

The Role of STING-Mediated Neuroinflammation in Traumatic Brain Injury

Lauren E. Fritsch

Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Translational Biology, Medicine, and Health

Alicia M. Pickrell, Chair
Michelle H. Theus
Irving Coy Allen
Pamela VandeVord

August 25th, 2022
Blacksburg, Virginia

Keywords: Traumatic brain injury, neuroinflammation, interferons
Copyright 2022, Lauren E. Fritsch

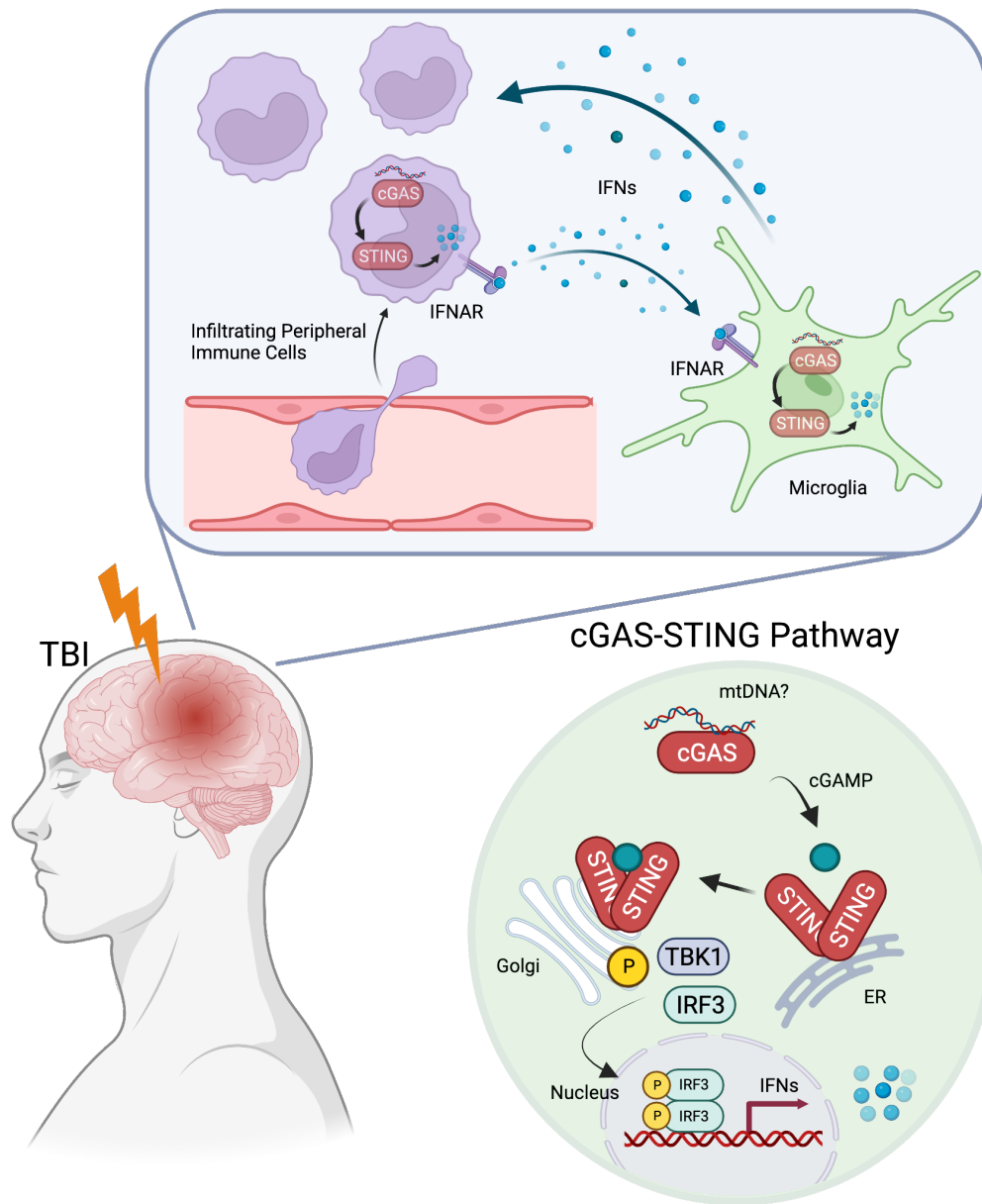
The Role of STING-Mediated Neuroinflammation in Traumatic Brain Injury

