ROLE OF THE DNA SENSORS cGAS AND ZBP1 IN DNA DAMAGE AND VIRAL INFECTION

by

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ABSTRACT

ALEXANDER JACOB SUPTELA. Role of the DNA Sensors cGAS and ZBP1 in DNA Damage and Viral Infection (Under the direction of DR. IAN MARRIOTT)

Genomic instability is a key driving force for the development and progression of many neurodegenerative diseases and central nervous system (CNS) cancers. The initiation of DNA damage responses (DDRs) is a critical step in maintaining genomic integrity and preventing such diseases. However, the absence of these responses or their inability to repair genomic or mitochondrial DNA damage resulting from insults, including ionizing radiation or oxidative stress, can lead to an accumulation of self-DNA in the cytoplasm. Resident CNS cells, such as astrocytes and microglia, are known to produce critical immune mediators following CNS infection due to the recognition of pathogen and damage-associated molecular patterns by specialized pattern recognition receptors (PRRs). Recently, multiple intracellular PRRs, including cyclic GMP-AMP synthase (cGAS), interferon gamma-inducible 16 (IFI16), absent in melanoma 2 (AIM2), and Z-DNA binding protein 1 (ZBP1), have been identified as cytosolic DNA sensors and play critical roles in glial immune responses to infectious agents. Intriguingly, some nucleic acid sensors have recently been shown to recognize endogenous DNA resulting from DNA damage and subsequently trigger immune responses in peripheral cell types, however, in the CNS, our understanding of these responses are limited. In this dissertation, we describe the functionality of the DNA sensors ZBP1, with regards to herpes simplex virus type-1 (HSV-1) infection in murine astrocytes and microglia, and cGAS with regards to DNA damage in human microglia.

Our laboratory has previously reported that ZBP1 contributes to neurotoxic immune responses to HSV-1 infection, however, the exact mechanisms behind such responses were unknown. Here, we demonstrate that ZBP1 acts as a restriction factor for HSV-1 in murine astrocytes and microglia, and that it does so via the induction of necroptotic and apoptotic cell death pathways. While it remains to be seen whether ZBP1-mediated activation of cell death in glia contributes significantly to host protection or, rather, exacerbates HSV-1 encephalitis pathology, the identification of such a role in resident CNS cells may represent a novel target for therapeutic intervention to reduce HSV encephalitis-associated morbidity and mortality.

Additionally, we report that cGAS serves as a mediator of proinflammatory immune responses following DNA damage in human microglia. We show that exposure to genotoxic insults such as ionizing radiation (IR) and hydrogen peroxide (H2O2) exposure not only elicit DNA damage, but also elicit production and secretion of proinflammatory cytokines in this glia. Furthermore, we show that cGAS deficiency results in markedly reduced levels of these cytokines following similar exposures. Surprisingly, we found that while cGAS deficient cells released lower levels of proinflammatory cytokines, they simultaneously expressed higher levels of DNA damage. Together, these data indicate a role for cGAS as a mediator of potentially neurotoxic inflammation following DNA damage. The mechanisms by which these responses occur require further study, as it is still unclear whether they are beneficial or detrimental. However, they may be targetable to augment glial responses that protect against tumorigenesis, or prevent responses that could initiate or exacerbate damaging neuroinflammation.

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AD	Alzheimer's disease
AGS	Aicardi-Goutières syndrome
AIM2	absent in melanoma 2
AKT	protein kinase B
ANOVA	analysis of variance
ASC	apoptosis-associated speck-like protein containing a CARD
A-T	ataxia telangiectasia
ATCC	American type tissue culture collection
ATM	ataxia-telangiectasia mutated
ATP	adenosine tri-phosphate
Aβ	amyloid beta
BBB	blood brain barrier
B-DNA	B-form DNA
BSA	bovine serum albumin
CARD	caspase activation and recruitment domain
Cas9	CRISPR-associated protein 9
CAT	catalase
CCL3	chemokine (C-C motif) ligand 3

CCL5	chemokine (C-C motif) ligand 5
cGAMP	2’3’ cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cyclic GMP-AMP synthase
CNS	central nervous system
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXCL10	chemokine (C-X-C motif) ligand 10
CXCL2	chemokine (C-X-C motif) ligand 2
DAI	DNA-dependent activator of IRFs
DAMP	damage associated molecular pattern
DDR	DNA damage response
DDX41	DEAD-box helicase
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	eimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
DNASE2	deoxyribonuclease 2
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA

EAE	experimental autoimmune encephalitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FVB-NJ	friend virus B-NIH Jackson
GOX	glucose oxidase
GSDMD	gasdermin D
HD	Huntington's disease
HRP	horseradish peroxidase
HSV-1	herpes simplex virus type 1
H_2O_2	hydrogen peroxide
IFI16	interferon gamma-inducible 16
IFI204	interferon-gamma inducible 204
IFN	interferon
IFN-β	interferon beta
IKKε	inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL-18	interleukin 18
IL-1β	interleukin 1 beta

IL-6	interleukin 6
IL-8	interleukin 8
IR	ionizing radiation
IRF3	interferon regulatory factor 3
IRF7	interferon regulatory factor 7
LGP2	laboratory of genetics and physiology 2
MAVS	mitochondrial antiviral-signaling
MDA5	melanoma differentiation-associated gene 5
MLKL	mixed lineage kinase domain-like
MOI	multiplicity of infection
MRE11	meiotic recombination 11 homolog A
MS	multiple sclerosis
mtDNA	mitochondrial DNA
NF-κB	nuclear factor kappa B
NIH	National Institute of Health
NLR	NOD-like receptor
NOD	nucleotide-binding and oligomerization domain
PAMP	pathogen associated molecular pattern

PARP1	poly(ADP-ribose) polymerase 1
PBS	phosphate buffered saline
PD	Parkinson’s disease
PFF	pre-formed fibrils
PRR	pattern recognition receptor
PYHIN	pyrin and HIN domain
RHIM	RIP homotypic interaction motif
RIG-I	retinoic acid-inducible gene I
RIP	receptor interacting serine-threonine protein
RIPK1	RIP kinase 1
RIPK3	RIP kinase 3
RLR	RIG-I-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
RP3	RNA polymerase III
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	silencing RNA

STAT1	signal transducer and activator of transcription 1
STING	stimulator of interferon genes
TANK	TRAF family member-associated nuclear factor-kappa-B activator
TBK1	TANK-binding kinase
TLR	toll-like receptor
TLR9	toll-like receptor 9
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor 2
TREX1	three prime repair exonuclease 1
ZBP1	Z-DNA binding protein 1
Z-DNA	Z-form DNA
α-syn	alpha synuclein
γH2AX	gamma-H2A histone family member X

CHAPTER 1: Introduction

1.1 Introduction

In 2022, there were an estimated 1.9 million new cancer cases and over 600,000 deaths due to these diseases in the United States (National Cancer Institute 2022a). Of these, approximately 25,000 were associated with the brain and the central nervous system (CNS), and these have a poor prognosis with only a 32.5% 5-year survival rate (National Cancer Institute 2022b). Additionally, lethal cases of neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) are becoming increasingly prevalent, and this is a serious concern as an estimated 6.5 million Americans were reported to live with AD in 2022 and 1 million having PD in 2019 (Rong et al. 2021; Centers for Disease Control and Prevention 2022). A key driving force for the development of these cancers and neurodegenerative diseases is genomic instability arising from excessive DNA damage and/or DNA repair mechanism dysfunction (Coppedè and Migliore 2015; Zhu et al. 2019).

High fidelity DNA repair is required to maintain genomic integrity. Breaks in DNA are a relatively common occurrence and can occur naturally up to tens of thousands of times per day, per cell. However, the number of such breaks can be drastically increased by exogenous insults such as ionizing radiation (IR) and oxidative stress. Exposure to high levels of IR can be tremendously detrimental and affects nearly every macromolecule in a cell. These effects can either occur directly or indirectly. For example, IR alone directly disrupts the structure of DNA, creating breaks in its molecular backbone (Reisz et al. 2014). Alternatively, IR can elicit radiolysis of cellular water whereby the chemical bonds of water molecules are broken down resulting in the generation of reactive oxygen species (ROS) including hydroxyl radicals, ionized water, superoxide anions, and hydrogen peroxide (Reisz et al. 2014). While ROS play essential

roles in many cellular processes, excessive levels of ROS disrupt redox homeostasis and can induce DNA lesions (Saikolappan et al. 2019). DNA lesions/breaks initiate DNA damage responses (DDR), a collection of mechanisms that ensure efficient DNA repair and maintain genomic integrity (Giglia-Mari et al. 2011; Abuetabh et al. 2022). However, DNA repair is not infallible. If cells are permitted to replicate with unrepaired or incompletely repaired DNA, an accumulation of mutations and DNA damage may lead to cancer development, neurodegenerative disorders, and other age-related diseases. Furthermore, DNA damage has recently been implicated in the generation of detrimental inflammation, and this effect is often associated with the presence of self-DNA in the cytoplasm (Horn and Triantafyllopoulou 2018; Li and Chen 2018a; Maekawa et al. 2019; Taffoni et al. 2021).

DNA damage due to exposure to IR, oxidative stress, or even chemotherapy, results in cytosolic DNA accumulation, often in the form of micronuclei (Krupina et al. 2021). Micronuclei are small nuclei-like structures containing lagging or damaged chromosome fragments that continue into the interphase following completion of mitosis or meiosis (Krupina et al. 2021) The nuclear envelopes surrounding micronuclei are typically defective and prone to rupture, after which, their DNA cargo is liberated into the cytosol. Additionally, micronuclei have been described as a source of complex genome rearrangements, including one-off catastrophic rearrangement events known as chromothripsis (Zhang et al. 2015). Furthermore, the presence of micronuclei is associated with many autoimmune diseases (Ballardin et al. 2004; Zúñiga-González et al. 2007; Donmez-Altuntas et al. 2007; Karaman et al. 2011; Silva et al. 2013), neurodegenerative diseases (Migliore et al. 2001; Petrozzi et al. 2001; Sathasivam et al. 2001; Trippi et al. 2001; Sprung et al. 2005; Wang et al. 2009), and aggressive cancers (Cortés-Ciriano et al. 2020) in affected tissues. Indeed, the presence of micronuclei has historically been

used as a means to assess the genotoxicity of chemicals and mutagens via the cytokinesis-block micronucleus assay, and their contribution to the initiation of innate immune responses is now becoming apparent (Fenech 2007).

While micronuclei are likely to be a major source of cytoplasmic genomic self-DNA, mitochondria may also serve as a source of DNA in the cytosol (Miller et al. 2021). Mitochondria primarily function to produce the ATP necessary for normal cell activity via oxidative phosphorylation. Outside of oxidative phosphorylation, mitochondria also perform many other metabolic and non-metabolic roles ranging from the regulation of apoptosis to the generation of ROS that are necessary for maintaining redox homeostasis (Andreyev and Kushnareva 2005; Shadel and Horvath 2015). What makes mitochondria truly unique, however, is that they contain their own circular genome due to their endosymbiotic origin (Roger et al. 2017). The various means by which mitochondrial contents, including mitochondrial DNA (mtDNA), are released have been extensively reviewed elsewhere (Riley and Tait 2020; De Gaetano et al. 2021; Wu et al. 2021; Moya et al. 2021; Zhao et al. 2021b; Carvalho et al. 2022), but generally involves passive/accidental release due to mitochondrial stress and dysfunction, and cell death pathways including necrosis and apoptosis (Wu et al. 2021).

Since DNA is normally sequestered in the nucleus and mitochondria, its presence in the cytosol can function as a damage associated molecular pattern (DAMP) and serve to trigger inflammatory innate immune responses. In the CNS, such responses to both endogenous and exogenous insults must be tightly regulated to avoid damaging, or even lethal, inflammation. While the recruitment of peripheral leukocytes to the CNS and their subsequent activation are important in the development of immune responses in disease states, it is now recognized that

resident glial cells, such as microglia and astrocytes, play a critical role in the initiation of detrimental neuroinflammation.

1.2 Glial cells play a critical role in the initiation and progression of immune responses in the CNS

In the healthy brain, microglia and astrocytes are essential for homeostasis. Microglia perform critical housekeeping functions such as synaptic remodeling and pruning (Paolicelli et al. 2011; Kettenmann et al. 2013), and removal of cellular debris and dead or dying cells (Tremblay et al. 2011), which is necessary for creating a regenerative environment (Neumann et al. 2008). Astrocytes are the most abundant glial cell in the brain and have a crucial role in synaptogenesis, synaptic transmission, and neurotransmitter recycling, and for the maintenance of the blood brain barrier (BBB) (Alvarez et al. 2011; Allen et al. 2012; Tsai et al. 2012; Chung et al. 2013; Molofsky et al. 2014). Importantly, it is now apparent that microglia and astrocytes both serve as sentinel cells that initiate and exacerbate immune responses associated with CNS pathology (Jack et al. 2005; Carpentier et al. 2008). Given their wide distribution throughout the CNS, they are ideally positioned to confront and respond to trauma or invading pathogens. In disease states, microglia and astrocytes are activated and produce a wide array of potent proinflammatory mediators, such as IL-6, IL-1β, and TNF, as well as chemokines that promote the recruitment of peripheral leukocytes across the BBB that can further contribute to potentially damaging neuroinflammation (Bsibsi et al. 2002; Heneka and O'Banion 2007; Griffiths et al. 2009; Cribbs et al. 2012). The initiation of microglial and astrocytic responses is now recognized to be mediated by multiple families of cell surface, endosomal, and cytosolic PRRs that are triggered by DAMPs and pathogen-associated molecular patterns (PAMPs). This subsequently results in the activation of transcription factors that precipitate the production of cell surface and secreted immune mediators.

Of these PRRs, perhaps the best studied are the cell surface and endosomal Toll-like receptors (TLRs) and the cytosolic nucleotide-binding and oligomerization domain-containing (NOD)-like receptors (NLRs), and these sensors have been exhaustively studied for their roles in antimicrobial and antiviral responses (Zhong et al. 2013; Platnich and Muruve 2019; Asami and Shimizu 2021; Johnston et al. 2021; Kienes et al. 2021; Li et al. 2021; Mokhtari et al. 2021; Danis and Mellett 2021; Wicherska-Pawłowska et al. 2021; Lind et al. 2022). The TLR and NLR families consist of at least 10 and 22 members in mammals, respectively, and these receptors are widely expressed throughout the body on/in peripheral leukocytes and non-leukocytic cell types (Takeda et al. 2002; Fritz et al. 2006; Ishii and Akira 2006). Importantly, we, and others, have demonstrated the constitutive and/or inducible expression of TLRs (Bsibsi et al. 2002; Rasley et al. 2002; Bowman et al. 2003; Olson and Miller 2004; Jack et al. 2005; Carpentier et al. 2008) and the NLRs, NOD1 and NOD2 (Sterka and Marriott 2006; Sterka et al. 2006; Chauhan et al. 2009), on/in both microglia and astrocytes.

These TLRs and NLRs can detect a variety of bacterial or viral extra- and intracellular PAMPs and DAMPs to activate downstream signaling cascades and initiate proinflammatory and/or antiviral activity by glial cells. However, NOD1/2 appears to be limited to the detection of bacterial cell wall components (Sterka and Marriott 2006). Furthermore, while several TLRs are able to detect and respond to nucleic acids in endosomal compartments (Alexopoulou et al. 2001; Latz et al. 2004; Ishii and Akira 2006), they are not well suited to detect compromised cytosolic sterility or damage. Rather, it now appears that cells utilize discrete cytosolic PRR families that are capable of responding to the presence of foreign and/or self-nucleic acids in the cytoplasm.

1.3 Detection of cytosolic nucleic acids by glial cells

PRR families, in addition to TLRs and NLRs, have recently been identified that serve as cytosolic sensors for foreign or altered self-nucleic acids (Roers et al. 2016). These include the retinoic acid-inducible gene I (RIG-I)-like family of receptors (RLRs) that can detect bacterial and viral nucleic acids and, more specifically, dsRNA (Yoneyama and Fujita 2007; Loo and Gale 2011; Rehwinkel and Gack 2020). There are currently three known RLRs: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). All three RLRs share a common central helicase domain and carboxy-terminal domain, although only RIG-I and MDA5 possess two caspase activation and recruitment domains (CARDs) that are required for downstream signal transduction (Yoneyama et al. 2005). In contrast, LGP2 lacks a CARD domain and, instead, negatively regulates RIG-I and MDA5 activity (Rothenfusser et al. 2005; Yoneyama et al. 2005). Upon RNA binding, RIG-I and MDA5 interact with the CARD domain found on mitochondrial antiviral-signaling (MAVS), a mitochondria-localized adaptor protein, which subsequently activates TRAF family member-associated nuclear factor-kappa-B activator (TANK)-binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε). TBK1 and IKKε, in turn, activate the transcription factors interferon regulatory factors 3 and 7 (IRF3 and IRF7), which finally induce the transcription of type-I interferons (IFNs) among other antiviral mediators. Importantly, we have shown that RIG-I and MDA5 are constitutively expressed in microglia and astrocytes and that such expression is upregulated following viral infection (Furr et al. 2008). Furthermore, we have recently demonstrated that RIG-I not only recognizes viral dsRNA in glia, but is also able to respond to bacterial dsRNA (Johnson et al. 2020).

Surprisingly, RIG-I may elicit immune responses to cytosolic DNA in addition to RNA, albeit in an indirect manner mediated by the actions of RNA polymerase III (RP3) (Ablasser et al. 2009; Chiu et al. 2009; Schlee and Hartmann 2010; Zevini et al. 2017; Zhao and Karijolich 2019; Johnson et al. 2020), wherein RP3 reverse transcribes cytosolic dsDNA into a 5' triphosphate-containing dsRNA ligand that then can subsequently be detected by RIG-I. Such a mechanism might explain the earlier, and perhaps erroneous, description of RP3 as a DNA sensor (Ablasser et al. 2009; Chiu et al. 2009).

However, it is now apparent that cells, including glia, possess a number of molecules that specifically serve as cytosolic DNA sensing molecules to initiate responses to foreign or self-DNA. The first of such molecules to be discovered was DNA sensor Z-DNA binding protein (ZBP1; previously known as DNA-dependent activator of IRFs (DAI)) in 2007 (Takaoka et al. 2007). Since then, our knowledge of cytosolic DNA sensing PRRs has expanded to include proteins such as cyclic GMP-AMP synthase (cGAS) (Sun et al. 2013), interferon gamma-inducible 16 (IFI16) (Unterholzner et al. 2010), and absent in melanoma 2 (AIM2) (Fernandes-Alnemri et al. 2009; Hornung et al. 2009; Roberts et al. 2009). Importantly, we, and others, have described the expression and activity of cGAS (Cox et al. 2015; Jeffries and Marriott 2017), IFI16 (Conrady et al. 2012; Coulon et al. 2019; Jeffries et al. 2020), ZBP1 (Furr et al. 2011; Daniels et al. 2019; Rothan et al. 2019; Jeffries et al. 2022), and AIM2 (Cox et al. 2015; Yogarajah et al. 2017; Song et al. 2019), in resident CNS cells including human and murine microglia and/or astrocytes.

As shown in Figure 1, cGAS and IFI16 use the endoplasmic reticulum-localized protein stimulator of interferon genes (STING) as an adaptor molecule to exert their responses, while ZBP1 and AIM2 recruit their own adaptor molecules, receptor interacting serine/threonine-

protein (RIP) kinases 1 and 3 (RIPK1/3), and apoptosis-associated speck-like protein containing a CARD (ASC), respectively (Roberts et al. 2009; Upton et al. 2012). Upon activation, STING facilitates the activation of transcription factors TBK1, IRF3, and nuclear factor kappa-B (NF-κB) that then initiate the transcription of type-I IFNs, proinflammatory cytokines, and chemokines (Ishikawa and Barber 2008). ZBP1 recruits RIPK1 and 3 via interactions with its RIP homotypic interaction motif (RHIM) domains, which subsequently activates NF-κB (Rebsamen et al. 2009). Both IFI16 and AIM2 can, like other receptors upstream of inflammasome formation, recruit ASC following DNA detection (Roberts et al. 2009; Kerur et al. 2011). AIM2 and IFI16 can both form an inflammasome complex with ASC to recruit the effector protein, caspase-1, that subsequently cleaves pro-IL-1β and pro-IL18 to their mature forms for release (Figure 1) (Roberts et al. 2009; Kerur et al. 2011). Additionally, AIM2-ASC inflammasome complex formation can lead to pyroptotic cell death via cleavage of gasdermin-D (GSDMD) (Fernandes-Alnemri et al. 2009; Hornung et al. 2009).

Finally, several other putative DNA sensors such as DNA-dependent protein kinase (DNA-PK) (Ferguson et al. 2012), DEAD-box helicase 41 (DDX41) (Zhang et al. 2011), and meiotic recombination 11 homolog A (MRE11) (Kondo et al. 2013) have been described in peripheral cell types, but their functions as PRRs for DNA in the CNS have not been investigated to date.

Peripheral and resident CNS cells exhibit inflammatory phenotypes following DNA damage (Murata and Kawanishi 2004; Bartsch and Nair 2006; Kiernan et al. 2016), although the mechanisms underlying the initiation of these responses have remained elusive. Interestingly, several cytosolic DNA sensors appear to be capable of detecting mitochondrial and genomic self-DNA resulting from insults such as oxidative stress and IR (Härtlova et al. 2015; Hu et al.

2016; Harding et al. 2017; Mackenzie et al. 2017; Banoth and Cassel 2018; Chatterjee et al. 2018; Forrester et al. 2018; Hou et al. 2018; Moya et al. 2021). As such, cytosolic DNA sensors may be the link between DNA damage and subsequent innate immune responses.

