS+/- microglia demonstrated lower levels of ISG mRNA at rest and following infection, supporting an antiviral role for this sensor.

In addition to confirming cGAS functionality in microglia, these studies provide the first demonstration of IFI16 protein expression in human glia. Surprisingly, despite the documented ability of IFI16 to induce IFN expression in response to foreign DNA in murine astrocytes and microglia (Cox et al., 2015), we observed no such responses following siRNA mediated IFI16 knockdown in human microglia. This finding cannot be explained by compensation via cGAS, as knockdown of IFI16 in cGAS+/- microglia did not further decrease IFN-β release compared to cGAS+/- microglia alone. Additionally, in contrast to peripheral cell types, knockdown of IFI16 had no effect on the number of viral particles released from HSV-1 infected microglia. Again, this observation was not due to a redundancy in DNA sensor function as IFI16 knockdown in

cGAS+/- microglia also did not reduce the number of viral particles released. Lastly, to begin to determine the mechanisms underlying the resistance of HSV-1 to cGAS and/or IFI16 mediated antiviral responses, we assessed the effect of this virus on expression of each sensor. We found that HSV-1 infection significantly reduced the expression of both cGAS and IFI16 protein in human glia. As such, this viral immune evasion mechanism may be responsible for the lack of effect observed during knockout of cGAS or knockdown of IFI16. Because both cGAS and IFI16 have demonstrated antiviral activity in non-CNS cell types, therapeutics targeting the stability of cGAS and/or IFI16 may have potential as therapeutics during CNS infections such as HSV-1.

6.3 ZBP1 mediates necroptotic and apoptotic cell death pathways in glia

In the present study, we have demonstrated that ZBP1 is a restriction factor for HSV-1 in murine glia. We have shown that loss of ZBP1 results in an increase in the number of viral particles released from infected cells and that this is not due to a change in the expression of the well-known antiviral mediator, IFN-β. Instead, we found that loss of ZBP1 results in a significant decrease in the rate of cell death for both astrocytes and microglia during infection with HSV-1. Furthermore, we demonstrated that astrocytes deficient in the expression of ZBP1 have significantly lower levels of cell death at 24 hours post-infection. These findings are consistent with those completed in non-CNS cell types, which demonstrate a role for ZBP1 in the initiation of cell death pathways such as necroptosis (Kuriakose et al., 2016; Thapa et al., 2016; Guo et al., 2018; Jiao et al., 2020).

To determine if the mechanisms underlying the observed differences in cell death were due to necroptosis we investigated the activation of the necroptotic marker, MLKL. Also, because the HSV-1 protein ICP6 can independently activate RIPK1 dependent necroptosis via its RHIM domain, we completed experiments using a strain of HSV-1 with mutations in the RHIM domain (ICP6 RHIM Mut) and compared this to the parental F strain (ICP6). We confirmed that the ICP6 strain was able to induce ZBP1 independent necroptosis while induction by the ICP6 RHIM Mut strain was significantly attenuated. Furthermore, inhibition of RIPK1 blocked the activation of MLKL in ZBP1 KO astrocytes during infection with either strain. Interestingly, there was no measurable difference in the percentage of cell death at 24hrs between viral strains in ZBP1 KO astrocytes, despite a significant reduction observed between WT and ZBP1 KO astrocytes. Similar trends were also observed between WT and ZBP1 KO microglia. Together, this suggests that the reduction in cell death was not dependent on necroptosis alone.