Lauren E. Fritsch

Abstract

Despite its prevalence, there are currently zero treatments available for traumatic brain injuries (TBI). Neuroinflammation is a key aspect of the secondary injury process, but remains poorly understood. Recent work has shown that Type I Interferons, inflammatory cytokines typically produced in response to viral infection, are present in the post-mortem brains of human TBI patients. The cyclic GMP-AMP Synthase- Stimulator of Interferon Genes (cGAS-STING) pathway is one of the primary methods of producing Type I IFNs; therefore, this work sought to evaluate the role of the cGAS-STING pathway in a murine controlled cortical impact (CCI) model of TBI. Using cGAS knockout (KO) or STING KO mice, we show that global loss of either protein results in substantial neuroprotection. One day after injury, animals have reduced lesion size, cell death, and inflammatory cytokine production, as well as reduced motor deficits several days after injury. We also determined that mitochondrial DNA (mtDNA) is present in the cytosol of injured cortical cells, indicating it is available to bind cGAS, a cytosolic pattern recognition receptor. To determine whether brain-resident or peripheral immune cells are responsible for detrimental cGAS-STING signaling after TBI, we utilized bone marrow chimeric animals lacking STING in either the brain or hematopoietic cells and animals lacking STING specifically in microglia. We found that both microglia and peripheral immune cells contribute to STING signaling after neurotrauma, and that loss of STING in either cell population is beneficial. Taken together, this work demonstrates that canonical, cGAS-dependent STING signaling occurs primarily in microglia and peripheral immune cells, resulting in detrimental neuroinflammatory events after TBI.

Graphical Abstract



The Role of STING-Mediated Neuroinflammation in Traumatic Brain Injury

Lauren E. Fritsch

General Audience Abstract

Traumatic brain injuries (TBI), including concussions and more severe injuries, are a leading cause of death and disability across the globe; yet, there are no Food and Drug Administration (FDA) approved treatments. There are two phases in the injury: primary injury, which is the immediate damage to brain cells upon impact, and secondary injury, which includes a wide range of cellular processes in the minutes to weeks after injury. Because the primary injury is so rapid, we utilize safety measures, such as helmets, to limit the severity of the TBI. The secondary injury, however, occurs over a longer period of time; therefore, this is where most research is focused for developing potential treatments. Inflammation in the brain, termed neuroinflammation, is a key part of this secondary injury. While some inflammation is useful for clearance of damaged cells, too much inflammation can cause additional damage. The goal of this work was to examine how a specific inflammatory pathway, the cGAS-STING (cyclic GMP-AMP synthase- stimulator of interferon genes) pathway, contributes to neuroinflammation after brain injury.

STING activation by cGAS results in the production of inflammatory proteins called interferons. These interferons cause additional inflammation that alerts nearby cells to potential danger. To investigate how STING activation affects brain injury outcome, we used a mouse model that mimics a moderate TBI. When either the cGAS or STING protein is genetically removed, animals have smaller injuries and fewer dying cells. These animals also perform better at motor tasks compared to control animals. Because many different cell types utilize STING signaling, we next tested which cells are responding in brain injury. We find that two populations of cells- immune cells living in the brain (termed microglia), and immune cells circulating in the bloodstream- contribute to the STING response after TBI. Taken together, these data show that limiting the activity of cGAS or STING may be a potential treatment for brain injury.

Dedication

To my mom. This work exists because of your unconditional love and support. Thank you.

Acknowledgments

Above all, thank you to my friends and family for always supporting me. Danny, I love the life we have built together and I would have never made it through graduate school without you. Mom, Kate, and Emily, thank you for listening to my science rants, even when you had no idea what I was talking about. To Nanny, thank you for reminding me to take a breath every once in a while. I hope this work has made you proud. To my friends, but especially Allie and Jamie, thank you for being the best support system and knowing when I just need time to rant. Finally, though I know they can't read it, thank you to Lucy and Honey for always brightening my day.

My deepest gratitude goes to my advisor, Dr. Alicia Pickrell. This work would not have been possible without her brilliance and passion for science. Thank you for teaching me how to think like a scientist, and always encouraging my goals. It's been an honor to be your first graduate student, and I will be forever grateful for everything you have taught me.

To Dr. Michelle Theus, thank you for taking a chance on me as a naive undergraduate student. Learning from you and your lab is what led me to pursue my doctorate in the first place, and your knowledge and support helped me complete it.

Thank you to my committee members, Dr. Coy Allen and Dr. Pamela VandeVord, as well as Dr. Paul Morton. Your questions and guidance have been instrumental in keeping me on track and helping me tell a complete story.

To my lab mates and undergraduate students, past and present, thank you for making lab a fun place to work and encouraging me when things weren't working and I had to do it again. I couldn't have asked for better coworkers.

I would also like to acknowledge all the staff of Life Sciences 1, the TBMH Graduate Program, and the School of Neuroscience. Thank you for supporting my work and making the administrative aspects of graduate school a breeze.

Finally, thank you to all the teachers who have helped me get to this point in my academic career. From high school physics teachers to advanced neuroscience professors, you have all taught me so much more than just your coursework.