1.4 Cytosolic DNA sensors lie at the intersection of DNA damage and innate immunity

The presence of DNA in the cytoplasm is indicative of cell compromise as it is normally confined to the nucleus or mitochondria in healthy cells. While cytosolic DNA sensors have been extensively studied with regard to bacterial and viral infections, it has only been recently recognized that they may also play a critical role in the generation of immune responses to cytosolic mtDNA and genomic self-DNA (Gao et al. 2015; Jakobs et al. 2015; Mackenzie et al. 2017; De Gaetano et al. 2021; Kumar 2021). In contrast to endosomal nucleic acid sensors such as TLR9 that detect prokaryotic DNA based on their distinct methylation patterns (Hiroaki Hemmi et al. 2000), cytosolic DNA sensors seem to be unable to discriminate between foreign and self-DNA, and so can detect and respond to either (Briard et al. 2020; Zhang et al. 2021). However, it remains unclear whether such responses to self-DNA are protective or detrimental, especially in the context of the CNS where there is strong potential for damaging neuroinflammation. Regardless, it is now apparent that glial cells express multiple sensors capable of initiating their immune functions in response to the presence of cytosolic DNA as described below.

1.4.1 cGAS

Of all the DNA sensors, cGAS has risen to the forefront of nucleic acid sensor research since it's discovery in 2013 (Sun et al. 2013). Over the past decade, our understanding of its role has expanded from the triggering of antiviral immunity to include the inhibition of homologous recombination-mediated DNA repair (Liu et al. 2018; Jiang et al. 2019), control of DNA replication dynamics (Chen et al. 2020), cellular senescence (Dou et al. 2017; Glück et al. 2017; Yang et al. 2017; Takahashi et al. 2018; Miller et al. 2021), cell death (Liang et al. 2014; Brault

et al. 2018; Chen et al. 2018; Gui et al. 2019; Liu et al. 2019; Sarhan et al. 2019), and tumorigenesis (Liu et al. 2018).

As shown in Figure 2A, following DNA binding, cGAS catalyzes the production of the secondary messenger molecule 2’3’cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), which subsequently binds to STING that is located on the surface of the endoplasmic reticulum (Ablasser et al. 2013; Gao et al. 2013; Sun et al. 2013). Following interaction with cGAMP, STING undergoes a conformational change that initiates the recruitment of TBK1, which then phosphorylates IRF3 and liberates NF-κB. Activation of this signaling cascade results in the production of potent proinflammatory cytokines and chemokines including IL-6, TNF, and IL-8, and the type-I IFNs such as IFN-β (Ishikawa and Barber 2008; Zhong et al. 2008a; Sun et al. 2009; Wu et al. 2013).

The discrimination, or lack thereof, between foreign and self-DNA has been recognized as a significant issue regarding cytosolic DNA sensors since indiscriminate DNA binding could lead to the development of autoimmune responses. Indeed, several autoimmune diseases, including Aicardi-Goutières syndrome (AGS), are associated with increased levels of cytoplasmic DNA and inflammatory mediator production (Crow and Rehwinkel 2009). While DNA-mediated activation of cGAS occurs in a length dependent manner, with robust activity occurring only with DNA longer than 45 bp or shorter DNA fragments with flayed ends (Herzner et al. 2015; Andreeva et al. 2017), its ability to discriminate between DNA fragments seems to end here.

DNA damage and genomic instability has long been known to induce inflammatory responses (Ahn et al. 2014; Härtlova et al. 2015; Paludan 2015), but it has only been recently that this phenomenon has been shown to be connected to cytosolic nucleic acid sensors including

cGAS. In 2017, Mackenzie et. al. (Mackenzie et al. 2017) discovered that a primary culprit in the induction of inflammatory responses following IR-induced DNA damage was the formation of micronuclei. The breakdown of the micronuclear envelope and subsequent exposure of self-DNA was associated with rapid cGAS translocation to the micronuclei and the onset of proinflammatory immune responses (Mackenzie et al. 2017), a result that has since been corroborated in other studies (de Oliveira Mann and Kranzusch 2017; Harding et al. 2017; Motwani and Fitzgerald 2017; Bakhoum et al. 2018; Sharma et al. 2020; Mohr et al. 2021; Zhao et al. 2021a). Additionally, DNA damaging events often precipitate the release of mtDNA to the cytoplasm and this can similarly be recognized by cGAS (West et al. 2015; Liu et al. 2016; Maekawa et al. 2019; Guo et al. 2020; Huang et al. 2020; Nadalutti et al. 2022; Zhang et al. 2022).

Importantly, mammalian cells appear to possess mechanisms that serve to limit cGAS activation to prevent excessive or prolonged activation that could lead to devastating autoimmunity. First, recent studies have determined that cGAS may primarily be localized to the nucleus in cells at rest (Volkman et al. 2019; Gentili et al. 2019), rather than being cytosolic as was initially thought (Sun et al. 2013). In the nucleus, cGAS is tightly bound to nucleosomes where its DNA binding sites are prevented from interacting with nucleosomal DNA (Boyer et al. 2020; Cao et al. 2020; Kujirai et al. 2020; Michalski et al. 2020; Pathare et al. 2020; Zhao et al. 2020). Furthermore, while nuclear cGAS activation can occur, cGAMP production resulting from such activation is approximately 500-fold less than that generated when DNA is administered to the cytoplasm (Gentili et al. 2019).

Second, cytoplasmic nucleases, such as three prime repair exonuclease 1 (TREX1) and deoxyribonuclease II (DNASE2) restrict cytoplasmic DNA accumulation thereby limiting cGAS

activation (Mohr et al. 2021). The impact of these nucleases is particularly apparent in inflammatory autoimmune disorders that occur due to mutations that result in their loss of function (Pokatayev et al. 2016; Bartsch et al. 2017; Takahashi et al. 2018). Together, these studies reinforce the importance of the regulation of DNA sensing proteins, especially cGAS.

1.4.2 ZBP1

ZBP1 was the first cytosolic DNA sensor discovered (Takaoka et al. 2007) and its importance in host responses to viral infection have been extensively described (Jeffries and Marriott 2020; Jeffries et al. 2022). ZBP1 was initially found to be a critical component in the detection of the dsDNA of HSV-1 (Takaoka et al. 2007) and other viruses in both peripheral and CNS cell types (Upton et al. 2012; Thapa et al. 2016; Daniels et al. 2019; Rothan et al. 2019; Jeffries et al. 2022). More recently, it appears that this sensor may also recognize RNA motifs in addition to DNA (Maelfait et al. 2017). Furthermore, this cytosolic sensor has been implicated in the initiation of the necroptotic pathway of immunogenic cell death (Furr et al. 2011; Upton et al. 2012; Pham et al. 2013; Lin et al. 2016; Kuriakose et al. 2016; Maelfait et al. 2017; Guo et al. 2018; Daniels et al. 2019; Rothan et al. 2019; Ingram et al. 2019; Hao et al. 2022; Jeffries et al. 2022).

As shown in Figure 2B, upon binding to DNA, ZBP1 recruits RIPK3 that subsequently activates NF-κB to induce proinflammatory cytokine production (Rebsamen et al. 2009). Additionally, ZBP1 has been shown to associate with TBK1 and IRF3 to regulate the activation of IRF3 and, thus, drive type-I IFN production (Takaoka et al. 2007). However, it is important to note that this receptor may act in a ligand and cell type specific manner, as ZBP1 knockdown was found to have no effect on exogenous DNA-induced IFN production in mouse embryonic

fibroblasts (Wang et al. 2008) or a lung epithelial cell line (Lippmann et al. 2008), while similar knockdown significantly reduced B-DNA-induced IFN-β production in an immortalized murine fibroblast cell line (Lippmann et al. 2008).

Importantly, our current knowledge of the role of ZBP1 in responses to self-DNA remains limited, especially since the precise identity of the ligand(s) for ZBP1 remains controversial (Wang et al. 2008; Thapa et al. 2016; Kuriakose et al. 2016; Maelfait et al. 2017; Semenova et al. 2019). However, there is data to support the notion that mtDNA serves as a ligand for ZBP1 (Szczesny et al. 2018; Baik et al. 2021). A recent study has indicated that mtDNA release induced by low level oxidative stress, in the absence of detectable damage to nuclear DNA, elicits a type-I IFN response by pulmonary epithelial cells (Szczesny et al. 2018). Interestingly, in this study, fragments of mtDNA were shown to be released in exosomes that were capable of initiating further inflammatory responses in naïve epithelial cells (Szczesny et al. 2018). Furthermore, a second study reported the ability of glucose deprivation to induce mtDNA release via the actions of NOXA in FVB/NJ mice following implantation of the MVT-1 mammary cancer cell line (Baik et al. 2021), which subsequently initiated necroptosis in a ZBP1-dependent manner (Baik et al. 2021).

1.4.3 IFI16

IFI16 is a DNA sensor that is a member of the pyrin and HIN domain (PYHIN) family. While it was first identified in 1992 (Trapani et al. 1992), it wasn't until 2010 that it was characterized as a cytosolic DNA sensor capable of inducing IFN-β production in response to transfected exogenous DNA (Unterholzner et al. 2010), and such responses have been reported to occur following direct interaction with STING (Cridland et al. 2012) (Figure 2C).

Interestingly, IFI16 exhibits both nuclear and cytosolic localization (Costa et al. 2011). In the nucleus, IFI16 has been shown to respond to viral DNA, which leads to the formation of an inflammasome that then translocates to the cytoplasm where it participates in the cleavage of pro IL-1β and IL-18 into their mature forms for release (Kerur et al. 2011; Monroe et al. 2014; Doitsh et al. 2014).

With regard to self-DNA detection, the murine ortholog of IFI16, IFI204, was found to mediate the detection of DNA released into the cytoplasm following DNA damage resulting from ataxia-telangiectasia mutated (ATM) deficiency in a murine model of ataxia telangiectasia (A-T) (Härtlova et al. 2015). Since the work of Mackenzie et. al, (Mackenzie et al. 2017) has indicated that cytosolic self-DNA recognition occurs due to the formation of micronuclei, it is possible that a similar mechanism underlies that ability of IFI204/IFI16 to perceive the presence of self-DNA, although further study will be necessary to confirm such a hypothesis.

While IFI16 is capable of binding self-DNA, it displays a preference for long non-self-DNA due to its ability to oligomerize into clusters, forming foci that are unable to bind nucleosomal self-DNA (Morrone et al. 2014; Dunphy et al. 2018). Alternatively, IFI16 may serve to detect DNA damage indirectly via the formation of a complex with DDR proteins that can subsequently initiate STING signaling (Figure 2C). Following etoposide-induced genotoxic stress, IFI16 has been found to combine with the DDR proteins ATM and poly(ADP-ribose) polymerase 1 (PARP1) to induce the formation of a STING signaling complex that results in the activation of NF-κB and proinflammatory cytokine production (Dunphy et al. 2018). This ATM-initiated nuclear mechanism results in far more rapid responses to DNA damage than those mediated by cGAS that require the formation and rupture of micronuclei (Dunphy et al. 2018). Furthermore, such responses predominantly result in the activation of NF-κB, rather than IRF3,

resulting in a pro-inflammatory response (Dunphy et al. 2018). However, it is important to note that most of this work has been performed in keratinocytes and it remains to be determined whether such mechanisms exist in resident CNS cell type.

1.4.4 AIM2

AIM2 is another member of the PYHIN family that appears to have DNA sensing capabilities. First identified in 1997 (DeYoung et al. 1997), its role in dsDNA detection was not recognized until a decade later (Bürckstümmer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009; Roberts et al. 2009). As shown in Figure 2D, AIM2 recruits the inflammasome adaptor protein ASC and procaspase-1 following binding to cytosolic DNA, forming an inflammasome complex that permits IL-1β and IL-18 maturation via the actions of caspase-1 (Bürckstümmer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009; Roberts et al. 2009). While AIM2, like ZBP1, can also initiate immunogenic cell death pathways, AIM2 appears to initiate pyroptosis rather than necroptosis via the cleavage of gasdermin-D and subsequent pore formation (Bürckstümmer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009; Roberts et al. 2009).

Importantly, AIM2 has been shown to detect cytosolic self-DNA and mtDNA (Jakobs et al. 2015; Baum et al. 2015; Micco et al. 2016; Dang et al. 2017). The interaction between AIM2 and cytosolic self-DNA has primarily been studied in the context of autoimmune diseases that characteristically entail cytosolic DNA accumulation, such as arthritis, psoriasis, and systemic lupus erythematosus (Kimkong et al. 2009; Jakobs et al. 2015; Baum et al. 2015). Furthermore, pharmacological disruption of the nuclear envelope and the liberation of self-DNA to the cytosol has also been shown to induce AIM2 activation (Micco et al. 2016). As such, it has been

inferred from these observations that AIM2 is capable of recognizing DNA and might be able to do so following micronuclei formation.

In addition, a limited number of studies suggest that AIM2 may also be localized to the nucleus in some cell types, such as macrophages (Hu et al. 2016), and can mediate responses to nuclear DNA damage (Diner et al. 2015; Hu et al. 2016). For example, AIM2 has been reported to co-localize with the DNA damage marker gamma-H2A histone family member X (γH2AX) at sites of double strand breaks (DSBs) following IR exposure in macrophages, where it subsequently forms an AIM2-ASC-caspase-1 inflammasome complex to trigger pyroptotic cell death (Hu et al. 2016).

While the relative importance of AIM2-mediated DNA detection in the cytosol versus the nucleus remains unclear, it is noteworthy that the localization of the AIM2 inflammasome complex to sites of nuclear DNA DSBs occurs as rapidly as 8 hours following IR exposure (Hu et al. 2016). Furthermore, it is interesting that these inflammasome complexes have been observed to accumulate in the perinuclear region (Hu et al. 2016). However, it remains to be determined whether such accumulations involve additional interactions of AIM2 with cytosolic self-DNA.

1.5 Self-DNA detection in the CNS

To date, the mechanisms by which resident CNS cells can detect and respond to self-DNA are understudied, and it remain unclear whether the net result of such responses are beneficial or detrimental. Neuroinflammation, while crucial in protecting the brain against infection, can result in serious neurological damage if it is of inappropriate intensity or duration. Indeed, neuroinflammation that stems from DNA damage and/or deficient or defective DNA repair can underlie or exacerbate neurodegeneration in CNS disease states (Coppedè and Migliore 2015; Shiwaku and Okazawa 2015; Ransohoff 2016; Subhramanyam et al. 2019). For example, the detection of cytosolic mtDNA and self-DNA by resident glial cells is a hallmark of CNS pathologies including AGS, AD, PD, A-T, and Huntington's disease (HD) (Bartsch et al. 2017; Nissanka and Moraes 2018; Xu et al. 2019; Song et al. 2019; Sharma et al. 2020; Zia et al. 2020; Paul et al. 2021; Hou et al. 2021; Zhao et al. 2021b; Healy et al. 2022), as summarized in Table 1.

In some CNS disorders, such as A-T and AGS, the origins of cytosolic DNA accumulation are clear. In A-T, a critical kinase in DDRs, ATM, is defective and results in the accumulation of DSBs leading to the presence of cytosolic DNA (Härtlova et al. 2015; Zhu et al. 2019), while in AGS, mutations in the genes encoding products that process/degrade nucleic acids, such as TREX1 and RNAse H2, lead to cytosolic DNA accumulation and lethal autoimmunity in neonates (Gray et al. 2015; Pokatayev et al. 2016).

However, the origin of cytosolic DNA in other neurodegenerative diseases is either unclear or unknown. For example, in AD, amyloid-β (Aβ) plaques have been shown to induce oxidative stress that can cause mitochondrial dysfunction and subsequent mtDNA release into the cytosol (Yoo et al. 2020). In contrast, the α-synuclein (α-Syn) fibrils that are commonly seen

in PD can induce genomic DNA damage (Hinkle et al. 2022), while affected striatal neurons in HD patients and murine models of this disease show significantly higher numbers of micronuclei. However, the mechanisms underlying DNA damage in HD remain unknown (Sharma et al. 2020).

We, and others, have shown that exogenous cytosolic DNA elicits reactive astrogliosis and microgliosis, and is associated with the production of proinflammatory and antiviral mediators by these cells (Booth et al. 2017; Jeffries and Marriott 2017, 2020; Nissanka and Moraes 2018; Gleichman and Carmichael 2020; Hou et al. 2021; Zhao et al. 2021b). Importantly, the recent demonstration that glial cells express multiple cytosolic sensors has provided the means by which they perceive DNA. We showed that ZBP1 is expressed in microglia and astrocytes in an inducible manner following HSV-1 infection (Furr et al. 2011). In addition, Cox et. al, (Cox et al. 2015), provided evidence that murine microglia and astrocytes express mRNA encoding cGAS, the p204 murine ortholog of IFI16, and AIM2 (Cox et al. 2015). Subsequently, we, and others, have extended these findings to demonstrate cGAS, IFI16, and AIM2, protein expression in human microglia and astrocytes, and have shown their ability to mediate glial immune responses to exogenous DNA administration (Wu et al. 2016; Jeffries and Marriott 2017; Jeffries et al. 2020, 2022).

Transfection of exogenous DNA into human and murine primary glia and cell lines elicits the production of type-I IFNs and proinflammatory cytokines including CCL3, CCL5, CXCL2, TNF, IFN-β, and IL-6 (87, 88). The importance of the cytosolic DNA sensors, cGAS, IFI16 (and murine ortholog p204), and AIM2, in the generation of such responses is implied by the demonstration that dsDNA transfection into primary murine microglia and astrocytes significantly upregulates the expression of mRNA encoding these sensors (Cox et al. 2015).

Importantly, we have shown that the responses of a human microglia cell line to intracellular exogenous DNA administration were significantly attenuated following CRISPR/Cas9 knockdown of cGAS expression (Jeffries et al. 2020). Furthermore, the secretion of mature IL-1β, downregulated dendritic growth, and enhanced axon extension of primary human and murine neurons elicited by dsDNA transfection was found to be dependent on the presence of AIM2 (Wu et al. 2016). As such, it is apparent that resident CNS cells are capable of responding to the presence of exogenous cytosolic DNA and do so via various sensor molecules.

With regard to the ability of resident CNS cells to perceive the presence of cytosolic self-DNA, glia have recently been shown to respond to mtDNA and self-DNA accumulation via sensors including cGAS and AIM2 (Nissanka and Moraes 2018; Sliter et al. 2018; Soni and Reizis 2019; Song et al. 2019; Zhao et al. 2021b; Healy et al. 2022; Hinkle et al. 2022; Zhang et al. 2022), as summarized in Table 1. For example, the addition of α-Syn preformed fibrils (PFF) to primary murine mixed glial cultures to simulate PD pathology resulted in DNA DSBs, an accumulation in cytosolic DNA, and subsequent activation of STING and TBK1 that resulted in type I IFN production (Hinkle et al. 2022). Importantly, these responses were attenuated by the pharmacological inhibition of STING activation (Hinkle et al. 2022). These findings are consistent with those in an in vivo mouse model where α-Syn-PFF similarly induced DNA damage, TBK1 activation, and IFN production by microglia in situ that preceded PD-like dopaminergic neurodegeneration, and the demonstration that substantia nigra pars compacta tissue from human PD patients show elevated STING protein levels that correlate with α-Syn-PFF accumulation (Hinkle et al. 2022).

The cGAS-STING axis has been implicated in the initiation of neurotoxic responses of primary microglial cultures to ATM mutations/deficiency that, again, results in the accumulation

of cytosolic self-DNA and models A-T (Song et al. 2019). This DNA sensing pathway has also been linked to striatal neuron cell death in HD (Sharma et al. 2020). In primary human and murine HD-affected striatal neurons, Sharma et. al (Sharma et al. 2020) reported a high incidence of micronuclei formation that coincided with cell death due to autophagy, and the increased expression of mRNA encoding CCL5 and CXCL10 that was abolished following cGAS depletion (Sharma et al. 2020). Additionally, studies in mouse models of other neurodegenerative disorders have similarly indicated a role for cGAS in their progression. Most notably, models of AGS development that feature mutations of TREX1 and RNAse H2 have revealed that cGAS is essential for the initiation of the autoimmune responses associated with this disorder (Gray et al. 2015; Pokatayev et al. 2016).

Interestingly, AIM2-mediated responses have also been associated with neurodegenerative diseases but, in contrast to cGAS, such response appear to play a protective rather than a detrimental role, as summarized in Table 1. In primary murine microglia, AIM2 has been shown to alleviate the damaging neuroinflammation seen in the experimental autoimmune encephalitis (EAE) model of multiple sclerosis (MS), and other mouse models of AD and PD (Wu et al. 2017; Ma et al. 2021; Rui et al. 2022a). In EAE, AIM2 was shown to negatively regulate DNA-PK activity in an inflammasome independent manner, and AIM2 deficiency was found to increase levels of microglial activation and peripheral leukocyte recruitment to the CNS (Ma et al. 2021). Furthermore, the inhibitory activity of AIM2 was found to be comparable to that of pharmacological DNA-PK inhibition (Ma et al. 2021).

In the 5XFAD model of AD, deletion of AIM2 resulted in a decrease in Aβ deposition, but caused an elevation in the production of the key inflammatory cytokines IL-6 and IL-18, further supporting a negative regulatory role for AIM2 in neuroinflammation (Wu et al. 2017).

Finally, in a neurotoxin (N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, MPTP)-induced PD mouse model, AIM2 activation served to limit cGAS activity via interference with protein kinase B (AKT)-mediated IRF3 phosphorylation, and conditional knockout of AIM2 in microglia, but not peripheral cells, exacerbated PD-like disease severity (Rui et al. 2022a).

As such, while it is clear that cytosolic DNA sensors play critical roles in the initiation and/or progression of CNS pathologies in animal models (as summarized in Table 1), their specific roles can often appear contradictory and so may be sensor and/or disease condition specific. Additionally, while light has been shed on the beneficial/detrimental effects mediated by these receptors in murine CNS cells and neurodegenerative disorder models, it remains unclear whether cytosolic DNA sensors exert similar functions in human disease. A better understanding of these DNA sensors in the context of human neurons/glia and patients with CNS disorders could identify new targets for therapeutic intervention to limit neuroinflammation and/or to promote beneficial immune responses.