To confirm that the differences in cell death were not due to necroptosis alone we employed inhibitors targeting either RIPK3, a key upstream mediator of necroptosis, or activators of apoptosis. Surprisingly, inhibition of either pathway alone did not result in a reduction in the percentage of cell death in WT astrocytes. In fact, treatment with a pan caspase inhibitor increased the percentage of cell death in ZBP1 KO astrocytes to levels similar to those seen in WT astrocytes. Again, similar trends were observed between WT and ZBP1 KO microglia. Interestingly, caspase inhibition appears to permit virally induced necroptosis that is independent of ZBP1, as treatment with a pan caspase inhibitor results in the activation of MLKL in ZBP1 KO astrocytes. Furthermore, this activation could be reduced by inhibiting RIPK1 activity, which also decreased the cell death for ZBP1 KO astrocytes that resulted from caspase inhibition. While inhibition of either pathway alone could not reduce cell death in WT astrocytes during infection, it could be reduced by simultaneous inhibition of both apoptosis and necroptosis. This suggests that ZBP1 contributes to both cell death pathways in murine astrocytes. These findings were also confirmed during infection with a neuroinvasive strain of HSV-1. Together, these data suggest ZBP1 may be a potential target during CNS infections with HSV. However, further investigation is needed to determine if activation of ZBP1 serves as a protective host response or, instead, contributes to pathophysiology during HSV-1 infection.

6.4 Importance and future directions

Viral CNS infections can lead to life threatening encephalitis and long-term neurological deficits in survivors (Xu et al., 2006). While it is known that glial cells can produce pro-inflammatory and antiviral mediators during infection, how glia perceive threats to initiate such responses are poorly understood. Because of this, determining the mechanisms that contribute to damaging inflammation or protective antiviral responses by glia are critical for the development of therapeutics to limit pathophysiology during CNS infections. In the present study, we begin to describe the mechanisms by which glia can sense neurotropic DNA viruses and highlight potential targets for therapeutic intervention.

The majority of encephalitis cases (48.2%) are attributed to viral infection (George et al., 2014) and, of these, most are caused by the neurotropic DNA virus HSV-1 (Xu et al., 2006). Current standard of care for HSE calls for treatment with antiviral drugs, however resistance to these medications have developed (Bacon et al., 2003; Venkatesan and Geocadin, 2014). Our results from the present study indicate that human glial cells express the DNA sensors IFI16 and cGAS, as well as the downstream adaptor molecule STING. Furthermore, we have determined that cGAS is functional in human glia and can initiate antiviral immune responses following challenge with a viral DNA mimetic. Additionally, evidence in non-CNS cell types indicates that both IFI16 and cGAS are capable of sensing HSV-1 and limiting viral replication (Thompson et al., 2014; Orzalli et al., 2015; Diner et al., 2016; Almine et al., 2017; Jønsson et al., 2017; Merkl et al., 2018). However, we found that in human microglia cGAS and IFI16 were not capable of limiting viral particle release. The discrepancy in these findings may be due to the ability of HSV-1 to downregulate the expression of both IFI16 and cGAS in human microglia. As such, therapeutics targeting the stability of these molecules may limit or contribute to the resolution of

CNS infections with HSV-1. Further research will need to be conducted to determine the validity of such an approach.

In addition to IFI16 and cGAS, we also investigated the role of ZBP1 during HSV-1 infection in glia. We found that ZBP1 was capable of restricting HSV-1 viral particle release in murine astrocytes and demonstrated a trend for restriction in microglia. Interestingly, this restriction did not appear to be dependent on the production of IFN-β, as both WT and ZBP1 KO glia demonstrated similarly low levels of this cytokine. Instead, we found that ZBP1 contributed to the activation of both necroptosis and apoptosis in astrocytes and appears to have a similar function in microglia. The induction of cell death pathways has the potential to limit viral infection by preventing the dissemination of virus (Nailwal and Chan, 2019). However, future studies will need to confirm whether activation of necroptosis and/or apoptosis contribute to HSV-1 restriction in glia. Furthermore, the induction of inflammatory death pathways, such as necroptosis, may exacerbate pathology in the context of CNS infections (Nailwal and Chan, 2019). In either case, it is tempting to speculate that ZBP1 may be an ideal candidate for targeted therapeutics against HSE, either blocking this molecule if proven detrimental or promoting responses if beneficial. Because of this, it will be important to determine what type of effect the induction of this pathway has on neighboring cells, especially neurons. It is also possible that with therapeutic intervention we may be able to skew the activation of different cell death pathways during infection. For instance, we have demonstrated that blocking RIPK3 kinase activity prevents the phosphorylation of MLKL while still allowing for reduced cell death in ZBP1 KO astrocytes via apoptosis. Thus, simultaneous activation of ZBP1 and inhibition of RIPK3 would skew the response towards apoptosis. Similarly, we have shown that inhibiting caspase activity results in the activation of necroptosis, although in a ZBP1 independent manner.