Contents

List of Figures	ix
List of Tables	x
1 Introduction	1
2 Review of Literature	4
2.1 Abstract	5
2.2 Discovery of STING	6
2.3 STING in Peripheral Viral and Bacterial Infection	7
2.4 STING in Central Nervous System Infection	8
2.5 STING in the Peripheral Nervous System	9
2.6 STING in the Central Nervous System	10
2.7 STING Signaling in Neurodegenerative Disease	13
2.8 Conclusions and Future Directions	18
2.9 References	19
3 Type I Interferon Response is Mediated by NLRX1-cGAS-STING Signaling in Brain Injury	29
3.1 Abstract	30
3.2 Introduction	31
3.3 Materials and Methods	31
3.4 Results	34
3.5 Discussion	39
3.6 Supplementary Materials	43
3.7 References	43

4	STING Signaling in Microglia and Peripheral Immune Cells Contributes to Neuroinflammation in Traumatic Brain Injury	46
4.1	Abstract	47
4.2	Introduction	48
4.3	Results	49
4.4	Discussion	56
4.5	Materials and Methods	59
4.6	References	63
5	Summary and Future Directions	70
5.1	Summary of Findings	71
5.2	Future Directions	72
5.3	Concluding Remarks	73
5.4	References	73

List of Figures

2.1	STING signaling pathway in TBI and stroke	17
3.1	CCI injury induces a biphasic ISG response in the damaged cortex	35
3.2	CCI injury induces the presence of cytosolic mitochondrial DNA in damaged cortex	36
3.3	Loss of cGAS-STING confers neuroprotection after CCI injury	37
3.4	Loss of cGAS-STING ameliorates pro-inflammatory gene expression after CCI injury	38
3.5	Microglia are the predominant cell type expressing cGAS and STING in the brain	39
3.6	NLRX1 negative regulates cGAS-STING activation after CCI injury	40
3.7	NLRX1-cGAS-STING summary figure	41
4.1	Loss of STING in the brain or peripheral immune cells confers neuroprotection	50
4.2	GFP+ cells in the brain of naive bone marrow chimeric animals are localized to the vasculature	51
4.3	Full body, brain, or hematopoietic loss of STING reduces peripheral immune cell infiltration acutely after TBI	52
4.4	WT BMDMs show robust immune response to STING activation	54
4.5	Loss of microglial-specific STING expression reduces lesion size 24 hours after injury	55
4.6	Loss of microglial-specific STING is neuroprotective	56
4.7	Loss of microglial STING reduces motor deficits and lesion size after TBI at more chronic timepoints	57

List of Tables

2.1	Key findings from STING-IFN studies in stroke	12
2.2	Key findings from STING-IFN studies in TBI	15
4.1	Complete blood count data from naive WT and STING KO animals	53

List of Abbreviations

BBB	Blood Brain Barrier
CCI	Controlled Cortical Impact
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP Synthase
CNS	Central Nervous System
DAMP	Damage Associated Molecular Pattern
IFN	Interferon
ISG	Interferon Stimulated Genes
KO	Knockout
mtDNA	Mitochondrial DNA
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
STING	Stimulator of Interferon Genes
TBI	Traumatic Brain Injury
WT	Wildtype

Chapter 1

Introduction

Introduction

Traumatic brain injury (TBI) is defined as brain dysfunction resulting from an external force to the head. These injuries include everything from minor concussions to severe, life-threatening injuries. An estimated 69 million people sustain TBIs globally each year (Dewan et al. 2018). While patients suffering from mild TBIs, commonly referred to as concussions, generally don't have permanent symptoms, moderate and severe TBIs frequently cause long-term complications. In the United States alone, the annual cost of these injuries is nearly \$80 billion (Seifert 2007), yet there are zero Food and Drug Administration (FDA) approved therapeutics for TBI. The goal for this work was to better understand how inflammatory events in the brain influence TBI outcome and evaluate whether a specific signaling pathway, termed the cGAS-STING pathway, may be a therapeutic target.

TBIs occur in two phases: a primary phase that causes immediate damage to the brain tissue, and a secondary injury that occurs over a longer period of time. Neuroinflammation (inflammatory events in the brain that occur as a result of the trauma) is a critical component of the secondary injury (Simon et al. 2017); thus, characterizing post-traumatic neuroinflammation may offer insight into novel therapeutics for TBI. Following injury, microglia, the brain's resident immune cells, produce proteins termed cytokines and recruit immune cells from the circulation to help repair the damage. However, these inflammatory events can cause excessive tissue clearance that exacerbates the injury. Recent work has shown that interferons, cytokines generally produced to combat viral infections, are present in the post-mortem brains of human TBI patients (Frugier et al. 2010; Karve et al. 2016). As one of the primary mechanisms for producing interferons, the cyclic GMP-AMP synthase- Stimulator of Interferon Genes (cGAS-STING) pathway (Ishikawa and Barber 2008) was postulated to play a role in TBI. Indeed, shortly after the discovery of interferons in human TBI patients, work by the same group demonstrated that STING is upregulated in the brains of human patients and in a mouse model of TBI (Abdullah et al. 2018). Additionally, genetic ablation of either STING (Abdullah et al. 2018), the interferon receptor, IFNAR (Karve et al. 2016), or interferon- β (Sen et al. 2020; Barrett et al. 2020) reduced tissue damage in a mouse model of TBI. Thus, the work presented here sought to build upon this knowledge by better characterizing the effects of loss of STING in TBI, as well as evaluate which cell types play a role in STING-mediated neuroinflammation.