1.6 Concluding remarks

Taken together, it has become apparent that resident CNS cells play a critical role in the protective and detrimental immune responses associated with infection of the CNS and the development/progression of neurological disorders. Furthermore, it is increasingly clear that such responses are initiated via the detection of DAMP and PAMP motifs that associated with cellular damage and infectious agents, respectively. The principal glial cells, microglia and astrocytes, express various cell-surface, endosomal, and cytosolic members of multiple PRR families that can trigger immune mediator production that can foster neuroinflammation and recruit leukocytes to the CNS. Importantly, glia can constitutively and/or inducibly express PRRs that can detect the presence of DNA in the cytosolic compartment. While these sensors were initially characterized as components in the detection of viral and bacterial nucleic acids by microglia and astrocytes, it is now recognized that molecules such as cGAS, IFI16, and AIM2, could play important roles in the generation of responses to the presence of self-DNA in the cytosol resulting from DNA damaging insults, such as IR or oxidative stress, or deficient/defective DNA repair. As such, these mechanisms might be targetable to either augment those glia-mediated responses that serve to protect against tumorigenesis, or prevent the inflammatory responses of these cells that initiate or exacerbate damaging neuroinflammation.

1.7 Hypotheses and goals of the present study

In the present study, we have tested the hypotheses that ZBP1 serves as a restriction factor for HSV-1 in murine microglia and astrocytes and that cGAS mediates human microglial responses to DNA damage. Previous research by our laboratory has provided evidence that ZBP1 initiates proinflammatory mediator production during HSV-1 infection and that such production subsequently results in neuronal death (Furr et al. 2011). Here, we expand on this work and describe mechanisms by which ZBP1 activation leads to such responses. In doing so, we tested the hypothesis that ZBP1 serves as a restriction factor for HSV-1 replication by initiating cell death pathways following activation.

Additionally, our laboratory has previously described the functional expression of the DNA sensor cGAS in human glial cells (Jeffries and Marriott 2017). Furthermore, cGAS has been shown to detect and initiate responses to self-DNA that results from DNA damage in peripheral human and murine cell types (West et al. 2015; Liu et al. 2016; de Oliveira Mann and Kranzusch 2017; Harding et al. 2017; Motwani and Fitzgerald 2017; Bakhoum et al. 2018; Maekawa et al. 2019; Sharma et al. 2020; Guo et al. 2020; Huang et al. 2020; Zhao et al. 2021a; Mohr et al. 2021; Zhang et al. 2022; Nadalutti et al. 2022). Here, we have expanded on these findings by testing the hypothesis that cGAS will also initiate responses to genotoxic insults in human microglia. Importantly, we have begun to describe mechanisms by which microglia perceive DNA damage. Specifically, we have elucidated and characterized early responses initiated by cGAS in response to DNA damage. In doing so, we begin to investigate how cGAS may be therapeutically targeted to modulate CNS responses to genotoxic events.

1.6 TABLES AND FIGURES

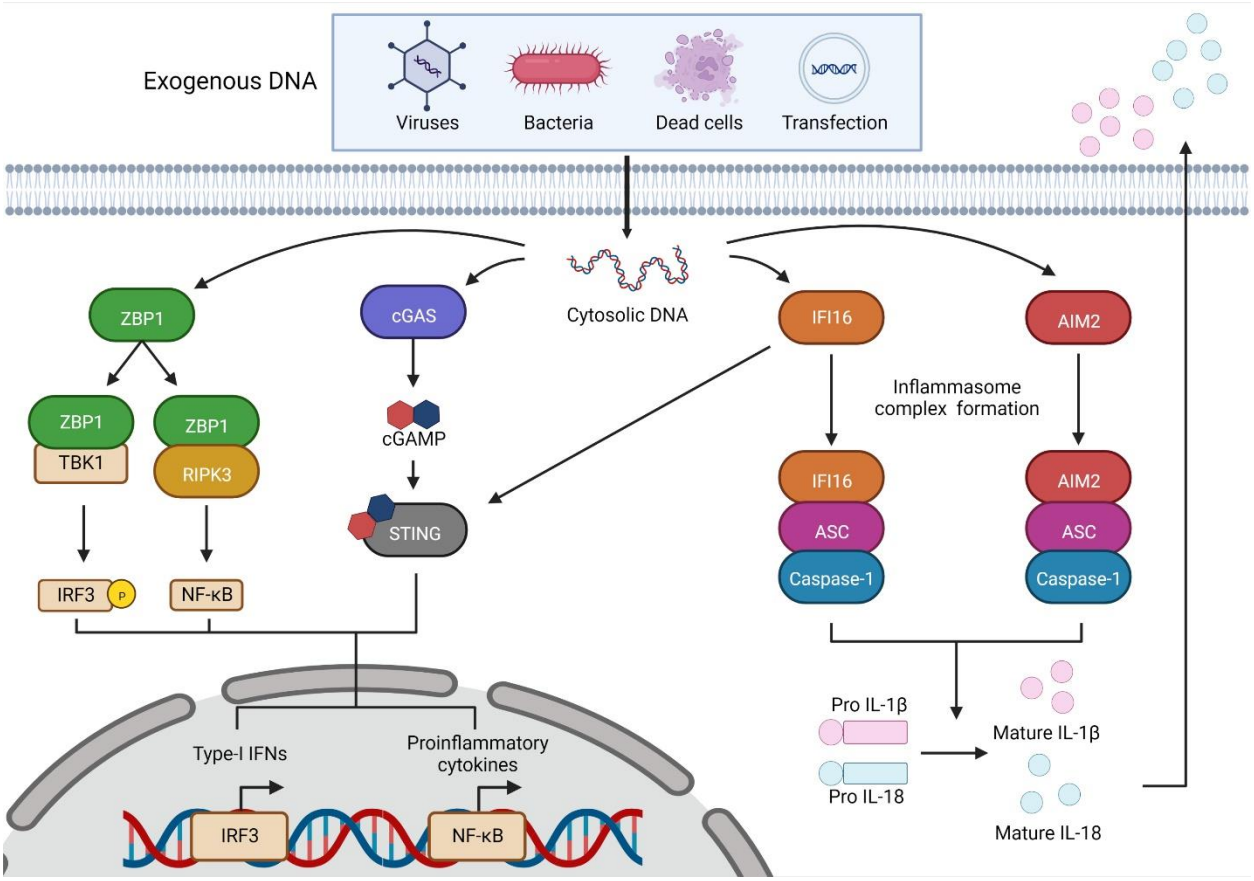

Figure 1. Cytosolic DNA sensors and their signaling pathways.
Sensing of dsDNA by ZBP1 induces its association with RIPK3 and subsequent activation of NF-κB to elicit pro-inflammatory cytokine production, and/or its interaction with TBK1 to induce IRF3 activation and type-I IFN expression. Sensing of dsDNA by cGAS catalyzes the production of cGAMP that activates STING and leads to TBK1 activation that induces IRF3 activation and type-1 IFN expression. IFI16 can directly interact with STING following DNA sensing resulting in NF-κB and IRF3 activation, or can associate with ASC to form an inflammasome complex resulting in caspase-1-mediated IL-1β and IL-18 release. AIM2 sensing of dsDNA also leads to inflammasome complex formation with ASC and mature IL-1β and IL-18 release.

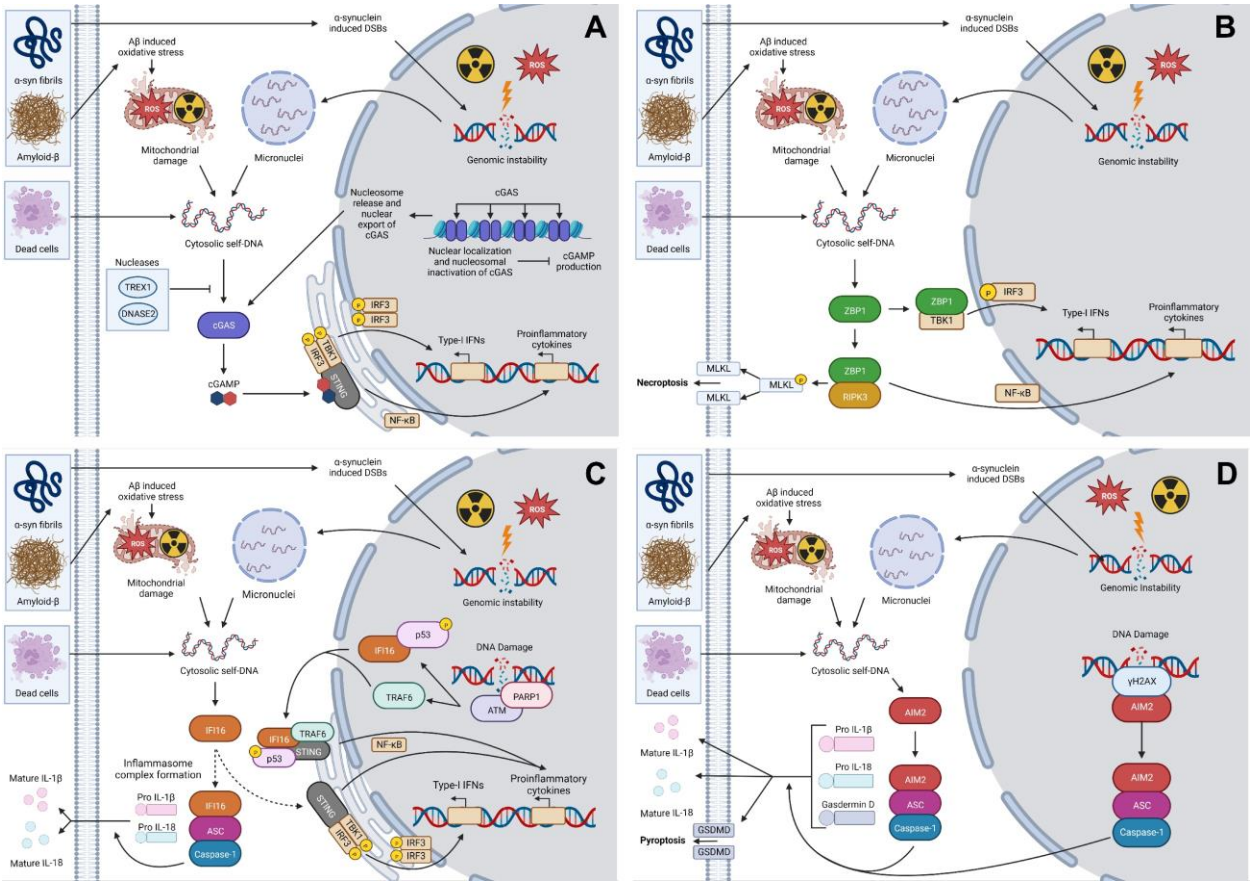

Figure 2. Cytosolic self-DNA sensing pathways.
In the CNS, the uptake of α-Syn fibrils, amyloid-β, and/or cellular debris by resident CNS cells, or exposure to exogenous oxidative stress or ionizing radiation (IR), can lead to liberation of self-DNA to the cytosol via mitochondrial damage or genomic instability leading to micronuclei formation. The presence of self-DNA in the cytoplasm can then be perceived via DNA sensing PRRs including cGAS (Panel A), ZBP1 (Panel B), IFI16 (Panel C), and/or AIM2 (Panel D), leading to sensor-specific signaling pathways that precipitate the production of inflammatory cytokines, type-I IFNs and/or immunogenic necroptotic and pyroptotic cell death pathways.

Table 1: DNA sensors and their disease associations in resident CNS cells

Cell Type		Species	Sensor(s)	Stimuli/Model	Disease Association	References
Microglia	Primary	Human	cGAS-STING	Ganciclovir treatment	N/A	(Mathur et al. 2017)
		Murine	cGAS-STING	ATM deficiency	Ataxia telangiectasia	(Song et al. 2019)
				DNA transfection	N/A	(Cox et al. 2015)
				Tau protein aggregation	Alzheimer's disease	(Jin et al. 2021)
			p204 (IFI16 ortholog)	DNA transfection	N/A	(Cox et al. 2015)
			AIM2	AIM2 deficient 5XFAD	Alzheimer's disease	(Wu et al. 2017)
				MPTP-induced PD	Parkinson's disease	(Rui et al. 2022a)
				EAE	Multiple sclerosis	(Ma et al. 2021)
	Cell line	Human	cGAS-STING	DNA transfection	N/A	(Jeffries and Marriott 2017)
Astrocytes	Primary	Murine	p204 (IFI16 ortholog)	DNA transfection	N/A	(Cox et al. 2015)
			AIM2	EAE	Multiple sclerosis	(Barclay et al. 2022)
Mixed Glia	Primary	Murine	cGAS-STING	α-synuclein preformed fibrils	Parkinson's disease	(Hinkle et al. 2022)
Neurons	Primary	Human	cGAS-STING	Micronuclei formation	Huntington's disease	(Sharma et al. 2020)
			AIM2	DNA transfection	N/A	(Wu et al. 2016)
		Murine	cGAS-STING	Micronuclei formation	Huntington's disease	(Sharma et al. 2020)
			AIM2	DNA transfection	N/A	(Wu et al. 2016)
	Cell line	Murine	cGAS-STING	Cytosolic mtDNA accumulation	Amyotrophic lateral sclerosis	(Yu et al. 2020)
Whole brain	N/A	Murine	cGAS-STING	TREX deficiency	Aicardi-Goutières syndrome	(Gray et al. 2015)
				$Rnaseh2a^{G37S/G37S}$ mutation	Aicardi-Goutières syndrome	(Pokatayev et al. 2016)

				Cytosolic mtDNA accumulation	Amyotrophic lateral sclerosis	(Yu et al. 2020)
				Tau protein aggregation	Alzheimer's disease	(Jin et al. 2021)
				APP/PS1	Alzheimer's disease	(Hou et al. 2021; Larrick and Mendelsohn 2021)
				EAE	Multiple sclerosis	(Lemos et al. 2014)
			AIM2	APP/PS1	Alzheimer's disease	(Chen et al. 2019; Cao et al. 2021)
				ME7	Chronic neurodegeneration	(Cox et al. 2015)
			p204 (IFI16 ortholog)	ME7	Chronic neurodegeneration	(Cox et al. 2015)
		Zebrafish	IFI16	Cytosolic mtDNA	Parkinson's disease	(Matsui et al. 2021)

CHAPTER 2: Materials and Methods

2.1 Source and propagation of cell lines

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 activities; 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.

2.2 Human and 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. 2015a). 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. 2015a). 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. 2015a), 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 Preparation of viral stocks and in vitro infection of glial cells

HSV-1 viral stocks were prepared by Dr. Austin Jeffries by infecting monolayer cultures of Vero cells (ATCC; CCL-81) with HSV-1 MacIntyre strain (HSV-1(MacIntyre)) from a patient with encephalitis (ATCC; VR-539), or an ICP6 RHIM mutant strain (HSV-1(F)-ICP6-RHIM Mut) or its parental F strain (HSV-1(F)) (kind gifts from Dr. Edward Mocarski of Emory University, Atlanta, GA) at a multiplicity of infection (MOI) of 0.01 and incubated for 48 to 72 hrs, at which time 100% of cells displayed cytopathic effects (Jeffries et al. 2022). 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 X 10^7 PFU/ml for HSV-1(MacIntyre) and HSV-1(F)-ICP6-RHIM Mut, and 1.5 X 10^7 PFU/ml for HSV-1(F). Murine astrocytes 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 hr 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 and/or whole-cell protein isolates. In experiments with inhibitors, following infection, the signal transducer and activator of transcription 1 (STAT1) inhibitor Fludarabine (10 μM; Selleckchem), RIPK1 inhibitors GSK963 and GSK547 (1 μM, 50 nM; Sigma, Selleckchem), RIPK3 inhibitors GSK872 and GSK843 (5 μM, 2μM; Sigma), the pan-caspase inhibitor Z-VAD-FMK (20 μM; InvivoGen), and the caspase-8 inhibitor Z-IETD-FMK (20 μM; InvivoGen), were reconstituted in DMSO and added to the cultures.

2.4 Immunoblot analysis

Whole-cell protein isolates were collected from microglia and astrocytes using Triton lysis buffer (10 mM Tris HCl pH 10.5, 5 mM $MgCl_2$, 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 (for ZBP1 and cGAS) or 5% BSA (for P-MLKL) for 1 h and then incubated overnight at 4°C with primary antibodies directed against ZBP1 (AdipoGen), cGAS (Abcam), or

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). Immunoblots shown are representative of at least three separate experiments using the Bio-Rad ChemiDoc imaging system, and quantification analysis was performed using ImageLab software (Bio-Rad).

2.5 Enzyme-linked immunosorbent assay

Specific capture enzyme-linked immunosorbent assays (ELISAs) were performed to quantify murine IL-6, IFN-β, or TNF, or human IL-6, IL-8, and CXCL1. 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 detection antibody (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 ELISA was conducted using a commercially available kit (R&D Systems DuoSet ELISA). 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 IL-8 and CXCL1 ELISAs were carried out using commercially available kits (R&D Systems DuoSet ELISA). Bound antibody was detected using streptavidin-HRP (BD Biosciences) followed by the addition of tetramethylbenzidine (TMB) substrate. H_2SO_4 or HCl were used to stop the reaction and absorbance was measured at 450 nm. Dilutions of murine IL-6, IFN-β, and TNF (BD Biosciences, Biolegend, R&D systems respectively) or human IL-6, IL-8, and CXCL1 (BD Biosciences and R&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.

2.6 Generation of heterozygous cGAS deletion microglial cell line

To investigate cGAS function, our laboratory created a heterozygous deletion microglial cell line that expresses cGAS at reduced levels, cGAS+/−, using CRISPR/Cas9 approaches (Jeffries et al. 2020). 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.

2.7 Irradiation of hμglia

WT and cGAS +/- hμglia cells were seeded (1 x 10^5 cells per well) in 12-well flat bottom tissue culture plates. Irradiation was carried out by utilizing the MultiRad 225 irradiation system (Precision X-Ray, Madison, CT). Cells were treated at approximately 6.5 Gy/min using the auto-

dose control program (225kV, 17.8mA, 37.0 SSD, Al 0.5mm filter) to the indicated target doses. Following incubation for 4, 8, 18, or 24 h post-irradiation, supernatants and whole-cell lysates were collected for ELISA and immunoblot analysis, respectively.

2.8 Oxidative stress treatments of hμglia

WT and cGAS +/- hμglia cells were seeded (1 x 10^5 cells per well) in 12-well flat bottom tissue culture plates. H_2O_2 dilutions were prepared at the indicated concentrations in Dulbecco's modified Eagle media supplemented with 5% FBS and 1% penicillin/streptomycin and immediately added to cells. Following a 2 h incubation at 37°C, media was aspirated and cells were washed once with 1x PBS prior to addition of fresh H_2O_2-free media. Cells were allowed to incubate for 4, 8, 18, or 24 h post-media change and then supernatants and whole-cell lysates were collected for ELISA and immunoblot analysis, respectively. Alternatively, cells were treated via the glucose oxidase/catalase (GOX/CAT) system as detailed by Mueller et al. (Mueller et al. 2009). A 1:1,000 dilution of glucose oxidase (Sigma Aldrich) and the indicated dilutions of catalase (Sigma Aldrich) were prepared in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin and added to cells simultaneously. Following a 24h incubation at 37°C, supernatants were collected for ELISA analysis.

2.9 Fluorescent immunohistochemical analysis

WT and cGAS +/- hμglia cells were seeded (1 x 10^5 cells per well) in 12-well flat bottom tissue culture plates. Following irradiation or H_2O_2 treatment as described, cells were gently washed 3 times with 1x PBS prior to fixation (4% PFA in 1xPBS) in the tissue culture plate. Cells were simultaneously permeabilized and blocked (0.1% NP-40 and 5% normal goat serum

in 1x PBS). Following permeabilization and blocking, cells were incubated with a monoclonal antibody directed against γH2AX (Novus Biologicals), then washed 3 times with 1x PBS prior to incubation with a secondary antibody conjugated to AlexaFluor 555 or 594 (Molecular Probes). Following secondary antibody incubation, cells were washed 3 times with 1x PBS then incubated briefly with DAPI (300nM in 1x PBS) before imaging with the Leica DM IL LED inverted microscope (Leica Microsystems, Wetzlar, Germany). Mean fluorescence of γH2AX was obtained via ImageJ software analysis.

2.10 Micronucleus analysis

WT hμglia cells were seeded (5 x 10^4 cells per well) on poly-D lysine treated glass coverslips in 24-well flat bottom tissue culture plates. Following irradiation as described, cells were gently washed 3 times with 1x PBS prior to fixation (4% PFA in 1xPBS) on the coverslips. Following fixation, cells were briefly incubated with DAPI (300nM in 1x PBS). Approximately 10 μL of ProLong™ Diamond Antifade Mountant (Invitrogen) was pipetted onto microscope slides prior to coverslip placement. Slides were protected from light and dried overnight at room temperature prior to imaging. Imaging was performed on the Olympus FV1000 (Olympus Life Science Solutions) confocal microscope. 10 images were taken from each coverslip and the total number of nuclei and the number of nuclei that had at least one surrounding micronucleus were counted by eye. The total number of micronucleus containing cells in each frame were divided by the total number of non-micronucleus containing cells to obtain a percentage of micronucleus containing cells per frame. The percentage of micronucleus containing cells in each frame were averaged together to obtain what was considered to be the total percentage of micronucleus containing cells per coverslip.

2.11 SiRNA transfection

WT hμglia cells were seeded (6 x 10^4 cells per well) in 6-well flat bottom tissue culture plates. Silencer® Select siRNA targeting human cGAS and scrambled RNA were purchased from ThermoFisher Scientific (Waltham, MA). Each was transfected into the hμglia human microglial cell line at a concentration of 15 nM using RNAiMAX transfection reagent (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. At 24 h, cGAS protein knockdown was confirmed by immunoblot assay and further treatments were performed as described.

2.12 Measuring cell viability and calculation of the percentage and kinetics of cell death

Cell viability was measured for 24 hrs post-infection in isolated murine astrocytes using RealTime-Glo^{TM} 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, with or without necroptosis or apoptosis pathway inhibitors, and added to glial cultures at one-hour post-infection. Luciferase activity was measured every two hours using the SpectraMax® iD5 plate reader for 24 hrs beginning at two hours following infection. Luciferase readings were normalized to uninfected controls and the resulting values were subtracted from a value of one and multiplied by 100% to calculate percentage cell death. Data was further normalized within experimental groups by subtracting the percentage of dead cells at 2 hrs from values at all subsequent time points. Negative values were recorded as zero. The slopes and standard deviations for each treatment were determined by linear regression using Microsoft Excel software.