Together, these data indicate that it is possible to target specific cell death pathways and this offers potential as an additional tool to combat virally induced encephalitis.

6.5 TABLES AND FIGURES

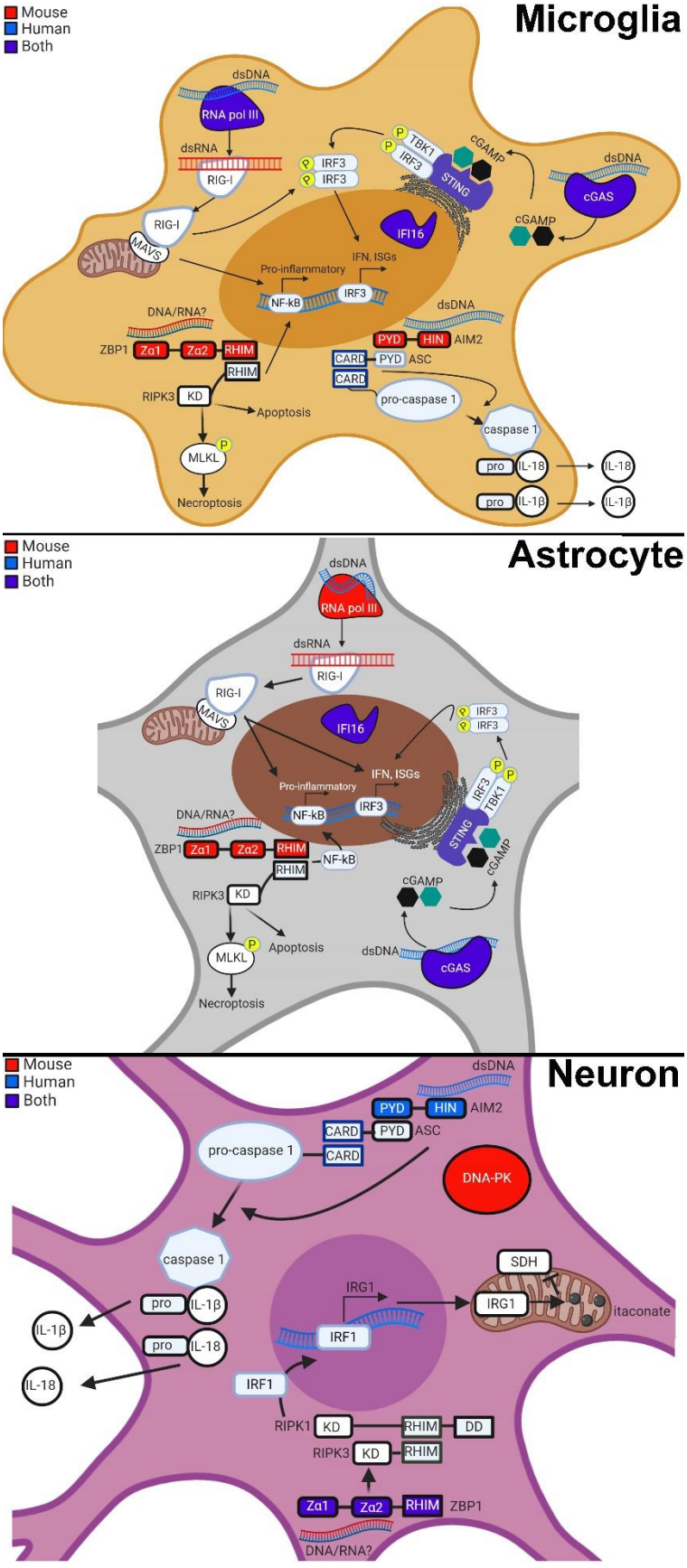

FIGURE 18: Intracellular DNA sensors in resident CNS cell types. Intracellular nucleic acid sensing by DNA sensors in microglia (A), astrocytes (B), and neurons (C). AIM2 sensing of dsDNA leads to the recruitment of apoptosis-associated speck-like protein containing a CARD (ASC) that then cleaves pro-caspase 1. Active caspase 1 then cleaves the precursor forms of IL18 and IL-1β, causing their maturation and release from the cell. ZBP1 sensing of either dsDNA or RNA causes it to associate with RIPK3, activate the transcription factor NF-κB, and phosphorylate mixed lineage kinase domain-like protein (MLKL) in microglia and astrocytes. This results in pro-inflammatory cytokine expression, execution of necroptosis, and induction of apoptosis. In neurons, ZBP1 sensing results in the activation of IRF1, expression of IRG1, production of itaconate, and a reduction in succinate dehydrogenase (SDH) activity. Sensing of dsDNA by cGAS leads to the production of cGAMP, which binds to and activates STING causing the phosphorylation and translocation of interferon regulatory factor 3 (IRF3) to the nucleus. This results in the expression of IFN and ISGs in microglia and astrocytes. RNA pol III senses dsDNA and converts it into dsRNA that can then be sensed by RIG-I in microglia and astrocytes. RIG-I sensing of dsRNA causes it to associate with mitochondrial antiviral-signaling protein (MAVS) leading to activation and translocation of IRF3 and NF-κB, resulting in the expression of IFN, ISGs, and pro-inflammatory cytokines.