Broadly, this work shows that cGAS-STING signaling plays a detrimental role in TBI outcome following a mouse model of TBI. Animals lacking cGAS or STING had significantly reduced inflammatory protein production, smaller lesion sizes, and reduced cell death (Chapter 3). Further, loss of STING in either the brain or peripheral immune cells resulted in reduced cell death and immune cell infiltration, indicating that both circulating immune cells and brain-resident microglia contribute to this response following TBI (Chapter 4). Together, this work suggests that the cGAS-STING pathway may be a therapeutic target for TBI (Chapter 5).

References

- Abdullah, Amar, Moses Zhang, Tony Frugier, Sammy Bedoui, Juliet M. Taylor, and Peter J. Crack. 2018. "STING-Mediated Type-I Interferons Contribute to the Neuroinflammatory Process and Detrimental Effects Following Traumatic Brain Injury." *Journal of Neuroinflammation* 15 (1). <https://doi.org/10.1186/S12974-018-1354-7>.
- Barrett, James P., Rebecca J. Henry, Kari Ann Shirey, Sarah J. Doran, Oleg D. Makarevich, Rodney R. Ritzel, Victoria A. Meadows, et al. 2020. "Interferon- β Plays a Detrimental Role in Experimental Traumatic Brain Injury by Enhancing Neuroinflammation That Drives Chronic Neurodegeneration." *The Journal of Neuroscience*, February, 2516–19. <https://doi.org/10.1523/jneurosci.2516-19.2020>.
- Dewan, Michael C., Abbas Rattani, Saksham Gupta, Ronnie E. Baticulon, Ya Ching Hung, Maria Punchak, Amit Agrawal, et al. 2018. "Estimating the Global Incidence of Traumatic Brain Injury." *Journal of Neurosurgery* 130 (4): 1080–97. <https://doi.org/10.3171/2017.10.JNS17352>.
- Frugier, Tony, Maria Cristina Morganti-Kossmann, David O'Reilly, and Catriona A. McLean. 2010. "In Situ Detection of Inflammatory Mediators in Post Mortem Human Brain Tissue after Traumatic Injury." *Journal of Neurotrauma* 27 (3): 497–507. <https://doi.org/10.1089/neu.2009.1120>.
- Ishikawa, Hiroki, and Glen N. Barber. 2008. "STING Is an Endoplasmic Reticulum Adaptor That Facilitates Innate Immune Signalling." *Nature* 455 (7213): 674–78. <https://doi.org/10.1038/nature07317>.
- Karve, Ila P., Moses Zhang, Mark Habgood, Tony Frugier, Kate M. Brody, Maithili Sashindranath, C. Joakim Ek, et al. 2016. "Ablation of Type-1 IFN Signaling in Hematopoietic Cells Confers Protection Following Traumatic Brain Injury." *ENeuro* 3 (1): 89–93. <https://doi.org/10.1523/ENEURO.0128-15.2016>.
- Seifert, Julia. 2007. "Incidence and Economic Burden of Injuries in the United States." *Journal of Epidemiology and Community Health* 61 (10): 926. <https://doi.org/10.1136/JECH.2007.059717>.
- Sen, Tanusree, Pampa Saha, Rajaneesh Gupta, Lesley M. Foley, Tong Jiang, Olena S. Abakumova, T. Kevin Hitchens, and Nilkantha Sen. 2020. "Aberrant ER Stress Induced Neuronal-IFN β Elicits White Matter Injury Due to Microglial Activation and T-Cell Infiltration after TBI." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 40 (2): 424–46. <https://doi.org/10.1523/JNEUROSCI.0718-19.2019>.
- Simon, Dennis W., Mandy J McGeachy, Hülya Bayır, Robert S B Clark, David J Loane, and Patrick M Kochanek. 2017. "The Far-Reaching Scope of Neuroinflammation after Traumatic Brain Injury." *Nature Reviews. Neurology* 13 (3): 171–91. <https://doi.org/10.1038/nrneurol.2017.13>.