2.13 Statistical analysis

Data is presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed by one or two-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: Z-DNA binding protein 1 mediates necroptotic and apoptotic cell death pathways in murine glia following herpes simplex virus-1 infection

3.1 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 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, such as Z-DNA binding protein 1 (ZBP1; also known as DNA dependent activator of interferon regulatory factors (DAI)), by glial cells is of particular interest as their ability to interact with nucleic acids in the intracellular environment suggests an important role in the detection of viral CNS pathogens such as herpes simplex virus type 1 (HSV-1) (Furr et al. 2011; Crill et al. 2015). Furthermore, ZBP1 has been demonstrated to be both protective and damaging to the host, depending on the context of the challenge (Wang et al., 2008; Ingram et al., 2019; Momota et al., 2019). This molecule was first identified as a DNA sensor capable of inducing

type one interferon (IFN) expression in response to cytosolic DNA or viral infection (Takaoka et al. 2007). However, ZBP1 was subsequently found 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 shown that these mediators cause neuronal cell death (Furr et al. 2011; Crill et al. 2015).

Recently, ZBP1 has also been found to initiate cell death pathways such as necroptosis in non-CNS cell types (Upton et al. 2012; Thapa et al. 2016; Kuriakose 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).

Here, we have investigated the relative contribution made by ZBP1 to HSV-1 mediated cell death in primary astrocytes and microglia using cells derived from mice genetically deficient in the expression of this sensor. We confirm that HSV-1 induces necroptosis in glia and have established the ability of ZBP1 to mediate this cell death pathway in these cells. Interestingly, while ZBP1 is best known for its role in necroptotic signaling, our findings indicate that this sensor can also contribute to virally-induced apoptosis in glia. Together, our findings suggest that ZBP1 serves as a restriction factor for HSV-1 infection via the induction of multiple cell

death pathways in non-neuronal CNS cells. While it remains to be seen whether ZBP1-mediated activation of cell death in glia contributes significantly to host protection or, rather, exacerbates HSV-1 encephalitis pathology, the identification of such a role in resident CNS cells may represent a novel target for therapeutic intervention to reduce HSV encephalitis-associated morbidity and mortality.

3.2 RESULTS

3.2.1 ZBP1 functions as an HSV-1 restriction factor in primary astrocytes

ZBP1 has previously been demonstrated to act as an HSV-1 restriction factor in peripheral myeloid cells (Guo et al. 2018). Here, we have investigated the ability of this sensor to limit infection in astrocytes derived from ZBP1+/+ and ZBP1-/- mice. Lack of ZBP1 protein expression was previously confirmed in astrocytes derived from ZBP1 knockout mice by immunoblot analysis (Jeffries et al. 2022) (Figure 3). ZBP1+/+ and ZBP1- astrocytes were infected with a clinical neuroinvasive HSV-1(MacIntyre) and the number of PFU released from infected cells was determined by conventional plaque assays in Vero cells. As shown in Figure 4A, infectious viral particle release by ZBP1 deficient astrocytes was significantly greater than that seen with wildtype cells, although it should be noted that we have not directly assessed the amount of cell-associated virus in these studies.

To determine whether the higher levels of viral release were due to a reduction in the production of antiviral mediators, our laboratory previously measured IFN-β secretion in astrocytes following HSV-1 infection (Jeffries et al. 2022). As shown in Figure 4B, both ZBP1+/+ and ZBP1-/- derived astrocytes produced only low levels of IFN-β production, with statistically significant amounts only being seen in ZBP1-/- derived astrocytes with HSV-1 at the higher MOI (2.0) (Jeffries et al. 2022). Furthermore, we determined that treatment of either ZBP1+/+ or ZBP1-/- derived astrocytes with the STAT1 inhibitor, Fludarabine, had no effect on infectious particle release (Figure 4A). In addition, our group previously assessed the effect of genetic ZBP1 deficiency on HSV-1-induced production of the inflammatory cytokines IL-6 and TNF by these cells, and we report that ZBP1-/- deficient astrocytes release demonstrable levels of IL-6 and TNF following HSV-1 infection at the higher MOI (Figure 4B) (Jeffries et al.

2022). However, such production was not significantly different from that seen by ZBP1 expressing astrocytes following infection (Figure 4B). Furthermore, the lower HSV-1 dose (0.2) failed to elicit significant production of any of these cytokines by either ZBP1+/+ or ZBP1-/- cells despite the significant difference seen in virus production at this MOI. As such, this data is inconsistent with ZBP1-mediated viral restriction being due to differences in cytokine production.

3.2.2 HSV-1 infection induces necroptosis in astrocytes

Several studies have shown that ZBP1 can mediate necroptosis in non-CNS cell types (Upton et al. 2012; Thapa et al. 2016; Kuriakose et al. 2016; Koehler et al. 2017; Maelfait et al. 2017; Sridharan et al. 2017; Guo et al. 2018; Jiao et al. 2020). To determine if this is also true in glia, we measured the rate of cell death in astrocytes derived from ZBP1+/+ and ZBP1-/- mice following HSV-1 infection. As shown in Figures 5 and 6A, there was significant difference between ZBP1+/+ and ZBP1-/- derived astrocytes in the rate and final percentage of cell death at 24 hrs following challenge with HSV-1(MacIntyre).

However, it has recently been discovered that the HSV-1 gene product ICP-6 has a RHIM domain that is capable of directly interacting with receptor interacting protein kinase 1 (RIPK1) and initiating necroptosis in mouse but not human cells (Wang et al. 2014; Huang et al. 2015; Guo et al. 2018)**.** To circumvent this possibility, and to more closely resemble responses in human cells, we have also assessed the ability of an HSV-1 strain with mutations in the ICP6 RHIM domain (HSV-1(F)-ICP6-RHIM Mut), and its parental F strain (HSV-1(F), to induce cell death in astrocytes in the presence and absence of ZBP1 expression. Interestingly, there was a significant difference between the rate and percentage of cell death at 24 hrs in ZBP1+/+ and ZBP1-/- derived astrocytes for both the HSV-1(F)-ICP6-RHIM Mut virus and the parental ICP6

expressing HSV-1(F) strain (Figures 5 and 6A). While the decreased cell death induced by HSV-1(F)-ICP6-RHIM Mut in ZBP1-/- astrocytes correlates with a significant reduction in the level of phosphorylated MLKL (Figure 6B), decreased cell death seen following infection with HSV-1(F) occurred despite similar levels of phosphorylated MLKL to those seen in ZBP1+/+ cells, suggesting that necroptosis is not solely responsible for HSV-1 induced cell death as demonstrated by Jeffries et. al, (Jeffries et al. 2022)**.**

To determine if RIPK1 mediates MLKL phosphorylation in ZBP1 deficient astrocytes following infection, Our group has previously treated ZBP1+/+ and ZBP1-/- derived astrocytes with the RIPK1 inhibitor, GSK963, during infection with the HSV-1(MacIntyre), HSV-1(F)-ICP6-RHIM Mut, and HSV-1(F) strains (Jeffries et al. 2022). The absence of a direct effect of GSK963, or other inhibitors used in this study, on cell death at 24 hrs and rate of cell death was confirmed in uninfected glial cells (data not shown) (Jeffries et al. 2022). As shown in Figures 5 and 6C, there remained a significant difference between ZBP1 expressing and ZBP1 deficient astrocytes in the rate and final percentage of cell death at 24 hrs in following challenge with HSV-1(MacIntyre) in the presence of GSK963. Similar results were obtained when another inhibitor of RIPK1, GSK547, was employed (Figure 7A). Furthermore, there was a significant difference between the rate and percentage of cell death at 24 hrs in ZBP1+/+ and ZBP1-/- derived astrocytes for both the HSV-1(F)-ICP6-RHIM Mut and HSV-1(F) strains (Figures 5 and 6C) following GSK963 treatment. Together, these data indicate that ZBP1 dependent differences in virally-induced astrocytes cell death are not mediated by RIPK1 kinase activity.

Interestingly, treatment of ZBP1-/- derived astrocytes with GSK963 significantly reduced levels of phosphorylated MLKL induced by HSV-1(F) (Figure 6D) (Jeffries et al. 2022), with similar trends seen in experiments using GSK547 (Figure 7B), suggesting that the MLKL

phosphorylation seen in the absence of ZBP1 (Figure 6B) is dependent on RIPK1 activity. Together, these data indicate that the HSV-1 is capable of inducing necroptosis in primary astrocytes by both a RIPK1 independent ZBP1-mediated pathway and a RIPK1-mediated ZBP1 independent pathway.

3.2.3 ZBP1 mediates both necroptotic and apoptotic cell death pathways in virally challenged astrocytes

Since both RIPK1- and ZBP1-mediated necroptosis have been shown to require RIPK3 activity to phosphorylate MLKL (Upton and Kaiser 2017; Jiao et al. 2020), we have inhibited RIPK3 with the inhibitor GSK872 to determine whether necroptosis is the primary mechanism underlying HSV-1-induced cell death. Surprisingly, a significant difference remained between ZBP1 expressing and ZBP1 deficient astrocytes in the rate of death following challenge with the neuroinvasive HSV-1(MacIntyre) clinical HSV-1 strain, the HSV-1(F)-ICP6-RHIM Mut virus, or the parental HSV-1(F) ICP6 expressing strain, and in the final percentage of cell death at 24 hrs for the HSV-1(MacIntyre) and HSV-1(F)-ICP6-RHIM Mut strains (Figures 5 and 8A) following treatment with the RIPK3 inhibitor, despite an absence of detectable phosphorylated MLKL expression our group previously showed (Figure 8B) (Jeffries et al. 2022). Similar results were obtained when another inhibitor of RIPK3, GSK843, was employed, with a significant difference remaining between ZBP1 expressing and ZBP1 deficient astrocytes in the final percentage of cell death at 24 hrs following challenge with HSV-1(MacIntyre) (Figure 7A) or HSV-1(F) (data not shown), despite very low levels of phosphorylated MLKL (Figure 7B). As such, these data indicate that necroptosis is not the sole mechanism underlying ZBP1-mediated astrocytic cell death.

To determine whether ZBP1 mediated astrocyte cell death also occurs via apoptosis, we performed parallel experiments using the pan caspase inhibitor zVAD-FMK or the caspase-8 inhibitor Z-IETD-FMK. As shown in Figures 5 and 8C, pan caspase inhibition prevented significant differences in the final percentage of cell death at 24 hrs between ZBP1+/+ and ZBP1-/- derived cells following infection with any HSV-1 strain. Similarly, caspase-8 inhibition also prevented significant differences in the final percentage of cell death at 24 hrs between ZBP1+/+ and ZBP1-/- derived cells following infection with HSV-1(MacIntyre) (Figure 7A) or HSV-1(F) (data not shown). In contrast, significant differences in the rates of cell death remained between cells expressing ZBP1 and those deficient in its expression following challenge with HSV-1(F) and HSV-1(F)-ICP6-RHIM Mut strains in the presence of the pan caspase inhibitor, but it is noteworthy that the lack of difference in final death percentage appears to be due primarily to a net increase in cell death in ZBP1-/- derived cells rather than a reduction in ZBP1+/+ cells (Figures 5, 7A, and 8C).

Some studies have suggested that caspase inhibition may promote RIPK1 activation leading to necroptosis (Degterev et al. 2005). To assess this possibility, we measured phosphorylated MLKL protein levels in HSV-1(F) and HSV-1(F)-ICP6-RHIM Mut infected astrocytes following caspase-8 or pan caspase inhibition. As shown in Figure 7B and 8D, both ZBP1+/+ and ZBP1-/- derived astrocytes showed similar levels of phosphorylated MLKL with either HSV-1 strain in the presence of Z-IETD-FMK or zVAD-FMK, respectively. These results demonstrate that ZBP1 independent necroptosis in astrocytes occurs in the absence of caspase activity.

To directly determine if RIPK1 activation is responsible for the higher cell death rate seen in ZBP1-/ derived astrocytes following caspase inhibition, we simultaneously treated

ZBP1+/+ or ZBP1-/- derived astrocytes with a RIPK1 inhibitor (GSK936) and a pan caspase inhibitor (zVAD-FMK) following infection with HSV-1(MacIntyre), HSV-1(F), and HSV-1(F)-ICP6-RHIM Mut strains. The percentage of cell death at 24 hrs (Figures 5 and 9A) and kinetics of cell death remained significantly different between ZBP1+/+ and ZBP1-/- derived astrocytes in the presence of GSK936 and zVAD-FMK following challenge with either HSV-1(F) or HSV-1(F)-ICP6-RHIM Mut, indicating that caspase inhibition permits RIPK1-mediated necroptosis following infection. Similarly, the percentage of cell death at 24 hrs remained significantly different between ZBP1+/+ and ZBP1-/- derived astrocytes following challenge with HSV-1(F)-ICP6-RHIM Mut in the presence of the alternate RIPK1 inhibitor GSK547 and a caspase-8 inhibitor, with a similar trend seen with the HSV-1(F) strain (data not shown). However, it should be noted that neither the GSK936 and zVAD-FMK (Figures 5 and 9A) nor the GSK547 and Z-IETD-FMK (Figure 7A) inhibitor combinations prevented significant differences in the final percentage of cell death or kinetics of cell death between ZBP1+/+ and ZBP1-/- derived astrocytes challenged with HSV-1(MacIntyre).

Since neither pan caspase nor RIPK3 inhibition alone significantly reduced the percentage of cell death at 24 hrs in virally challenged ZBP+/+ derived astrocytes (Figures 5 and 8), we investigated whether simultaneous activation of both pathways could account for the differences in cell death seen between ZBP1+/+ and ZBP-/- astrocytes. To accomplish this, we treated ZBP1+/+ and ZBP1-/- derived astrocytes with both a RIPK3 inhibitor (GSK872) and a pan caspase inhibitor (zVAD-FMK) following infection with HSV-1(MacIntyre), HSV-1(F), and HSV-1(F)-ICP6-RHIM Mut strains. The percentage cell death at 24 hrs and the death rates following infection of ZBP1+/+ cells with all strains were all significantly lower in the presence of this inhibitor combination (Figures 5 and 9B). Interestingly, while the kinetics of cell death

remained significantly different between ZBP1+/+ and ZBP1-/- derived cells following infection with the HSV-1(F) and HSV-1(F)-ICP6-RHIM Mut strains, the percentage cell death induced by all HSV-1 strains at 24 hrs was reduced in ZBP1+/+ derived astrocytes to levels that were not significantly different from those seen in ZBP1-/- astrocytes (Figures 5 and 9), and co-treatment significantly increased PFU release from wild type ZBP1 expressing astrocytes as assessed by plaque assays in Vero cells (13.93 +/- 0.52 X 10^5 PFU/ml versus 0.64 +/- 0.06 X 10^5 PFU/ml in GSK872/ zVAD-FMK treated versus untreated cells, respectively, $p < 0.05$, $n = 3$). However, it should be noted that this was not the case for HSV-1(MacIntyre) infected cells treated with GSK843 and Z-IETD-FMK (Figure 7B) and the reason for this disparity is unclear. Taken overall, however, these studies indicate that ZBP1 mediates both apoptotic and necroptotic cell death pathways in virally challenged primary murine astrocytes that can serve to restrict HSV-1 replication.

3.2.4 ZBP1 mediates apoptotic and necroptotic cell death in microglia

As shown in Figure 10A, ZBP1+/+ derived microglia displayed a significantly higher rate of cell death compared to ZBP1 deficient microglia for MacIntyre, ICP6, and ICP6 RHIM Mut viruses (MacIntyre: 3.85 ± 1.15% death·hrs^{-1} vs. 3.51 ± 1.61% death·hrs^{-1}, ICP6: 2.03 ± 0.10% death·hrs^{-1} vs. 1.17 ± 0.07% death·hrs^{-1}, ICP6 RHIM Mut: 2.06 ± 0.10% death·hrs^{-1} vs. 0.96 ± 0.07% death·hrs^{-1}, respectively, $p < 0.05$) (Figure 10A). Similar to astrocytes, treatment with the RIPK1 inhibitor had no effect on the rate or percentage of microglial cell death at 24 hrs (Figure 10B) (MacIntyre: 3.60 ± 1.47% death·hrs^{-1} vs. 2.64 ± 2.30% death·hrs^{-1}, ICP6: 2.19 ± 0.07% death·hrs^{-1} vs. 0.70 ± 0.07% death·hrs^{-1}, ICP6 RHIM Mut: 1.90 ± 0.06% death·hrs^{-1} vs. 0.54 ± 0.05% death·hrs^{-1}, $p < 0.05$). Furthermore, ZBP1+/+ and ZBP1-/- derived microglia treated with a RIPK3 inhibitor continued to have significant differences in the rate of cell death for all strains

(MacIntyre: 3.50 ± 1.25% death·hrs^{-1} vs. 3.15 ± 1.81% death·hrs^{-1}, ICP6: 1.80 ± 0.10% death·hrs^{-1} vs. 1.11 ± 0.03% death·hrs^{-1}, ICP6 RHIM Mut 1.56 ± 0.1% death·hrs^{-1} vs. 0.85 ±0.04% death·hrs^{-1}, $p < 0.05$) (Figure 10C), suggesting that necroptosis may not be the only pathway activated by ZBP1. Finally, treatment with the pan caspase inhibitor zVAD-FMK appears to diminish the rate of cell death in infected ZBP1+/+ derived cells (MacIntyre: 2.70 ± 0.41% death·hrs^{-1}, ICP6: 1.58 ± 0.10% death·hrs^{-1}, ICP6 RHIM Mut: 1.35 ± 0.05% death·hrs^{-1}) while increasing cell death rate in infected ZBP1 deficient microglia to comparable levels (MacIntyre: 2.55 ± 0.89% death·hrs^{-1}, ICP6: 1.37 ± 0.10% death·hrs^{-1}, ICP6 RHIM Mut: 1.04 ± 0.06% death·hrs^{-1}) (Figure 10D), possibly due to RIPK1 activity as observed in astrocytes (Figure 7). As such, it appears likely that ZBP1 mediates virally-induced cell death in microglia in a similar manner to that seen in astrocytes.

3.3 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 HSV encephalitis is attributable either to a 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 determined that murine glia express ZBP1 and showed that this cytosolic nucleic acid sensor contributes to glial inflammatory responses to HSV-1 that are damaging to neuronal cells (Furr et al. 2011; Crill et al. 2015b). In the present study, we demonstrate a role for this sensor in HSV-1 restriction and the initiation of cell death path- ways in glia. We show that the loss of ZBP1 in primary murine astrocytes results in a significant increase in the release of PFUs following infection with a neuroinvasive clinical strain of HSV-1 (Krinke and Dietrich 1990). Interestingly, this increase in infectious viral particle release does not appear to result from changes in IFN or inflammatory cytokine production as evidenced by the minimal production of IFN-β by astrocytes following infection or the lack of a significant effect of ZBP1 deficiency on HSV-1-induced release of this antiviral mediator or the key inflammatory cytokines IL-6 and TNF (Jeffries et al. 2022), in addition to the lack of effect of STAT1 inhibition on PFU increases.

While the present results might seem at odds with our prior studies utilizing siRNA approaches (Furr et al. 2011), it must be noted that we previously showed that infection of astrocytes with HSV-1(McIntyre) at a high dose (MOI of 10) elicits robust IL-6 and TNF production that is sensitive to siRNA-mediated ZBP1 knockdown, while lower MOIs do not. As

such, the present demonstration that infection of murine astrocytes with HSV-1 at lower doses (MOI of 0.2–2.0) elicits low level inflammatory cytokine release that is not significantly different between wild-type and ZBP1-deficient cells is consistent with our previous findings (Jeffries et al. 2022).

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 previously demonstrated that HSV-1 can initiate necroptosis in a ZBP1-dependent manner in astrocytes, as evidenced by the activation of the necroptotic marker MLKL (Jeffries et al. 2022). Additionally, our results are also consistent with an ability of the HSV-1 product ICP6 to activate necroptosis in murine astrocytes independent of ZBP1 via RIPK1, an ability that has previously been described in other mouse cell types (Wang et al. 2014; Huang et al. 2015; Guo et al. 2018). The detection of ICP6 and subsequent initiation of necroptosis could serve to restrict viral replication and/or exacerbate inflammatory host responses in mice. However, this HSV-1 product does not appear to induce necroptosis in human cells, and so we have additionally employed the HSV-1(F)- ICP6-RHIM Mut strain to circumvent direct ICP6- induced necroptosis in the present study to distinguish ZBP1-mediated effects that might be relevant to human cells.

Surprisingly, inhibition of necroptosis using RIPK3 inhibitors failed to significantly reduce the rate or percentage of cell death in ZBP1-expressing astrocytes to the levels seen in ZBP1-deficient cells following challenge with laboratory and clinical strains of HSV-1. Furthermore, inhibition of apoptosis with a pan caspase or caspase-8 inhibitors also failed to reduce virally induced cell death in ZBP1+/+ astrocytes. However, while inhibition of either pathway alone could not reduce cell death in WT astrocytes during infection, it was reduced by

simultaneous inhibition of both apoptosis and necroptosis, and this was associated with a significant increase in infectious particle release as assessed by conventional plaque assays. This suggests that ZBP1 contributes to both cell death pathways in astrocytes and these serve to restrict replication of this DNA virus.

Interestingly, our group has previously shown that inhibition of apoptotic pathways appears to permit HSV-induced necroptosis in astrocytes that is independent of ZBP1, as treatment with a pan caspase or a caspase-8 inhibitor did not reduce levels of phosphorylated MLKL following infection in ZBP1-deficient cells (Jeffries et al. 2022). Indeed, inhibition of caspase-8 led to a seemingly paradoxical increase in the rate of death in ZBP1−/− derived astrocytes. However, prior studies have indicated that caspase-8 can act as a negative regulator of RIPK1-medi-ated necroptosis (Pasparakis and Vandenabeele 2015; Silke et al. 2015; Weinlich et al. 2017; Grootjans et al. 2017). Furthermore, Mandal and coworkers (Mandal et al. 2014) have previously demonstrated that RIPK3 can initiate apoptosis independent of its kinase activity. In these studies, they showed that RIPK3 can associate with RIPK1 via their respective RHIM domains in the presence of RIPK3 kinase inhibitors, leading to the recruitment and activation of caspase-8 that results in apoptosis (Mandal et al. 2014). In the present study, we have shown that increases in the rate of virally induced death in ZBP1−/− derived astrocytes following pan caspase inhibition are abolished by the simultaneous inhibition of RIPK1, consistent with a similar role for caspases as negative regulators of RIPK1-induced necroptosis in glia.