Table 4: Expression and antiviral activity of intracellular DNA sensors in CNS cell types

Sensor	CNS cell type	Antiviral activity	References
ZBP1	Primary mouse whole brain tissue, cortical neurons, microglia, and astrocytes	Neuronal immunometabolism regulation, antiviral and proinflammatory cytokine production	Furr et. al, 2011, Daniels et. al, 2019, Rothan et. al, 2019
cGAS	Primary murine neurons, astrocytes, and microglia Primary human astrocytes and microglia Human cell lines U87-MG and hμglia	IFN and ISG expression	Cox et. al, 2015, Reinert et. al, 2016, Jeffries and Marriott. 2017
IFI16	Primary mouse epithelial cells Primary human astrocytes Human cell lines, corneal epithelial, astrocytes, microglia	Inflammasome activation	Conrady et. al, 2012, Coulon et. al, 2019, Jeffries et. al, 2020
AIM2	Primary mouse astrocytes and microglia Human cell line, SK-N-SH	Inflammasome activation	Cox et. al, 2015, Yogarajah et. al, 2017. Song et. al, 2019
DDX41	Zebrafish whole brain	IFN expression	Ma et. al, 2018
DNA PK	Primary mouse cerebral cortex and neurons	Unknown	Vemuri et. al, 2001, Chechlacz et. al, 2001
RNA pol III	Primary mouse astrocytes and microglia Mouse cell line, EOC13.31 Human cell line, hμglia	IFN expression and NF-κB activation via RIG-I	Crill et. al, 2015, Johnson et. al, 2020

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APPENDIX: Publications

Martins, A.N., Jeffries, A.M., and Marriott, I. (2016). Human astrocytes and microglia-like cells constitutively express the novel cytosolic viral DNA sensor cGAS. *J. Immunol.* 196:217.22 Abstract

Jeffries, A.M., and Marriott, I. (2017). Human astrocytes and microglia-like cells express cGAS-STING viral sensing components. *J. Immunol.* 198:129.6.6 Abstract

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