Chapter 2

Review of Literature

This article is under review as an invited review to WIREs Mechanisms of Disease.

The Role of STING Signaling in Neuroinflammatory Disease

Lauren E. Fritsch¹ and Alicia M. Pickrell^{2#}

¹Graduate Program in Translational Biology, Medicine, and Health, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

²School of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Correspondence should be addressed to:

Alicia M. Pickrell, 970 Washington Street SW, Life Science I Rm 217, Blacksburg, VA 24061

Tel: 540-232-8465; Fax: 540-231-1475; Email: alicia.pickrell@vt.edu

Keywords: STING, neuroinflammation, interferons

Abbreviations: Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), blood brain barrier (BBB), controlled cortical impact (CCI), cyclic GMP-AMP (cGAMP), cGAMP synthase (cGAS), central nervous system (CNS), cerebral spinal fluid (CSF), cerebral venous sinus thrombosis (CVST), danger associated molecular pattern (DAMP), dorsal root ganglion (DRG), double stranded DNA (dsDNA), hepatitis B virus (HBV), Huntington's disease (HD), human immunodeficiency virus (HIV), herpes simplex encephalitis (HSE), herpes simplex virus 1 (HSV-1), huntingtin (HTT), interferon (IFN), interferon α/β receptor (IFNAR), interferon regulatory factor (IRF3), interferon stimulated gene (ISG), knockdown (KD), knockout (KO), ligand binding domain (LBD), middle cerebral artery occlusion (MCAO), mouse embryonic fibroblast (MEF), matrix metalloprotease 9 (MMP9), mitochondrial permeability transition pore (mPTP), mitochondrial DNA (mtDNA), nicotinamide adenine dinucleotide (NAD⁺), neutrophil extracellular trap (NET), natural killer (NK), pathogen associated molecular pattern (PAMP), Parkinson's disease (PD), pattern recognition receptor (PRR), subarachnoid hemorrhage (SAH), spinal cord injury (SCI), single stranded RNA (ssRNA), stimulator of interferon genes (STING), traumatic brain injury (TBI), tank binding kinase 1 (TBK1), transactive response DNA binding protein 43 (TDP-43), wildtype (WT)

Abstract

The cyclic GMP-AMP Synthase-Stimulator of Interferon Genes (cGAS-STING) pathway is a critical innate immune mechanism for detecting the presence of double-stranded DNA (dsDNA) and prompting a robust immune response. Canonical cGAS-STING activation occurs when cGAS, a predominately cytosolic pattern recognition receptor (PRR), binds viral DNA to promote STING activation. Upon STING activation, transcription factors enter the nucleus to cause production of Type I interferons (IFNs), inflammatory cytokines whose primary function is priming the host for viral infection by producing a number of antiviral interferon stimulated genes (ISGs). While the pathway was originally described in viral infection, more recent studies have

implicated cGAS-STING signaling as playing a role in a number of different contexts, including autoimmune disease, cancer, injury, and neuroinflammatory disease.

This review focuses on how our understanding of the cGAS-STING pathway has evolved over time with an emphasis on the role of STING-mediated neuroinflammation in the nervous system. We discuss recent findings on how STING signaling contributes to the pathology of pain, traumatic brain injury, and stroke, as well as how mitochondrial DNA may promote STING activation in neurodegenerative disease. We conclude by commenting on the current knowledge gaps that should be filled before STING can be an effective therapeutic target in neuroinflammatory disease.

Discovery of STING

Interferons were first described in the 1950s as factors released during infection to interfere with viral growth (Vilcek 2006; Isaacs and Lindenmann 1957). While three subsets of interferons exist (Type I, II, and III), Type I IFNs (primarily IFN α and IFN β) are of particular interest because nearly all nucleated cells are able to produce and respond to Type I IFNs (McNab et al. 2015). In contrast, Type II IFNs (IFN γ) are produced predominantly by natural killer (NK) cells during infection (Lee and Ashkar 2018), whereas Type III IFNs (IFN λ) are produced only at the mucosal surfaces (J. H. Zhou et al. 2018), limiting their applicability to other body systems such as the peripheral or central nervous systems. While IFNs were originally described for their role in infection, it is now known that IFNs are produced in response to a number of different stimuli and cause pleiotropic effects. STING was first identified by several different groups (Ishikawa and Barber 2008; Zhong et al. 2008; Sun et al. 2009) as a key mediator of the Type I IFN response with possible roles in mediating apoptosis (Jin et al. 2008). Initial reports suggested that STING functions downstream of the cytosolic RNA sensor RIG-I and showed that knockout (KO) or knockdown (KD) of STING resulted in blunted IFN β response to single strand RNA (ssRNA) viruses (Ishikawa and Barber 2008; Zhong et al. 2008). Overexpression experiments showed increased viral resistance, increased production of IFN β , as well as dimerization and translocation of the transcription factor, interferon regulatory factor 3 (IRF3), indicating its activation (Ishikawa and Barber 2008; Zhong et al. 2008). Tank Binding Kinase 1 (TBK1) was shown to be necessary for STING activation (Ishikawa and Barber 2008), likely due to STING phosphorylation by TBK1 (Zhong et al. 2008). Interestingly, using similar methods the groups reported different cellular localizations and mechanisms of action: (Zhong et al. 2008) reported that STING was located in the mitochondria, a finding that was also noted by (Jin et al. 2008), and did not see a change in NF- κ B in overexpression models. In contrast, (Ishikawa and Barber 2008) showed that STING is located on the ER, which was also shown by (W. Sun et al. 2009), and is able to induce NF- κ B expression when overexpressed. Critically, both groups noted that STING plays a role in the Type I IFN response to dsDNA (Ishikawa and Barber 2008; Zhong et al. 2008), though the mechanism of this response remained unclear. This was further confirmed in a subsequent study which verified that loss of STING in mouse embryonic fibroblasts (MEFs) abolished the IFN β response to viral DNA or synthetic dsDNA and showed that STING KO mice displayed an extreme susceptibility