As such, we propose that ZBP1 functions as an intracellular sensor for DNA viruses, such as HSV-1, and induces both apoptotic and necroptotic cell death signaling pathways in mouse astrocytes via multiple signaling pathways. In this model, the release of genetic material or transcription of HSV-1 genes leads to nucleic acid recognition by ZBP1 and its subsequent

association with RIPK3 via their respective RHIM domains, which results in phosphorylation of MLKL and the execution of necroptosis. In addition, ZBP1 association with RIPK3 can also induce apoptosis via an, as yet, unknown mechanism. Alternatively, the HSV-1 viral protein ICP6 can directly interact with RIPK1 and/or RIPK3 via each of their RHIM domains causing RIPK1 and RIPK3 to associate, thereby initiating necroptosis and apoptosis in a similar manner to that caused by ZBP1/RIPK3. Lastly, viral infection elicits the production of TNF that can act in an autocrine/paracrine manner to initiate apoptosis in the presence of functional caspase-8, or necroptosis via the association of RIPK1 with RIPK3 in its absence, as demonstrated by Jeffries et al, (Jeffries et al. 2022).

Finally, we have shown that such virally-induced cell death mechanisms also appear to be present in microglia. Similar to astrocytes, we found that ZBP1 deficient microglia show a reduced rate of cell death following HSV-1 infection, consistent with a significant role for this sensor in these responses. Furthermore, cell death rate is increased in ZBP1 deficient microglia in the presence of a pan caspase inhibitor following infection with either laboratory or clinical neuroinvasive HSV-1 strains, likely in a similar manner to that seen in astrocytes. However, while rates of cell death showed statistically significant differences in these studies, there were no significant differences in the final percentages of cell death at 24 hrs and so cell type specific differences may exist between astrocytes and microglia with regard to the degree to which these viral stimuli initiate these cell death pathways. Regardless, it is clear that ZBP1 can mediate cell death in HSV-1 infected microglia via similar mechanisms to those in astrocytes.

It should be noted that the present study does not preclude the involvement of other nucleic sensors, such as cGAS-STING, or even RIG-I, in glial immune and cell death responses to HSV-1, perhaps in a cooperative or cell-type specific manner. Indeed, a recent study has

suggested that cGAS-STING can mediate type I IFN-independent apoptotic cell death in murine micro- glia in vitro and in vivo following HSV-1 infection at high doses (Reinert et al. 2016). Although, our studies suggest that this DNA sensor system fails to mediate effective antiviral immune responses to HSV-1 in human microglial cells, despite being important in their IFN-β responses (Jeffries et al. 2020). Furthermore, the relative importance of such ZBP1-mediated responses in host defense against DNA virus challenge remains to be determined in vivo and it is, as yet, unclear whether such mechanisms operate in human glia in response to HSV-1 infection.

3.4 FIGURES

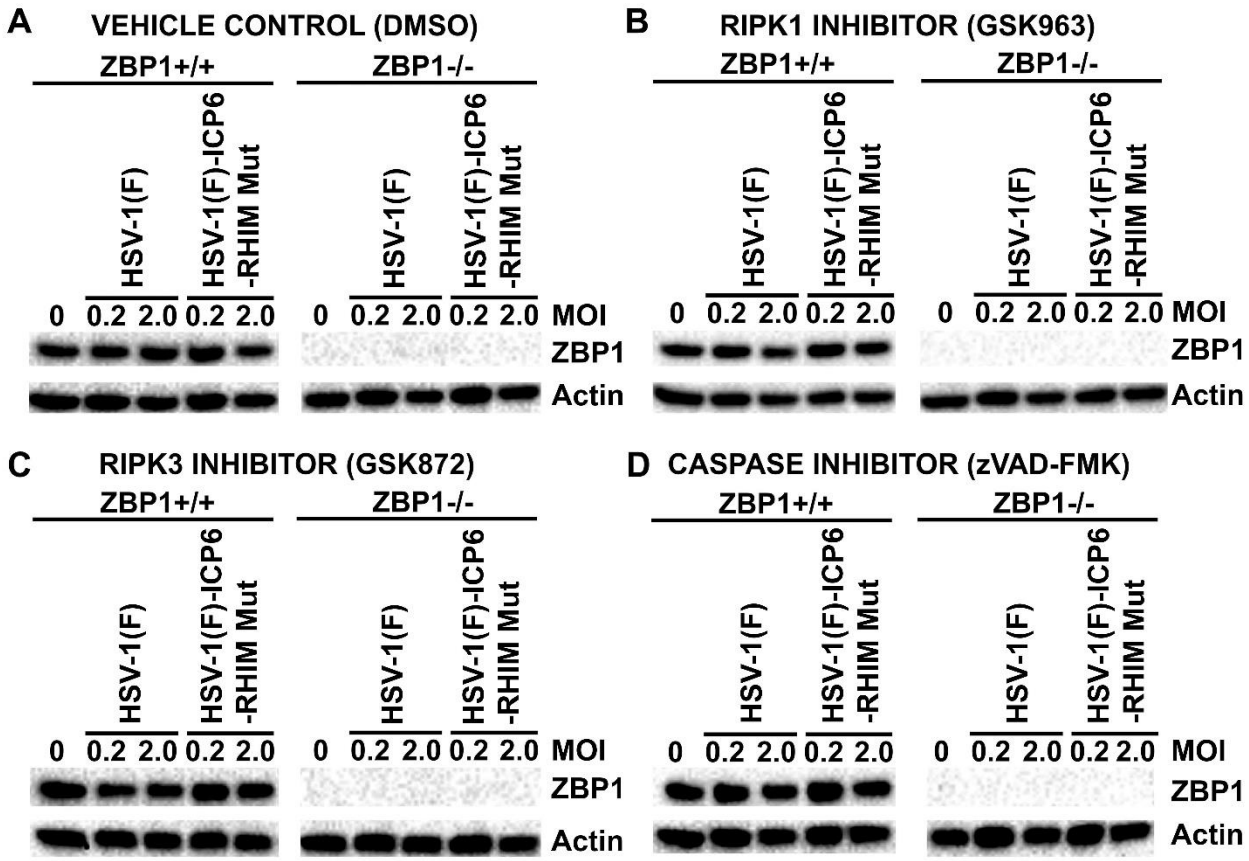

Fig. 3 Confirmation of ZBP1 deficiency in ZBP1-/- derived astrocytes.
ZBP1+/+ and ZBP1-/- derived primary murine astrocytes were infected with HSV-1(F) or HSV-1(F)-ICP6-RHIM Mut and, one hour following infection, were treated with (A) DMSO (vehicle), (B) GSK963 (1 μM), (C) GSK872 (5 μM), (D) zVAD-FMK (20 μM). At 24 hours following treatment total cell lysates were collected and analyzed for the presence of ZBP1 or the house keeping gene β-actin by immunoblot analysis (Jeffries et al. 2022).

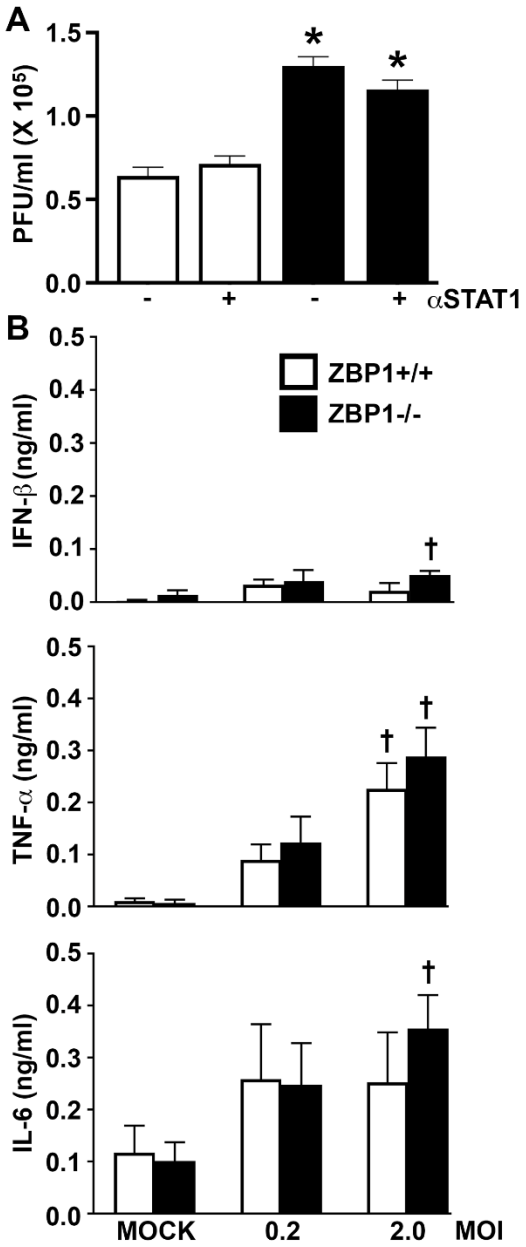

Fig 4. ZBP1 restricts HSV-1 replication in astrocytes in a manner that is independent of interferon production.

Panel A: Murine astrocytes derived from wild type mice (ZBP1+/+) or animals deficient in the expression of ZBP1 (ZBP1-/-) were infected with HSV-1(MacIntyre) at an MOI of 0.2 or 2.0 for 60 min and then untreated or treated with the STAT1 inhibitor Fludarabine (10 μM). At 24 hours, cell free supernatants were collected and the number of plaque-forming units (PFU) released from HSV-1 infected astrocytes were determined by conventional plaque assays in Vero cells. Panel B: Murine astrocytes derived from ZBP1+/+ or ZBP1-/- mice were infected with HSV-1 at an MOI of 0.2 or 2.0. At 24 hours, the concentration of IFN-β, IL-6, and TNF, in cell free supernatants was quantified by specific capture ELISAs (Jeffries et al. 2022). Data is presented as the mean of at least three independent experimental replicates +/- the SEM. An asterisk indicates a significant difference from similarly treated ZBP1+/+ cells and dagger symbols indicate a significant difference from mock infected cells ($p < 0.05$; $n = 3$).

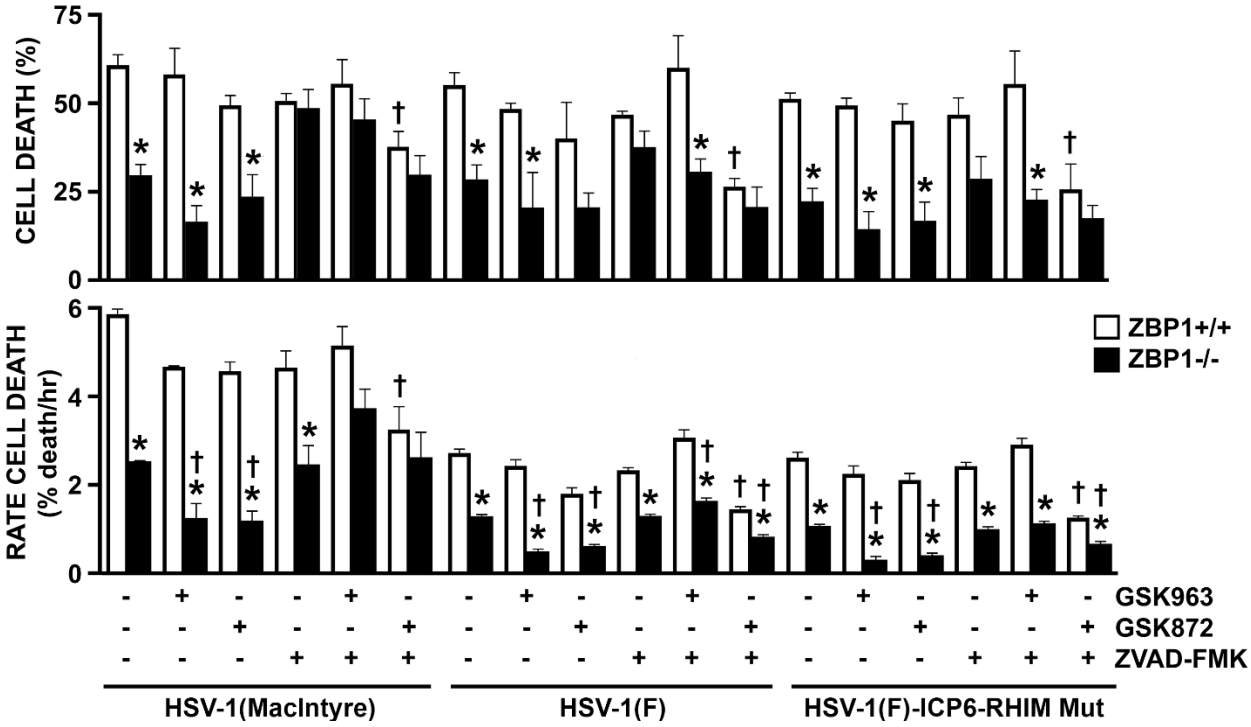

Fig. 5 ZBP1 mediates HSV-1 induced cell death in primary murine astrocytes.
ZBP1+/+ and ZBP1-/- murine astrocytes were infected with HSV-1(MacIntyre), HSV-1(F)-ICP6-RHIM Mut, or its parental ICP6 expressing parental strain (HSV-1(F)). One hour following infection, cells were treated with DMSO vehicle control, the RIPK1 inhibitor GSK963 (1 μM), the RIPK3 inhibitor GSK872 (5 μM), and/or the pan caspase inhibitor zVAD-FMK (20 μM). Cell viability was measured every two hours with a RealTime-Glo™ MT assay beginning at two hours following infection and data is reported as the percentage of dead cells at 24 hrs relative to non-infected controls (cell death) and as the rate of cell death. Data is shown as the mean of 3-6 independent experiments +/- SEM. Asterisks indicate a significant difference from similarly treated ZBP1+/+ cells while dagger symbols indicate significant difference from similarly challenged cells treated with DMSO vehicle only.

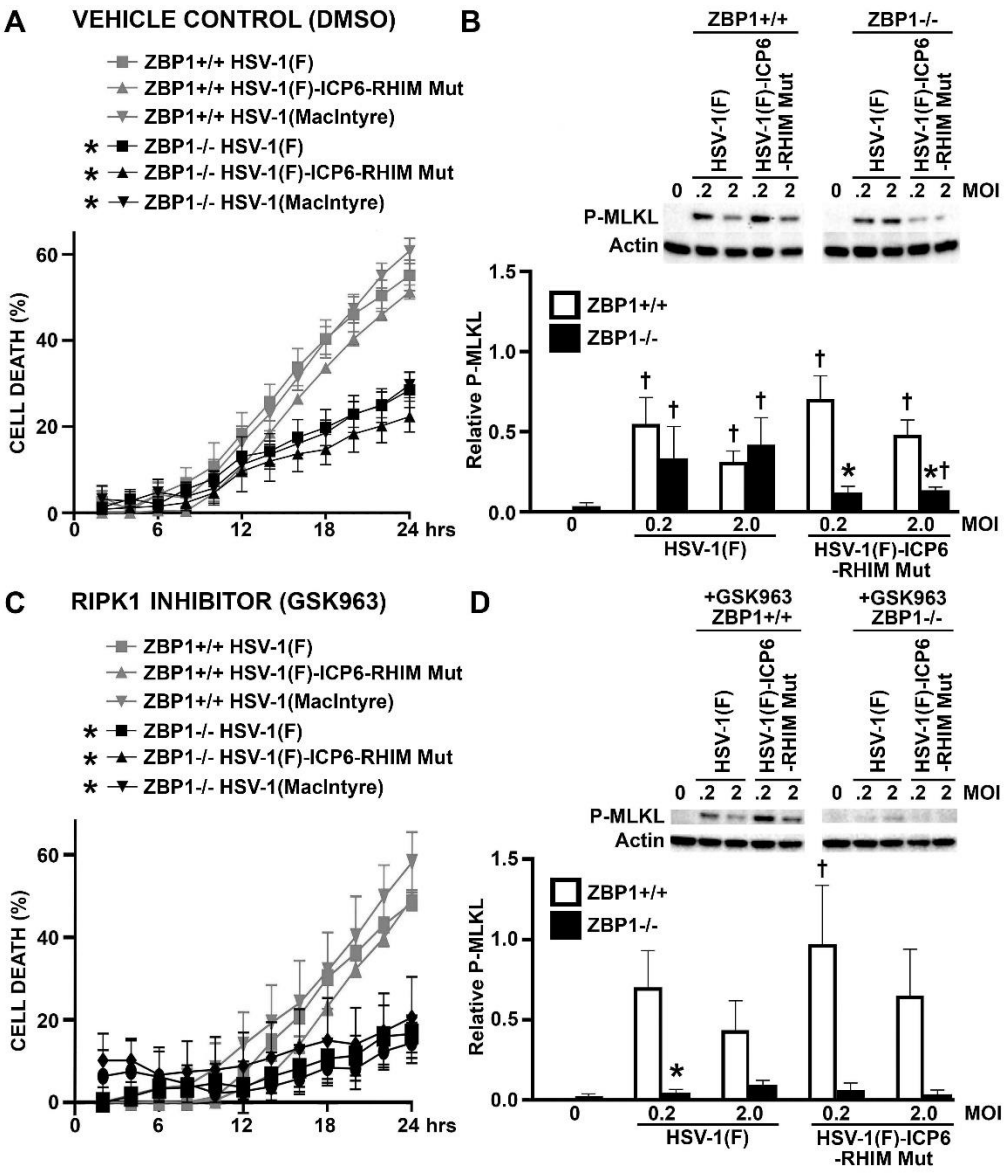

Fig. 6 HSV-1 induces necroptotic cell death in primary astrocytes by both a RIPK1 independent ZBP1-mediated pathway and a RIPK1-mediated ZBP1 independent pathway. ZBP1+/+ and ZBP1-/- murine astrocytes were infected with HSV-1(MacIntyre), HSV-1(F)-ICP6-RHIM Mut, or its parental ICP6 expressing parental strain (HSV-1(F)). One hour following infection, cells were treated with either DMSO vehicle control (A and B) or the RIPK1 inhibitor GSK963 (1 μM) (C and D). Panels A and C: Cell viability was measured every two hours with a RealTime-Glo™ MT assay beginning at two hours following infection. Panels B and D: At 24 hrs, total cell lysates were collected and analyzed for the presence of phosphorylated MLKL (P-MLKL) or the housekeeping gene product β-actin (Actin) by immunoblot analysis (Jeffries et al. 2022). Relative P-MLKL expression was determined by densitometric analysis and normalized to β-actin expression levels. Data is shown as the mean of three independent experiments +/- SEM. An asterisk indicates a significant difference in final cell death or P-MLKL expression from similarly treated ZBP1+/+ cells and dagger symbols indicate a significant difference from uninfected cells.

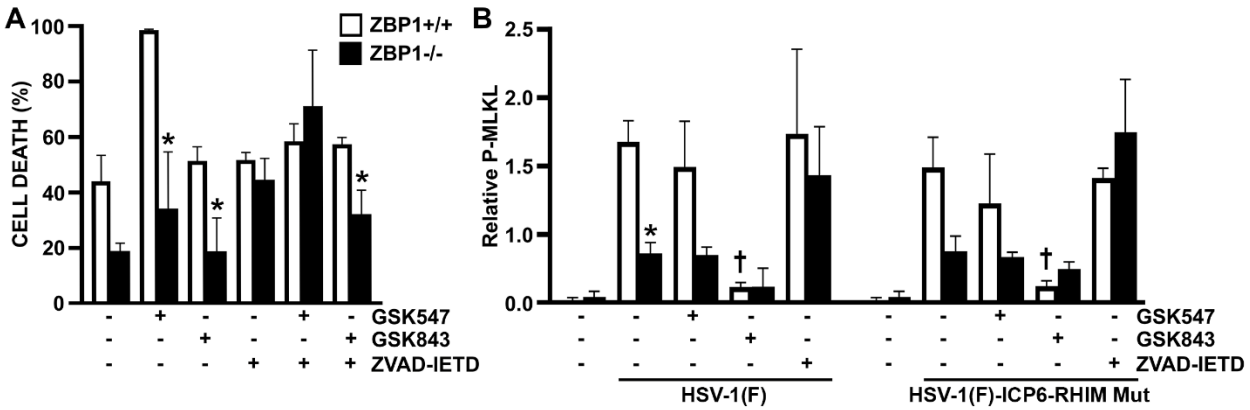

Fig. 7 The notion that ZBP1 mediates multiple cell death pathways in HSV-1 challenged primary murine astrocytes is supported by similar results obtained using alternative pharmacological inhibitors.

ZBP1+/+ and ZBP1-/- murine astrocytes were infected with HSV-1(MacIntyre) (Panel A), or HSV-1(F)-ICP6-RHIM Mut or its parental ICP6 expressing parental strain (HSV-1(F)) (Panel B). One hour following infection, cells were treated with either DMSO vehicle control, the RIPK1 inhibitor GSK547 (50 nM), the RIPK3 inhibitor GSK843 (2 μM), and/or the caspase-8 inhibitor Z-IETD-FMK (20 μM). Panel A: Cell viability was measured at 24 hrs following infection with a RealTime-Glo^{TM} MT assay. Panel B: At 24 hrs, total cell lysates were collected and analyzed for the presence of phosphorylated MLKL (P-MLKL). Relative P-MLKL expression determined by densitometric analysis is shown normalized to β-actin expression levels. Data is shown as the mean of three independent experiments +/- SEM. An asterisk indicates a significant difference in final cell death or P-MLKL expression from similarly treated ZBP1+/+ cells and dagger symbols indicate a significant difference from untreated infected cells.