to herpes simplex virus 1 (HSV-1) infection (Ishikawa, Ma, and Barber 2009). These findings prompted further studies to identify an upstream cytosolic DNA sensor that would mediate STING activity (Burdette and Vance 2013).

Following the discovery that STING is able to bind cyclic dinucleotides (Burdette et al. 2011), it was confirmed that cyclic GMP-AMP (cGAMP) strongly binds STING to promote its activation and downstream signaling (Wu et al. 2013). The same group identified cGAMP synthase (cGAS), a previously uncharacterized nucleotidyltransferase, as the protein responsible for binding DNA and synthesizing cGAMP *in vitro* (Sun et al. 2013). By generating a cGAS KO mouse, they also demonstrated that cGAS and STING were both critical in host defense against HSV-1 *in vivo*, though they did note that some cell types may have compensatory mechanisms for detecting viral DNA (X. D. Li et al. 2013).

The discovery of the cGAS-cGAMP-STING signaling pathway led to a flurry of studies to elucidate the structure of these molecules and clarify the specific mechanism of activation. cGAS binds the sugar-phosphate backbone of viral dsDNA, resulting in conformational changes that catalyze the synthesis of the second messenger cGAMP (Civril et al. 2013; Ablasser et al. 2013; X. Zhang et al. 2014; X. Li et al. 2013). Upon its production, cGAMP binds the ligand binding domain (LBD) of the ER-resident STING dimer with high affinity (X. Zhang et al. 2013). This binding causes a 180° rotation in the LBD that forms a β -sheet “lid” over the ligand binding pocket and enables STING oligomerization and translocation to the Golgi (Shang et al. 2019; P. Gao et al. 2013). At the Golgi, TBK1 is recruited and activated by STING, resulting in STING phosphorylation which facilitates recruitment and phosphorylation of the interferon regulatory factor IRF3 (Tanaka and Chen 2012; Dobbs et al. 2015; Mukai et al. 2016; C. Zhang et al. 2019), a transcription factor long-known to be crucial for the Type I IFN response (Stetson and Medzhitov 2006). Phosphorylated IRF3 dimers translocate to the nucleus where they prompt transcription of Type I IFNs (Sato et al. 2000). While alternative mechanisms of activation have been proposed (Dunphy et al. 2018; Unterholzner and Dunphy 2019), the above is the presently-accepted canonical STING signaling mechanism.

STING in Peripheral Viral and Bacterial Infection

STING was first described as a mediator of the response to cytosolic RNA, specifically through its interaction with RIG-I. However, it was quickly determined that STING plays a critical role in response to intracellular DNA viruses, though evidence also indicates that cGAS is able to detect retroviruses such as human immunodeficiency virus (HIV) (D. Gao et al. 2013). Early work in MEFs demonstrated a STING-dependent IFN β response to DNA from adenovirus, HSV-1, and HSV-2 (Ishikawa, Ma, and Barber 2009). cGAS-STING signaling has also been shown to be activated in response to adenoviruses in human cell lines (Lam and Falck-Pedersen 2014; Lam, Stein, and Falck-Pedersen 2014). The role of cGAS-STING in detecting and inhibiting hepatitis B virus (HBV) has also been well-studied. STING activation by cGAMP or DMXAA, a STING agonist, (Prantner et al. 2012) suppresses HBV replication and increases IFN β production both *in vitro* and *in vivo* (He et al. 2016; F. Guo et al. 2015). There is also evidence that the viral

polymerase, Pol, is able to interact with and prevent STING activation by disrupting its ubiquitination (Yinghui Liu et al. 2015). STING ubiquitination facilitates its dimerization, which is necessary for TBK1 recruitment (Tsuchida et al. 2010); therefore, this disruption allows HBV to counteract the host's STING-mediated immune response. Furthermore, by packaging its genome and producing non-immune stimulatory RNAs, HBV is able to evade detection by cGAS in hepatocytes (Lauterbach-Rivière et al. 2020; Verrier et al. 2018). Based on these findings, cGAS-STING signaling is now a promising therapeutic target for treating chronic HBV infection (Alexopoulou, Vasilieva, and Karayiannis 2020).