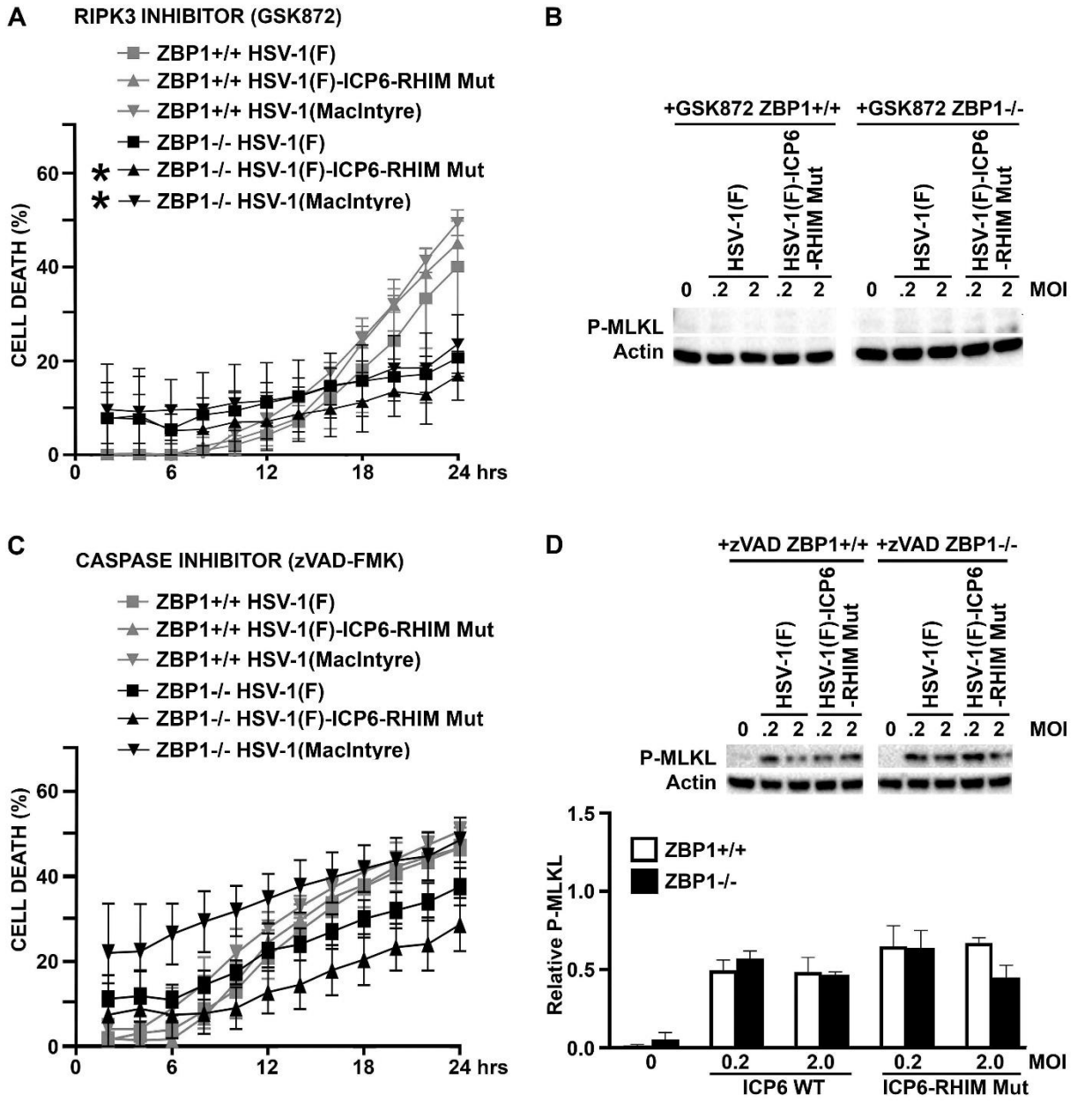

Fig. 8 ZBP1-mediated cell death does not exclusively occur in HSV-1 infected astrocytes via necroptosis.

ZBP1+/+ or ZBP1-/- murine astrocytes were infected with HSV-1(MacIntyre), HSV-1(F)-ICP6-RHIM Mut, or its parental ICP6 expressing parental strain (HSV-1(F)). One hour following infection, cells were treated with either the RIPK3 inhibitor GSK872 (5 μM) (A and B) or the pan caspase inhibitor zVAD-FMK (20 μM) (C and D) . Panels A and C: Cell viability was measured every two hours with a RealTime-Glo™ MT assay beginning at two hours following infection. Panels B and D: At 24 hrs, total cell lysates were collected and analyzed for the presence of phosphorylated MLKL (P-MLKL) or the housekeeping gene product β-actin (Actin) by immunoblot analysis (Jeffries et al. 2022). Relative P-MLKL expression was determined by densitometric analysis and normalized to β-actin expression levels. Data is shown as the mean of three independent experiments +/- SEM. Asterisks indicate a significant difference in final cell death from similarly treated ZBP1+/+ cells.

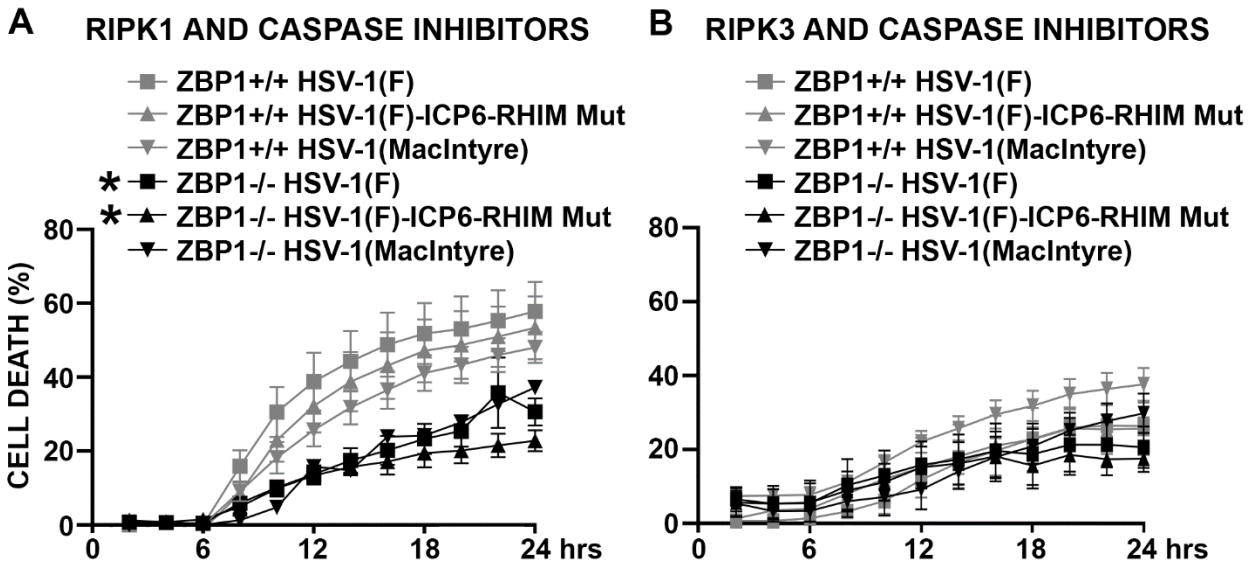

Fig. 9 ZBP1 mediates both apoptotic and necroptotic cell death pathways in HSV-1 challenged primary murine astrocytes.
ZBP1+/+ or ZBP1-/- derived astrocytes were infected with HSV-1(MacIntyre), HSV-1(F)-ICP6-RHIM Mut, or its parental ICP6 expressing parental strain (HSV-1(F)). One hour following infection, cells were treated with either the RIPK1 inhibitor GSK963 (1 μM) plus the pan caspase inhibitor zVAD-FMK (20 μM) (Panel A) or the RIPK3 inhibitor GSK872 (5 μM) plus zVAD-FMK (20 μM) (Panel B). Cell viability was measured every two hours with a RealTime-Glo^{TM} MT assay beginning at two hours following infection. Data is shown as the mean of 4-6 independent experiments +/- SEM. Asterisks indicate a significant difference in final cell death from similarly treated ZBP1+/+ cells.

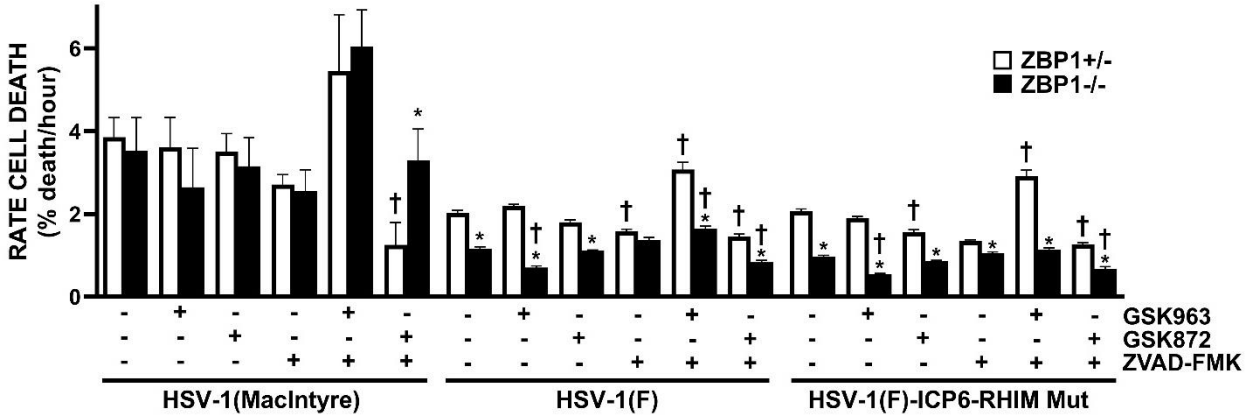

Fig. 10 ZBP1 mediates HSV-1 induced cell death in primary murine microglia.
ZBP1+/+ or ZBP1-/- derived microglia were infected with HSV-1 MacIntyre strain (Mac), ICP6 RHIM mutant virus (ICP6-RHIM Mut), or its parental ICP6 expressing parental strain (ICP6). One hour following infection, cells were treated with either DMSO vehicle, the RIPK1 inhibitor GSK963 (1 μM), the RIPK3 inhibitor GSK872 (5 μM), the pan caspase inhibitor zVAD-FMK (20 μM), or a combination of GSK963 and zVAD-FMK or GSK872 and zVAD-FMK. Cell viability was measured every two hours with a RealTime-Glo^{TM} MT assay beginning at two hours following infection and data is reported as the rate of cell death. Data is shown as the mean of 3-8 independent experiments +/- SEM. Asterisks indicate a significant difference from similarly treated ZBP1+/+ cells while dagger symbols indicate significant difference from similarly challenged cells treated with DMSO vehicle only.

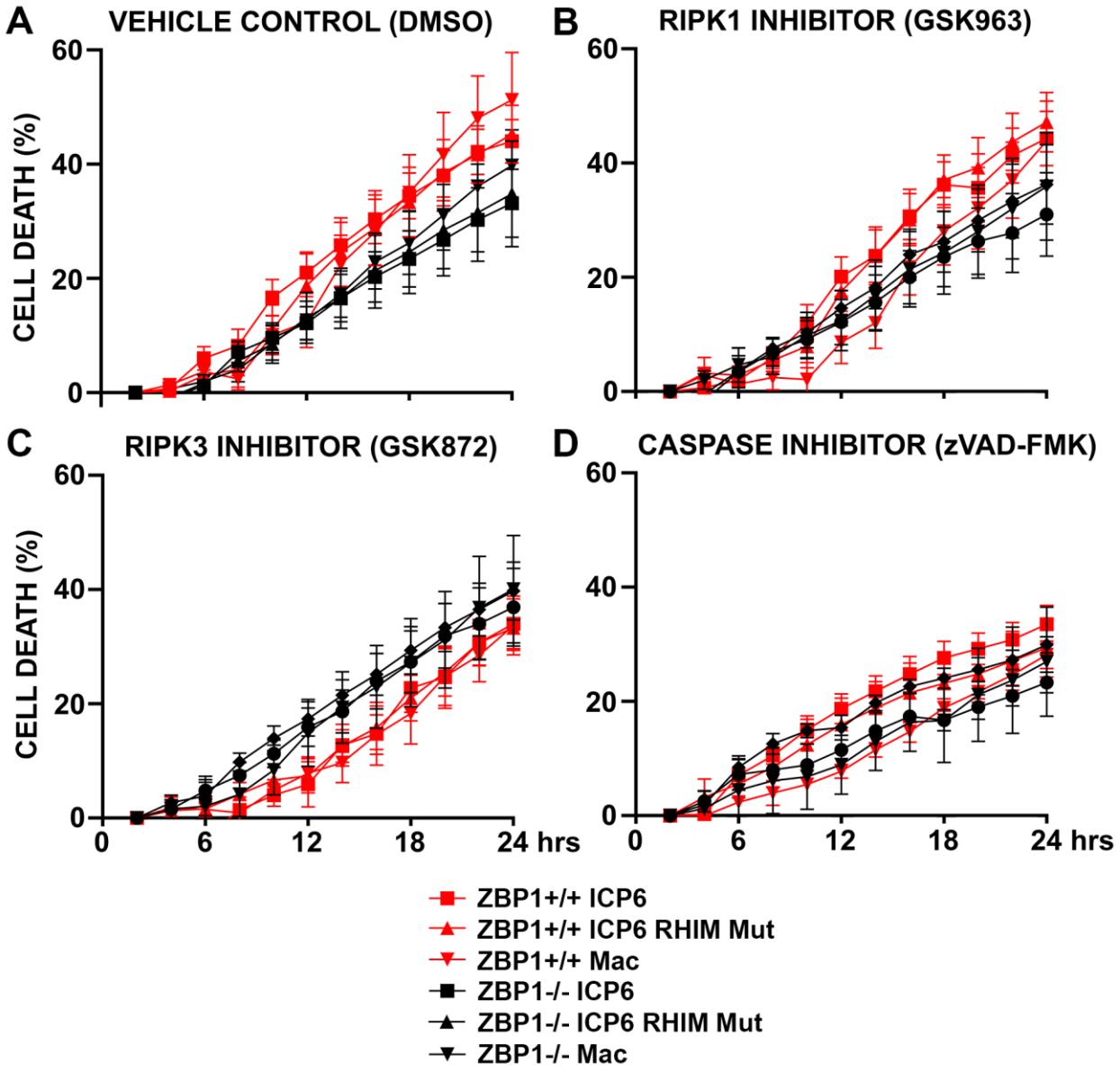

Fig. 11 ZBP1 mediates both apoptotic and necroptotic cell death pathways in HSV-1 challenged primary murine microglia.
ZBP1+/+ or ZBP1-/- derived microglia were infected with HSV-1 MacIntyre strain (Mac), ICP6 RHIM mutant virus (ICP6-RHIM Mut), or its parental ICP6 expressing parental strain (ICP6). One hour following infection, cells were treated with either DMSO vehicle (Panel A), the RIPK1 inhibitor GSK963 (1 μM) (Panel B), the RIPK3 inhibitor GSK872 (5 μM) (Panel C), or the pan caspase inhibitor zVAD-FMK (20 μM) (Panel D). Cell viability was measured every two hours with a RealTime-Glo™ MT assay beginning at two hours following infection. Data is shown as the mean of 3-8 independent experiments +/- SEM.

CHAPTER 4: cGAS mediates inflammatory responses to DNA damage in human microglia

4.1 RATIONALE

Genomic instability that results from excessive DNA damage and/or dysfunctional DNA repair is a major driving force in the development of cancers and fatal age-related diseases, such as Alzheimer's disease and Parkinson's disease (Coppedè and Migliore 2015; Zhu et al. 2019). While breaks in DNA are a relatively common occurrence, exposure to genotoxic insults such as ionizing radiation (IR) or oxidative stress can drastically exacerbate DNA damage and disrupt cellular homeostasis. Accumulating evidence suggests that DNA damage and genomic instability can result in the generation of detrimental inflammatory responses that are often associated with the presence of mitochondrial (mtDNA) and genomic self-DNA in the cytoplasm (Horn and Triantafyllopoulou 2018; Li and Chen 2018b; Krupina et al. 2021; Miller et al. 2021; Taffoni et al. 2021). In healthy cell states, DNA is sequestered to the nucleus or mitochondria. As such, the presence of DNA in the cytoplasm serves as a damage associated molecular pattern (DAMP) to trigger inflammatory innate immune responses. To perceive DAMPs, our cells express specialized pattern recognition receptors (PRRs), such as the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS). Importantly, cGAS has been implicated in the detection of cytosolic mtDNA and genomic self-DNA that arise from DNA damage and genomic instability, making it of particular interest (Gao et al. 2015; Jakobs et al. 2015; Mackenzie et al. 2017; De Gaetano et al. 2021; Kumar 2021). Upon DNA binding, cGAS catalyzes the production of the secondary messenger molecule 2'3'cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), which subsequently binds to the adaptor protein stimulator of interferon genes (STING). cGAMP-STING interaction results in the activation of the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa-B (NF-κB) that mediate transcription of

type-1 IFNs and proinflammatory cytokines and chemokines (Zhong et al. 2008b; Sun et al. 2009; Wu et al. 2013).

In the central nervous system (CNS), it is now appreciated that resident glial cells play a critical role in the initiation of immune responses to DAMPs and pathogen associated molecular patterns (PAMPs) via the expression and activation of multiple families of PRRs, including cytosolic DNA sensors, such as cGAS. (Sterka and Marriott 2006; Sterka et al. 2006; Furr et al. 2008; Cox et al. 2015; Jeffries and Marriott 2017; Wu et al. 2017; Song et al. 2019; Ma et al. 2021; Jin et al. 2021; Rui et al. 2022b).

While the role of cGAS as a mediator of anti-bacterial and viral innate immunity is well documented in both peripheral and resident CNS cells (Ablasser et al. 2013; Gao et al. 2013; Sun et al. 2013; Wu et al. 2013; Cox et al. 2015; Jeffries and Marriott 2017; Jeffries et al. 2020), the mechanisms surrounding self-DNA detection by this molecule and the properties of subsequent downstream proinflammatory responses in the CNS have remained elusive. Here, we have investigated the contribution made by cGAS to inflammatory responses following oxidative stress and IR-mediated DNA damage in human microglia. We confirm that DNA damage results in the release of several key proinflammatory mediators and that cGAS is responsible for such responses in microglia. Additionally, our findings suggest that cGAS-mediated DNA damage responses are potentially detrimental in nature, although more research is needed to further confirm this hypothesis in microglia.

4.2 RESULTS

4.2.1 Ionizing radiation and oxidative stress elicit DNA damage in human microglia

IR and oxidative stress have been consistently demonstrated to elicit DNA damage (As reviewed here: (Einor et al. 2016)). Nonetheless, we confirmed the ability of these treatments to induce DNA damage in a human microglial cell line (hμglia). Wild type (WT) hμglia were exposed to a single accumulating dose of IR (0, 10, 20, or 30 Gy at 6.5 Gy/min) or a bolus dose of hydrogen peroxide (H_2O_2, 0, 25, 100, or 250 uM) and γH2AX fluorescence was determined by immunofluorescent microscopy and subsequent ImageJ analysis. As shown in Figs. 12A and B, mean γH2AX fluorescence was significantly greater 30 min., 1-hr, and 2-hrs post treatment in cells exposed to 10, 20, or 30 Gy IR. Additionally, γH2AX fluorescence was significantly greater in cells 1-hr post treatment with 250 uM H2O2, and 2-hrs post treatment with 100 uM H_2O_2 or 250 uM.

Furthermore, we assessed the ability of IR to induce micronucleus formation in WT hμglia. WT hμglia were exposed to a single accumulating dose of IR (1 Gy at 6.5 Gy/min) and subsequently stained with DAPI for nucleus visualization at 24- and 36 hours post treatment. As shown in Fig. 12C, IR exposure resulted in significantly higher percentages of cells that possess micronuclei (5.5% in untreated vs. 27.4% in IR-treated at 24 hours and 2.5% in untreated vs. 28.2% in IR-treated at 36 hours, $p < 0.05$, $n = 3$). Taken together, these data are consistent with other reports (Einor et al. 2016) that IR and oxidative stress induce DNA damage, and that IR induces micronucleus formation in the human microglial cell line, hμglia.

4.2.2 DNA damage elicits proinflammatory mediator production in human microglia challenged with ionizing radiation and oxidative stress

Recent studies have shown that DNA damage is capable of eliciting innate immune responses in peripheral cell types (Horn and Triantafyllopoulou 2018; Li and Chen 2018b; Maekawa et al. 2019; Taffoni et al. 2021). To determine if such responses are present in microglia, we exposed WT hμglia to IR, H_2O_2, and the oxidative stress inducing GOX/CAT system (as described in (Mueller et al. 2009)), and measured proinflammatory mediator release. As shown in Fig 13A, IL-8 secretion was significantly higher 24 hours post exposure to 75 and 100 Gy IR in comparison to untreated cells. Additionally, similar release was seen at 24 hours post treatment with 25 uM bolus H_2O_2 and 1:50K CAT dilutions (Fig. 13B and C). Taken together, these results confirm that DNA damage elicits proinflammatory immune responses in human microglia, similar to previous studies detailing this effect in peripheral cells (Horn and Triantafyllopoulou 2018; Li and Chen 2018b; Maekawa et al. 2019; Taffoni et al. 2021).

4.2.3 cGAS mediates proinflammatory immune molecule production in human microglia following DNA damage

Several studies have shown that cGAS can mediate immune responses to genotoxic stress in human and murine peripheral cell types (Liu et al. 2016; de Oliveira Mann and Kranzusch 2017; Mackenzie et al. 2017; Taffoni et al. 2021; Wu et al. 2021; Zhao et al. 2021b). Accordingly, we have examined whether these same responses are present in human microglia. To achieve this, we utilized a human cGAS knockdown (cGAS +/-) microglial cell line previously generated by our laboratory via CRISPR/Cas9 technology (Jeffries et al. 2020) to determine the effects of cGAS deficiency on proinflammatory mediator production following the

same genotoxic insults seen in Figure 15. As shown in Figure 15A, cGAS +/- hμglia showed significantly reduced secretion of the neutrophil recruitment factor, IL-8, 24h post-exposure to 75 and 100 Gy IR. Interestingly, this reduction was not seen in cGAS +/- cells treated with the bacterial ligand, lipopolysaccharide (LPS) (Fig. 15A). Additionally, similar reduction was found 24h post-treatment with bolus H_2O_2 and treatment with the GOX/CAT oxidative stress inducing system at 15 and 25 uM H_2O_2 and 50K CAT dilution, respectively (Fig. 15B and C). Again, this reduction was not present in LPS treated microglia (Fig. 15B and C). Together, these data indicate that cGAS is, at least in part, responsible for the proinflammatory immune responses seen following genotoxic stress in human microglia.

4.2.4 siRNA targeting cGAS lessens proinflammatory immune molecule secretion in human microglia challenged with ionizing radiation

While previous studies in our laboratory have shown the CRISPR/Cas9 mediated knockdown of cGAS in our microglial cell line was successful and had limited off-target effects (Fig. 14, (Jeffries et al. 2020)), we nonetheless aimed to verify the knockdown data seen in Figure 15 with an alternative approach. To achieve this, cGAS +/+ hμglia were transfected with siRNA targeting cGAS for 24 hours. Successful knockdown was verified via immunoblot assay and densiometric analysis (Fig. 16). Interestingly, siRNA mediated knockdown of cGAS successfully attenuated production of the proinflammatory cytokine IL-6 and the neutrophil recruiting factor IL-8 compared to wild type microglia transfected with a scramble siRNA negative control, as shown in Figure 17A and B. Together, these data verify the previously obtained results contending that cGAS mediates microglial responses to IR exposure.

4.2.5 cGAS knockdown permits higher γH2AX expression following IR and H_2O_2 exposure in human microglia

To determine if previous results were due to a reduction in DNA damage, cGAS +/- hμglia were exposed to the same IR and H2O2 treatments as in Figure 12. Similar to cGAS +/+ cells, cGAS +/- hμglia showed significantly higher mean fluorescence of γH2AX 30 min., 1-hr, and 2-hrs post treatment in cells exposed to 10, 20, or 30Gy IR compared to untreated cells, in most experimental groups (Fig. 18A-E). Intriguingly, cGAS +/- hμglia expressed significantly higher mean fluorescence of γH2AX than cGAS +/+ cells 30 minutes post treatment with 10 and 20 Gy IR (Fig. 18A), 1hr post treatment with 10, 20, and 30 Gy IR (Fig. 18B), 2hr post treatment with 10 and 30 Gy IR (Fig. 18C), and 1hr post treatment with 250 uM H_2O_2 (Fig. 18D). We hypothesized that these results could be due to the cGAS +/- cells having higher background levels of γH2AX, however, mean γH2AX fluorescence between untreated cGAS +/+ and cGAS +/- hμglia only reached significance at the 30-minute time-point in the IR experimental group (Fig. 18A), which does not explain the significant differences in the other experimental groups at different time points. These data could be explained by a recent publication that suggests a role for cGAS in suppressing genomic instability by slowing down DNA replication dynamics at the replication fork (Chen et al. 2020). Together, these results indicate that cGAS deficiency may permit higher levels of DNA damage in human microglia.

4.3 DISCUSSION

Genomic instability is a key driving force for the development of cancers and age-related diseases (Coppedè and Migliore 2015; Zhu et al. 2019). Recently, DNA damage and genomic instability have been found to generate potentially detrimental inflammatory responses, most commonly via the presence of DAMPs such as mtDNA and genomic self-DNA in the cytosol (Horn and Triantafyllopoulou 2018; Li and Chen 2018b; Maekawa et al. 2019; Taffoni et al. 2021). Such DAMPs are recognized by cytosolic PRRs, such as cGAS, that detect foreign and self-DNA and initiate responses to them. As neuroinflammation can prove incredibly detrimental, if not fatal, if proinflammatory responses are not tightly regulated, it is critical to determine the mechanisms by which resident glial cells, such as microglia, perceive and respond to DNA damaging events via sensors like cGAS.

Our laboratory has previously demonstrated that human microglia and astrocytes express cGAS and that it is capable of detecting and responding to exogenous sources of cytosolic DNA (Jeffries and Marriott 2017; Jeffries et al. 2020). Furthermore, cGAS' role in the detection of mtDNA and self-DNA detection in peripheral cells is well documented (West et al. 2015; Liu et al. 2016; de Oliveira Mann and Kranzusch 2017; Harding et al. 2017; Motwani and Fitzgerald 2017; Bakhoum et al. 2018; Maekawa et al. 2019; Sharma et al. 2020; Guo et al. 2020; Huang et al. 2020; Zhao et al. 2021a; Mohr et al. 2021; Zhang et al. 2022; Nadalutti et al. 2022). In the present study, we describe cGAS' role in mediating human microglial inflammatory responses to DNA damage. First, we show that cGAS deficiency results in significantly decreased secretion of the proinflammatory cytokine IL-8. Importantly, we also show that this response is specific to DNA damaging treatments, as treatment with LPS did not show similar reduction. Second, we provide evidence of cGAS playing a neuroprotective role, with regards to genomic instability, as

cGAS +/- hμglia expressed significantly higher mean fluorescence of the DNA damage marker γH2AX when compared to cGAS +/+ hμglia treated with IR and H_2O_2. Interestingly, although DNA damage was seemingly exacerbated in cGAS deficient cells, proinflammatory mediator secretion was significantly lower following similar treatments.

Taken together, we have shown that the DNA sensor cGAS, at least in part, mediates microglial responses to DNA damage. We demonstrate that, in a human microglial cell line, hμglia, cGAS deficiency results in significantly reduced secretion of the neutrophil recruitment factor, IL-8, specifically in response to DNA damaging treatments. Furthermore, we show that cGAS deficiency seemingly permits more DNA damage to occur, which is consistent with one study that details cGAS' importance in deceleration of replication forks, thus enhancing genomic stability (Chen et al. 2020), but inconsistent with other studies that suggest cGAS inhibits HR-mediated DNA repair and would, in turn, induce tumorigenesis and exacerbate genomic instability (Liu et al. 2018; Jiang et al. 2019). As such, we will need to further examine the mechanisms behind cGAS' apparent role with regards to DNA damage occurrence. Furthermore, we will need to examine other cytokines and chemokines that may have their production and secretion hampered by cGAS deficiency. In doing so, we will gain a better understanding into the downstream effects of cGAS activation following DNA damage, in addition to whether such responses to DNA damage are beneficial or detrimental. This will be particularly important work to perform in an *in vivo* setting where other resident CNS cells and recruited leukocytes will act in concert with microglia. These studies will allow future researchers to identify potential targets for therapeutic intervention that could dampen neuroinflammatory responses while still allowing beneficial responses to thrive.

4.4 FIGURES

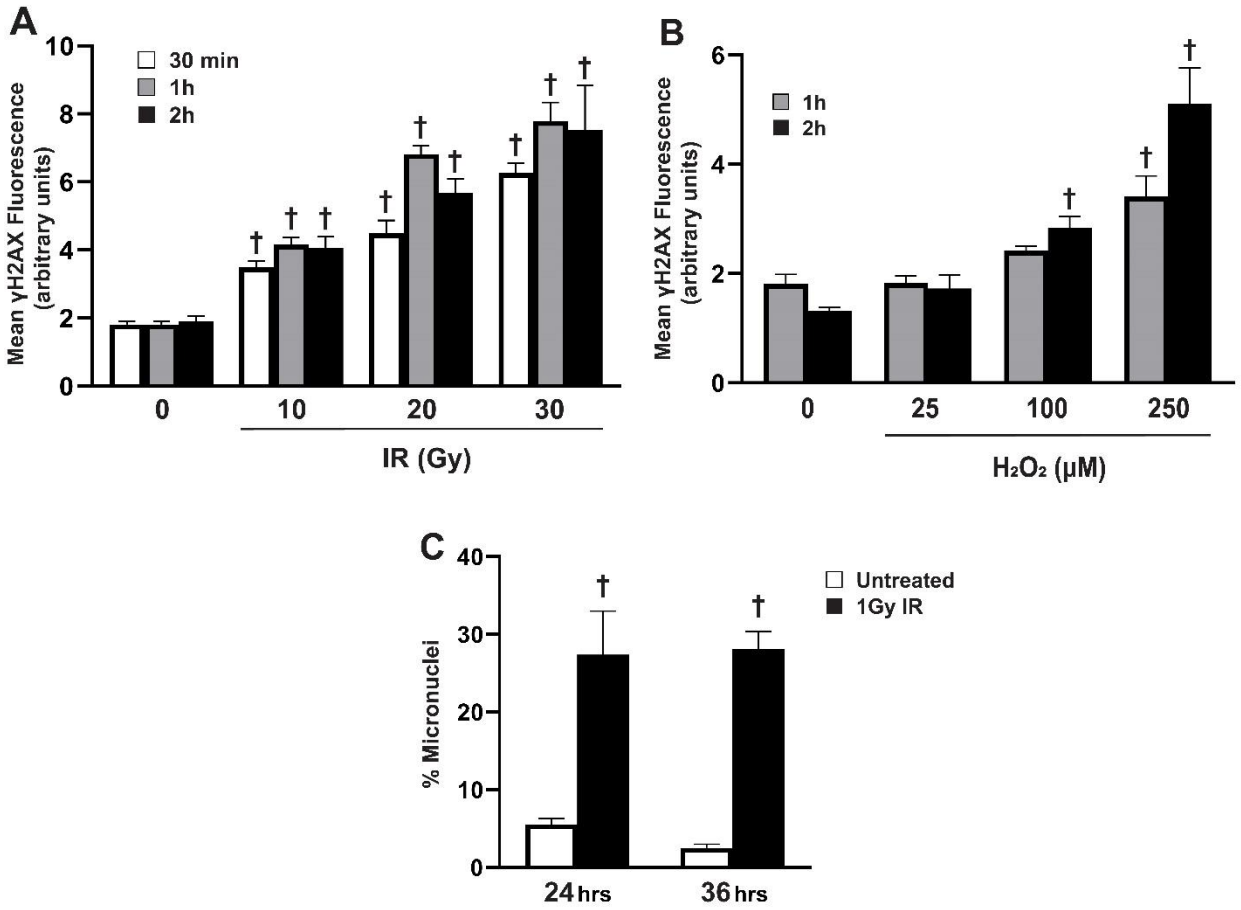

Figure 12: Ionizing radiation and hydrogen peroxide exposure elicit DNA damage in human microglia. Panel A: Wild type hμglia were exposed to a single accumulating dose of ionizing radiation (0, 10, 20, or 30 Gy at 6.5 Gy/min). 30 minutes, 1-hour, or 2-hours post-treatment, cells were fixed and fluorescently probed for nuclear visualization (DAPI) and γH2AX expression. Panel B: Wild type hμglia were treated with bolus doses of H_2O_2 (0, 25, 100, or 250 uM). 1-hour or 2-hours post-treatment, cells were fixed and fluorescently probed for nuclear visualization (DAPI) and γH2AX expression. Results are presented as mean fluorescence of γH2AX, as determined via ImageJ analysis, from 3 independent experiments +/- SEM. Panel C: Wild type hμglia were exposed to a single accumulating dose of ionizing radiation (1Gy at 6.5 Gy/min). 24- or 36-hours post-treatment, cells were fixed and fluorescently probed for nuclear visualization (DAPI) and the number of nuclei possessing micronuclei were counted by eye. Results are presented as percent of total nuclei that had at least one associated micronucleus from 3 independent experiments +/- SEM. Dagger symbols indicate significant difference from untreated cells ($p < 0.05$; $n = 3$).

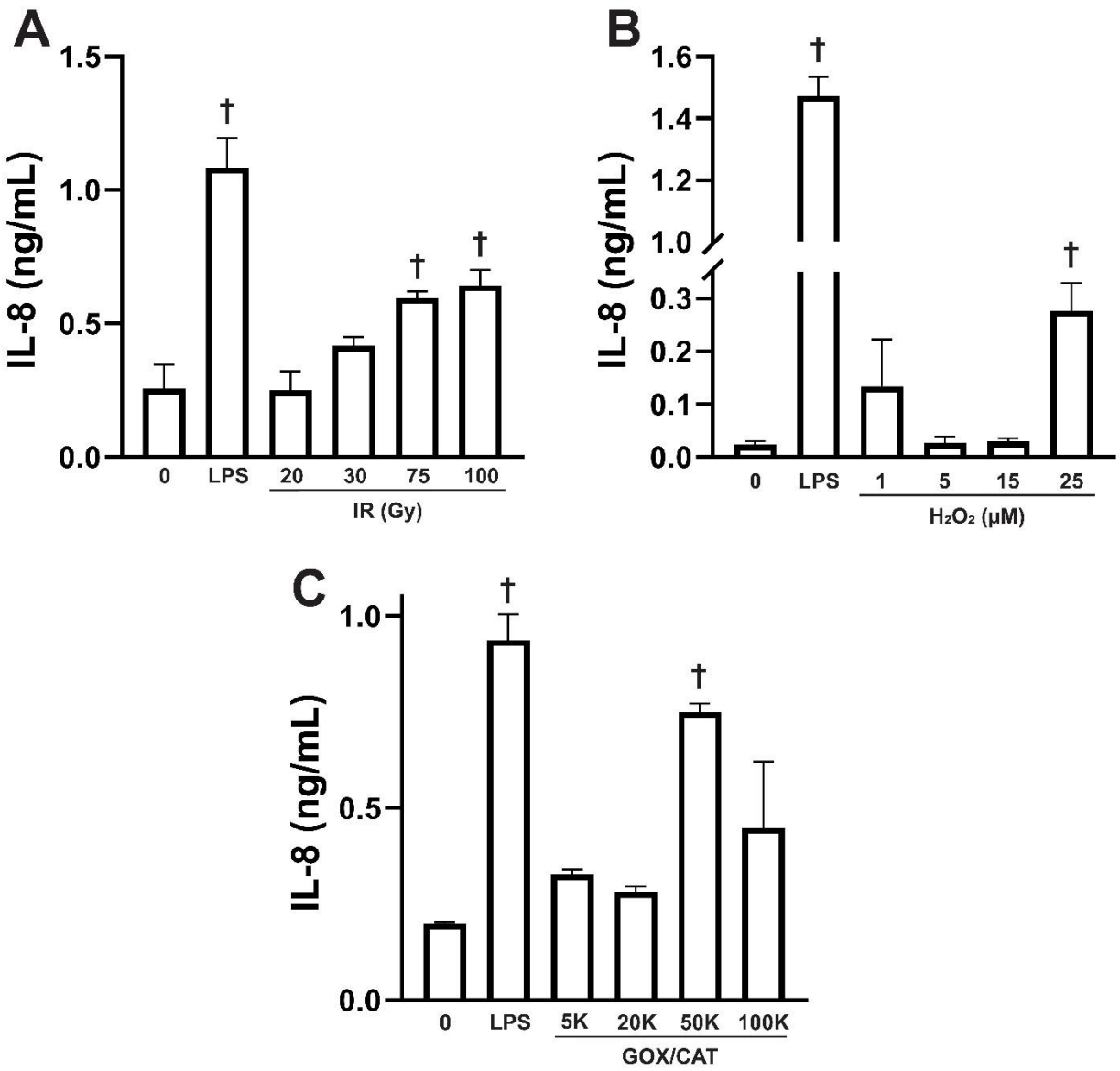

Figure 13: DNA damage elicits proinflammatory mediator production in human microglia. Panel A: Wild type hμglia were exposed to a single accumulating dose of ionizing radiation (0, 20, 30, 75, or 100 Gy) or LPS (5ng/mL). At 24 hours, the concentration of IL-8 in cell free supernatants was quantified by specific capture ELISA. Panel B: Wild type hμglia were treated with a single bolus dose of H_2O_2 (1, 5, 15, or 25 uM) or LPS (5ng/mL). At 24 hours, the concentration of IL-8 in cell free supernatants was quantified by specific capture ELISA. Panel C: Wild type hμglia were treated with the oxidative stress inducing GOX/CAT system (GOX 1:1000 dilution in all GOX/CAT treatment groups, CAT 1:5K, 1:20K, 1:50K, or 1:100K dilutions). At 24 hours, the concentration of IL-8 in cell free supernatants was quantified by specific capture ELISA. Dagger symbols indicate significant difference from untreated cells ($p < 0.05$; $n = 3$).

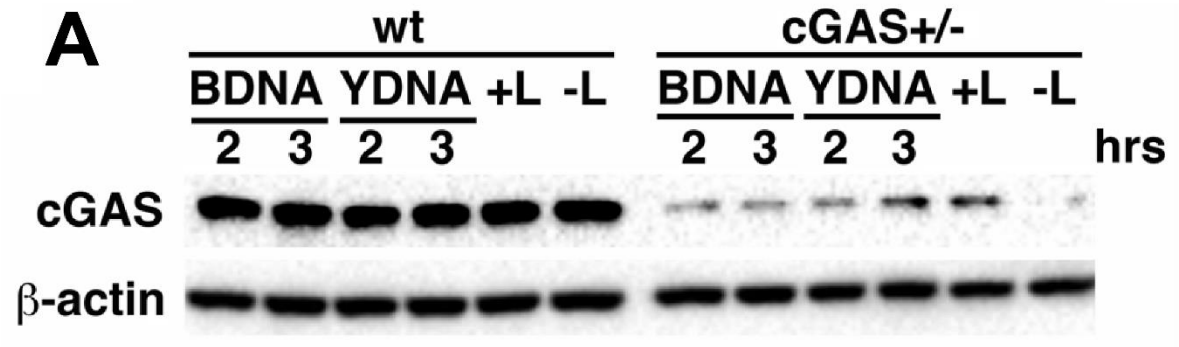

Figure 14: Confirmation of CRISPR/Cas9-mediated cGAS deficiency in human microglia (Adapted from Jeffries et al. 2020).
Panel A: wild type (wt) or heterozygous cGAS deletion (cGAS+/−) immortalized human microglia (hμglia) were untreated (−L) or exposed to transfection reagent alone (+L) for 3 h, or were challenged with intracellular B-form DNA (BDNA; 0.1 μg/ml) or Y-form DNA (YDNA; 1 μg/ml) for 2 or 3 h (Jeffries et al. 2020). Whole-cell lysates were subsequently collected and analyzed for the expression of cGAS, phosphorylated IRF3 (pIRF3) (Data not shown), or the housekeeping gene product β-actin by immunoblot analysis (n = 4-6) (Jeffries et al. 2020).

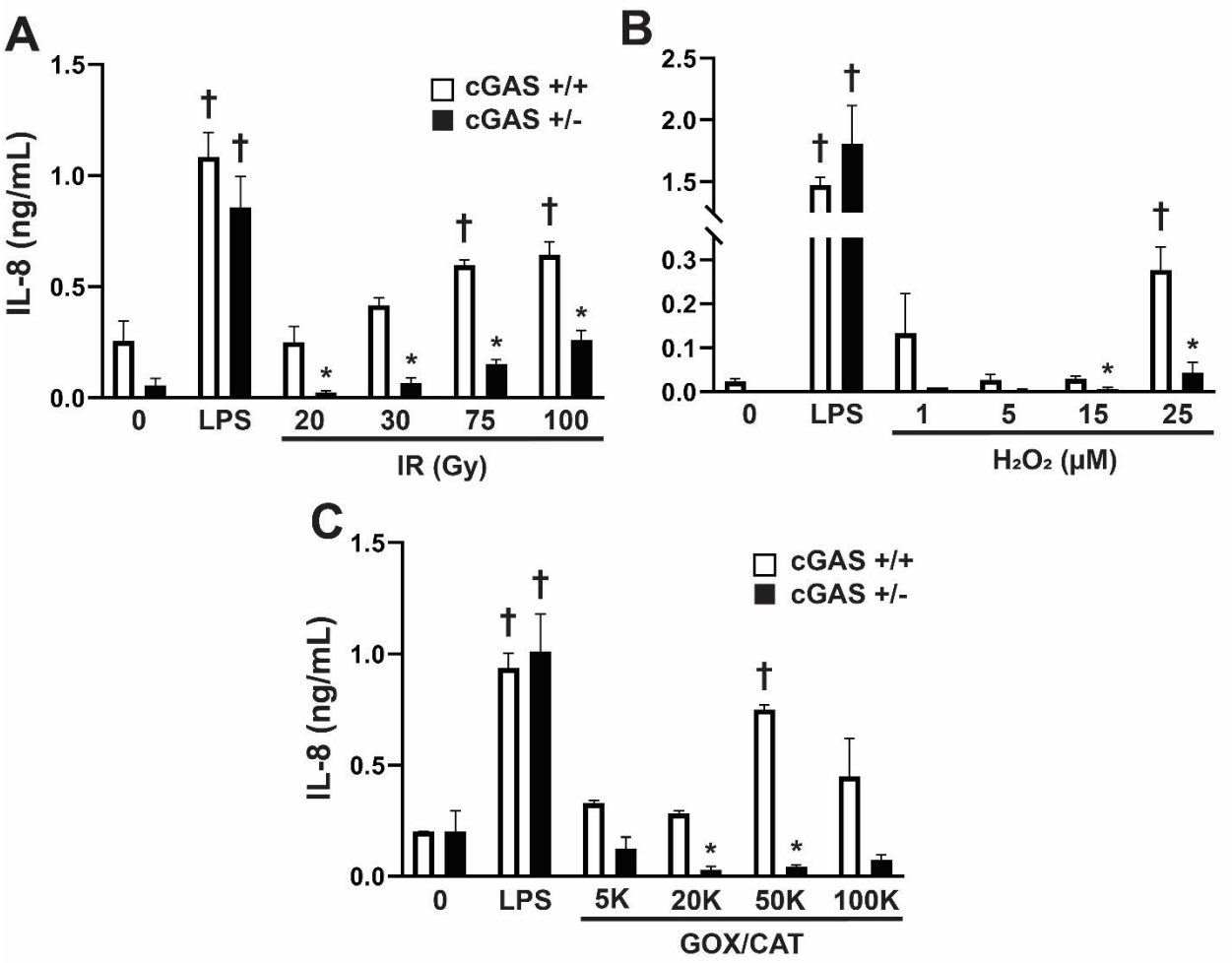

Figure 15: cGAS mediates IL-8 secretion following genotoxic damage in human microglia. Panel A: cGAS +/+ and cGAS +/- hμglia were exposed to a single accumulating dose of ionizing radiation (0, 20, 30, 75, or 100 Gy) or LPS (5 ng/mL). At 24 hours, the concentration of IL-8 in cell free supernatants was quantified by specific capture ELISA. Panel B: cGAS +/+ and cGAS +/- hμglia were treated with a single bolus dose of H_2O_2 (1, 5, 15, or 25 uM) or LPS (5 ng/mL). At 24 hours, the concentration of IL-8 in cell free supernatants was quantified by specific capture ELISA. Panel C: cGAS +/+ and cGAS +/- hμglia were treated with the oxidative stress inducing GOX/CAT system (GOX 1:1000 dilution in all GOX/CAT treatment groups, CAT 1:5K, 1:20K, 1:50K, or 1:100K dilutions). At 24 hours, the concentration of IL-8 in cell free supernatants was quantified by specific capture ELISA. Asterisks indicate a significant difference from similarly treated cGAS+/+ cells while dagger symbols indicate significant difference from similar untreated cells ($p < 0.05$; $n = 3$).