cGAS has been shown to bind a variety of different nucleic acids independent of sequence (Civril et al. 2013; L. Sun et al. 2013), so unsurprisingly cGAS-STING signaling has been shown to also play a role in the response to a number of bacteria (reviewed extensively in (N. Liu et al. 2022; Marinho et al. 2017; Guimarães et al. 2021)). Interestingly, the production of IFN β is detrimental to clearance of many of these bacterial infections, including *Brucella abortus* (de Almeida et al. 2011) and *Listeria monocytogenes* (Sauer et al. 2011; Archer, Durack, and Portnoy 2014; Jin et al. 2013), suggesting the effects of STING signaling are highly context-dependent.

STING in Central Nervous System Infection

One of the first reports of STING activity possibly playing a role in the central nervous system (CNS) described its interaction with RIG-I when neuroblastoma cells were infected with the ssRNA flavivirus, Japanese encephalitis virus (Nazmi et al. 2012). Subsequent studies have also implicated STING in restricting CNS spread of other flaviviruses, including West Nile virus (Wuertz et al. 2019) and Zika virus (Yuan Liu et al. 2018; Q. Ding et al. 2018). Type I IFN signaling in neurons has been shown to be particularly important. Primary neurons lacking the Type I IFN receptor, IFNAR, are more susceptible to rabies virus infection (Chopy et al. 2011), and IFN β treatment of WT primary neurons reduces replication of vesicular stomatitis virus (Trottier, Palian, and Reiss 2005).

Other work has confirmed a critical role for cGAS-STING signaling in DNA viruses within the CNS, most notably herpes simplex virus 1 (HSV-1). It is estimated that 67% of the global population harbors HSV-1 virions, yet no cure exists (James et al. 2020). While initial symptoms are generally mild, HSV-1 is neurotropic and remains dormant in neuronal tissue until an immune stressor stimulates proliferation. Herpes simplex encephalitis (HSE) can be life-threatening, and there is increasing evidence that brain-resident HSV-1 contributes to neurodegenerative disease in patients both with and without clinical symptoms of the infection (Duarte et al. 2019).

While it was established early on that STING is critical for host response to HSV-1 (Ishikawa, Ma, and Barber 2009), it was later shown that STING's protective role is at least in part based on whether virus was introduced directly to the brain or peripherally. STING KO animals were highly susceptible to central (intravenous or intracerebral) HSV-1 challenge, whereas animals deficient in STING had similar survival as WT animals when challenged peripherally (via the cornea) (Parker, Murphy, and Leib 2015), suggesting that CNS, rather than peripheral, STING may be particularly beneficial for combatting HSV-1. Other work demonstrated that STING KO

mice had increased CNS spread of HSV-1 and showed that microglia are the primary brain cell type responsible for the STING-dependent type I IFN response to HSV-1 infection (Reinert et al. 2016; Katzilieris-Petras et al. 2022). In contrast, others have shown that neuronal IFN signaling is critical for restricting HSV-1 production in the brain (Rosato and Leib 2015), and IFN β treatment of primary neuronal cultures increases cell survival (Low-Calle, Prada-Arismendy, and Castellanos 2014). It is possible that microglia are responsible for STING activation and produce IFNs that bind neuronal IFNAR to restrict viral replication. Of note, it has been shown that the outcome of cGAS-STING signaling in HSV-1 infection is dose-dependent: When mice or BV2 microglia-like cells are infected with low doses of HSV-1, a robust Type I IFN response occurs. In contrast, when the HSV-1 dose was tripled, immune cells underwent apoptosis (Reinert et al. 2021). This suggests the immune cells have evolved innate mechanisms for blunting the Type I IFN response to prevent excessive tissue damage, a particularly critical mechanism in the brain where substantial tissue regeneration does not occur.