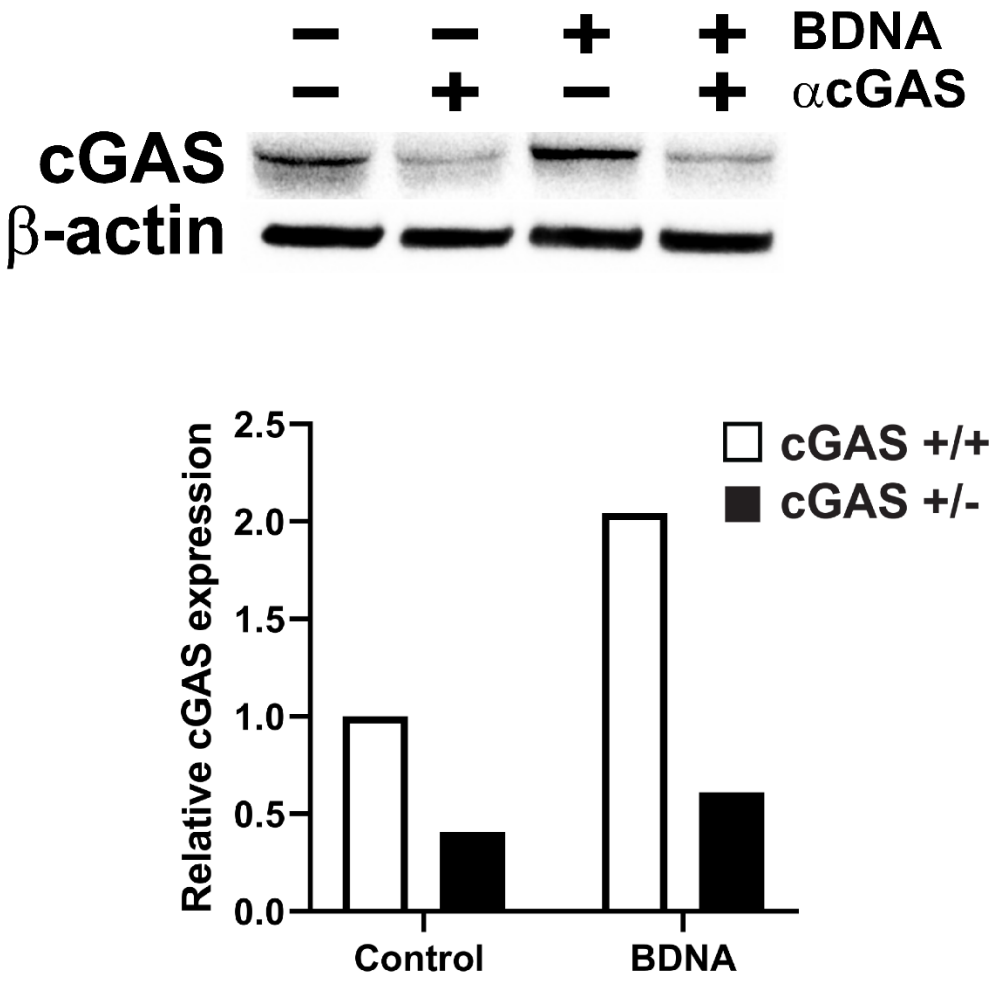

Figure 16: Confirmation of siRNA-mediated knockdown of cGAS in human microglia. WT microglia were transfected with αcGAS siRNA for 24 hours prior to further transfection with BDNA (0.1 µg/mL). At 4 hours post-BDNA treatment, whole-cell lysates were collected and analyzed for the expression of cGAS or the housekeeping gene product β-actin by immunoblot analysis (n = 1).

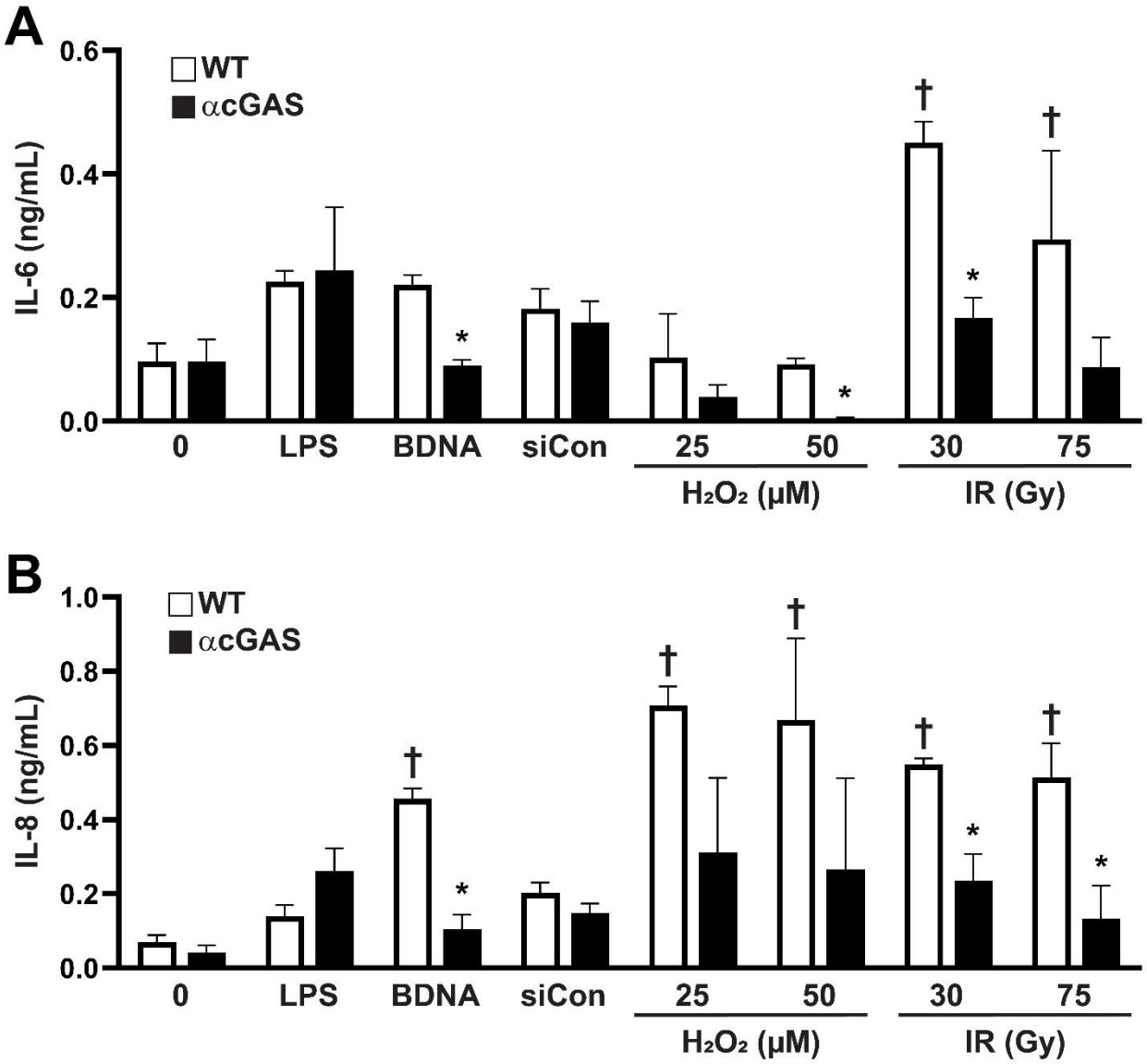

Figure 17: siRNA-mediated knockdown of cGAS diminishes responses to genotoxic damage.

Panel A and B: WT microglia were transfected with αcGAS siRNA for 24 hours prior to treatment with LPS (5 ng/mL), BDNA transfection (0.1 μg/mL), scrambled RNA transfection (siCon, 15 nM), H_2O_2 (25 or 50 μM), or IR (30 or 75 Gy). At 6 hours post-treatment, the concentration of IL-6 (Panel A) and IL-8 (Panel B) in cell free supernatants was quantified by specific capture ELISA. Asterisks indicate a significant difference from similarly treated WT cells while dagger symbols indicate significant difference from similar untreated cells ($p < 0.05$; $n = 3$).

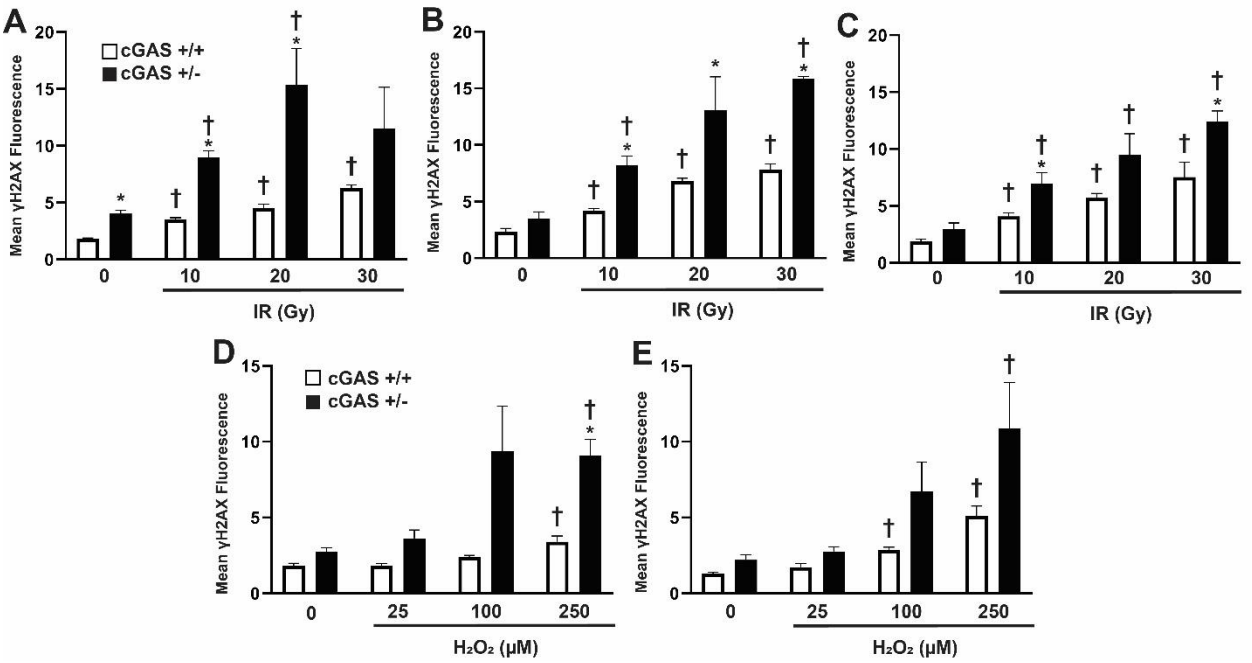

Figure 18: DNA damage in response to ionizing radiation and hydrogen peroxide exposure is significantly increased in cGAS deficient human microglia.
Panels A-C: cGAS +/+ and cGAS +/- hμglia were exposed to a single accumulating dose of ionizing radiation (0, 10, 20, or 30 Gy at 6.5 Gy/min). 30 minutes (Panel A), 1-hour (Panel B), or 2-hours (Panel C) post-treatment, cells were fixed and fluorescently probed for nuclear visualization (DAPI) and γH2AX expression. Panels D and E: cGAS +/+ and cGAS +/- hμglia were treated with bolus doses of H_2O_2 (0, 25, 100, or 250 uM). 1-hour (Panel D) or 2-hours (Panel D) post-treatment, cells were fixed and fluorescently probed for nuclear visualization (DAPI) and γH2AX expression. Results are presented as mean fluorescence of γH2AX, as determined via ImageJ analysis, from 3 independent experiments +/- SEM. Asterisks indicate a significant difference from similarly treated cGAS+/+ cells while dagger symbols indicate significant difference from similar untreated cells ($p < 0.05$; $n = 3$).

CHAPTER 5: Summary and Conclusions

5.1 Z-DNA binding protein 1 mediates necroptotic and apoptotic cell death pathways in murine glia following herpes simplex virus-1 infection

In the present study, we have demonstrated that ZBP1 serves as a restriction factor for HSV-1 infection in murine microglia and astrocytes by inducing necroptotic and apoptotic cell death. Our laboratory has previously shown that this sensor's activity as a restriction factor is independent of interferon production, as IFN-β production was higher in ZBP1 -/- astrocytes compared to ZBP1 +/+ astrocytes following HSV-1 infection (Jeffries et al. 2022). Additionally, treatment with the STAT1 inhibitor, fludarabine, following infection with HSV-1 did not significantly alter the amount of plaque forming units in ZBP1 deficient astrocytes. Furthermore, we showed that ZBP1 deficient astrocytes had significantly lower levels of cell death 24-hours following infection.

To determine the mechanisms surrounding the ability of ZBP1 to induce cell death in murine glia, the activity of the necroptosis marker, MLKL, was investigated (Jeffries et al. 2022). Due to the ability of the HSV-1 protein, ICP6, to independently induce RIPK1-dependent necroptotic cell death via its RHIM domain, we employed the use of an HSV-1 strain with a mutant RHIM domain (HSV-1(F)-ICP6-RHIM Mut) in addition to the parental F strain (HSV-1(F)) for comparison. Additionally, all findings were also confirmed during infection with a neuroinvasive strain of HSV-1 (HSV-1 (MacIntyre)). Notably, we previously demonstrated that the parental HSV-1 strain was capable of inducing necroptotic cell death independent of ZBP1 activity, while the ability of HSV-1(F)-ICP6-RHIM Mut strain to do the same was significantly reduced (Jeffries et al. 2022). Furthermore, inhibition of RIPK1 prevented activation of the necroptosis marker, MLKL, in ZBP1 deficient astrocytes, regardless of which HSV-1 strain cells

were infected with (Jeffries et al. 2022). Interestingly, while there was no significant difference in the percentage of cell death at 24-hours post infection between viral strains, a significant reduction in cell death was observed between ZBP1 expressing and ZBP1 deficient astrocytes, with a similar trend present between ZBP1 expressing and ZBP1 deficient microglia. Taken together, this insinuates that the reduction in cell death is not completely due to necroptosis.

To test the hypothesis that differences in cell death were not due to necroptosis alone, we tested a pharmacological inhibitor that targets an upstream mediator of necroptosis, RIPK3 (GSK872), or a pan-caspase inhibitor (zVAD-FMK) to prevent apoptosis. Interestingly, the use of GSK872 and zVAD-FMK individually did not reduce the percentage of cell death in ZBP1 deficient astrocytes, however, the pan-caspase inhibitor did increase the percentage of cell death in ZBP1 expressing astrocytes to similar levels that were seen in ZBP1 deficient astrocytes. Additionally, a similar trend was also found in murine microglia. Interestingly, while the inhibition of RIPK1 and apoptosis alone did not reduce cell death in ZBP1 expressing astrocytes, the concurrent inhibition of RIPK1 and apoptosis did reduce cell death in these cells. To further confirm this hypothesis, alternative pharmacological inhibitors of RIPK1, RIPK3, and caspase-8 (GSK547, GSK843, and zVAD-IETD, respectively) were employed, and the use of these inhibitors yielded similar results. In concert, these data suggest that ZBP1 is capable of mediating both necroptotic and apoptotic cell death pathways in murine astrocytes. Further investigation is needed, as it remains largely unknown whether the demonstrated responses are a protective host response during HSV-1 infection or contribute to the pathophysiology associated with HSV-1 infection.

5.2 cGAS mediates inflammatory responses to DNA damage in human microglia

In the present study, we have demonstrated that cGAS initiates inflammatory responses following DNA damage in a human microglial cell line, hμglia. Consistent with previous studies, we have shown that IR and oxidative stress elicit DNA damage and, subsequently, expression of the DNA damage marker, γH2AX (Einor et al. 2016). Furthermore, we have demonstrated that IR induces the formation of micronuclei.

To determine if DNA damage induces proinflammatory cytokine release in human microglia, we exposed a human microglial cell line to IR, H2O2, and the oxidative stress-inducing GOX/CAT system (Mueller et al. 2009). In doing so, we found that these treatments resulted in significantly elevated secretion of the neutrophil recruitment factor IL-8 in comparison to untreated cells. To investigate whether cGAS is involved in such responses, we employed cGAS +/- hμglia that were previously created by our laboratory using CRISPR/Cas9 technology (Jeffries et al. 2020). Interestingly, following treatment with IR, H2O2, and the GOX/CAT system, we found that cGAS +/- microglia secreted significantly lower levels of IL-8 compared to similarly treated WT microglia. Importantly, these responses are specific to DNA damaging treatments, as exposure to LPS did not result in a decrease of IL-8 secretion. Furthermore, preliminary experiments involving siRNA knockdown of cGAS resulted in similarly diminished secretion of both IL-6 and IL-8. Together, these data suggest that cGAS mediates proinflammatory responses to DNA damage in human microglia. Future in vivo experiments will be necessary, as further research is needed to determine whether such responses are beneficial or detrimental with regards to the CNS as a whole, rather than an isolated microglial cell line.

To investigate whether the reductions in cytokine secretion were due to lower levels of DNA damage, we treated cells with IR and examined expression of γH2AX. Interestingly, cGAS +/- microglia express higher levels of γH2AX, even though cytokine secretion was markedly diminished in the same cells. Previous studies have reported that cGAS could play a role in the nucleus with regards to DNA damage and tumorigenesis, although these studies are seemingly in conflict (Liu et al. 2018; Jiang et al. 2019; Chen et al. 2020). For instance, in one study, cGAS was found to suppress homologous recombination-mediated DNA repair (Jiang et al. 2019), thus increasing DNA damage, while in another, cGAS was found to slow replication fork dynamics and prevent genomic instability (Chen et al. 2020). As such, more research is needed to determine the exact mechanisms behind this increase in DNA damage in cGAS deficient microglia and whether such responses are beneficial or detrimental.

5.3 Importance and future directions

Proinflammatory molecule production by glia following infection has been well documented (Jack et al. 2005; Carpentier et al. 2008). However, the mechanisms by which these resident CNS cells perceive cell damage and pathogenic threats are still largely unknown, and responses may be cell- and disease-specific. As such, a better understanding of the sensors that initiate these responses is critical to the development of potentially life saving therapeutics that could limit damaging neuroinflammation by preventing these sensors from initiating responses.

To date, several small molecule inhibitors and agonists have been developed for cGAS (Decout et al. 2021) and AIM2 (Jiao et al. 2022), as well as for the downstream adaptor proteins STING (Haag et al. 2018; Liu et al. 2020; Wu et al. 2020; Hong et al. 2021; Tian et al. 2022) and RIPK1/3 (Speir et al. 2021). These agents have shown good efficacy in both peripheral cells and isolated glia where pharmacological inhibition of these components has led to significant reductions in the production of immune mediators including IFN-β, IL-1β, and IL-6, following activation (Zorman et al. 2016; Vincent et al. 2017; Haag et al. 2018; Liu et al. 2020; Wiser et al. 2020; Zhang et al. 2020; Jeffries et al. 2022; Jiao et al. 2022). Indeed, the STING agonists, TAK-676 (NCT04420884, NCT04879849) and E7766 (NCT04144140), are currently being tested clinically for the treatment of advanced or metastatic solid tumors, lymphomas, and leukemias, as adjunctive agents to conventional chemotherapy. As such, it might be possible in the future to target the DNA sensors or their downstream adaptor proteins associated with CNS cancers and neurodegenerative diseases.

However, considerable hurdles remain for the development and use of such agents as the available data regarding the specific role of each cytosolic DNA sensor in CNS disorders can be unclear or even contradictory. It may be that the glial responses initiated by the presence of

cytosolic DNA are sensor, cell type, or even disease stage, dependent. In addition, the ability of many small molecule inhibitors and agonists that target cytosolic DNA sensors to cross the BBB and their efficacy in the brain have not been determined. Indeed, marked differences in the effectiveness of such agents has been noted between studies in isolated glia and those in murine model or clinical settings, primarily due to poor BBB penetration, as reviewed elsewhere (Akhtar et al. 2021). While various methods have been successfully employed to circumvent this issue, including BBB disruption and intracerebral, intrathecal, or intranasal, delivery, each carries its own problems, such as neurotoxicity (BBB disruption) or a high degree of invasiveness (intrathecal delivery) (Akhtar et al. 2021). Finally, the potential for adverse side-effects of agents targeting DNA sensor mechanisms remains unclear, as no DNA sensor inhibitors or agonists are currently undergoing clinical trials for their efficacy in the treatment of CNS tumors or neurodegenerative disease.

Clearly, more study of these novel cytosolic DNA sensor pathways is warranted given our current lack of understanding of the role of each in glial functions in the context of specific CNS disorders and their stages. Furthermore, successfully establishing the *in-situ* efficacy of agonists/antagonists of these DNA sensor pathways in the CNS, and solving the issue of delivery across the BBB, could represent an exciting new therapeutic modality that might be used alone or in conjunction with existing approaches to improve the treatment of a wide range of CNS pathologies.

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APPENDIX: Publications

Jeffries AM, Suptela AJ, Marriott I (2022) Z-DNA binding protein 1 mediates necroptotic and apoptotic cell death pathways in murine astrocytes following herpes simplex virus-1 infection. *J Neuroinflammation* 19:1–13. https://doi.org/10.1186/s12974-022-02469-z

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