While the host immune system has established ways to maintain a delicate balance of viral-clearing Type I IFN production and tissue homeostasis, HSV-1 has developed its own mechanisms to evade detection (reviewed extensively in (Verzosa et al. 2021)). HSV-1 proteins prevent IFN signaling across the entire cGAS-STING pathway by degrading cGAS mRNA (Su and Zheng 2017), preventing cGAMP production (Huang et al. 2018), and inhibiting TBK1 dimerization (You et al. 2019). A number of other proteins have been proposed to be involved in the STING-mediated response to HSV-1, including RIG-I (Yiliu Liu et al. 2016), ZDHHC1 (Q. Zhou et al. 2014), and NLRP3 (W. Wang et al. 2020), offering additional proteins for HSV-1 to target to limit the immune response. Some HSV-1 proteins have been shown to interact with STING to promote its deubiquitination (Bodda et al. 2020), thus preventing STING dimerization, or bind STING to counteract its activation (Deschamps and Kalamvoki 2017), though the mechanisms of these interactions are not entirely clear. Interestingly, HSV-1 has also been shown to stabilize STING protein in a cell-type specific manner and export STING and viral mRNA to nearby cells via exosomes (Kalamvoki and Roizman 2014; Kalamvoki, Du, and Roizman 2014). Taken together, these data suggest a highly complex interplay between various immune modulators and CNS viruses that we are only just beginning to understand.

STING in the Peripheral Nervous System

Nociceptive pain is sensed through peripheral neuro-immune mechanisms. Given that many painful stimuli are associated with immune activation, such as in the context of infection, it is unsurprising that this connection between immune cells and pain-detecting neurons exists. Peripheral sensory neurons (termed nociceptors) detect noxious stimuli, causing action potentials that are relayed to the brain via the dorsal horn of the spinal cord (Basbaum et al. 2009). These neurons are able to detect stimuli from a variety of sources, such as heat, mechanical damage, or by binding cytokines and other factors produced by immune cells (Baral, Udit, and Chiu 2019). Furthermore, nociceptors also express their own pattern recognition receptors (PRRs) that allow

them to directly detect pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) and activate immune cells (reviewed extensively in (Donnelly, Chen, and Ji 2020)).

STING was identified as a regulator of nociception (Donnelly et al. 2021). In an elegant and thorough study utilizing rodents and non-human primates, Donnelly and colleagues show that STING in sensory neurons plays a substantial role in nociception. Mice lacking STING had increased pain sensitivity when compared to WT animals, and intrathecal treatment with STING agonists produced a dose-dependent reduction in this hypersensitivity. These effects were entirely abolished in animals with IFNAR conditionally knocked-out in sensory neurons, confirming that the antinociceptive effects of STING activation is mediated by neurons (Donnelly et al. 2021). Of translational relevance, treatment with the STING agonist ADU-S100 produced increased cerebral spinal fluid (CSF) levels of IFN β and decreased action potential firing of nociceptors in the dorsal root ganglia (DRG) of non-human primates, suggesting that targeting STING may be promising for treating pain in humans (Donnelly et al. 2021). In a separate study, the same group also showed that systemic treatment with STING agonists reduced bone cancer pain and improved locomotor function in mice (K. Wang et al. 2021). Together, these studies highlight a novel role for neuronal STING in peripheral nociception.

STING in the Central Nervous System

Expression of STING in the CNS

There is currently disagreement about which cell types in the brain express STING. As an immune protein, it is logical to assume that microglia have robust STING expression. In fact, many studies have shown that microglia are the primary brain cell type expressing STING (Katzilieris-Petras et al. 2022; Reinert et al. 2016; Fritsch et al. 2022; R. Ding et al. 2022; Peng et al. 2020). An RNA-sequencing study in a brain injury model showed that while astrocytes mount a Type I IFN response following trauma, the microglial response is significantly larger (Todd et al. 2021). RNA sequencing databases also indicate that STING expression is predominately found in microglia (Y. Zhang et al. 2014, 2016). However, others have suggested that neurons and astrocytes also express STING in high levels (Jeffries and Marriott 2017; Gamdzyk et al. 2020; Abdullah et al. 2018; Donnelly et al. 2021). One key problem is that many of these studies rely upon immunofluorescence, which may produce non-specific signal. However, even when validated in STING KO tissue, studies using the same antibodies have shown different staining patterns across cell types in different studies (Chu et al. 2021; Peng et al. 2020), highlighting the need for more specific antibodies. Knowledge on the neuronal or glial subtype(s) that STING expression may be restricted to is relatively unexplored. Nevertheless, loss of STING either in neurons or glial cells both increase ZIKA viral particle replication in *Drosophila*, indicating it functions across many cell types in the brain (Yuan Liu et al. 2018). The use of conditional KO animals may help elucidate the roles of STING across CNS cell types and neuronal subtypes in different disease and infection paradigms. However, a majority of studies as of yet have restricted study to global KO animals.

Stroke/Ischemia

Stroke is the second most common cause of death globally (Johnson et al. 2019) and occurs when blood flow to the brain is interrupted due to blood clots or hemorrhage. The alteration of blood flow results in regions of ischemia and neuronal excitotoxicity, causing rapid cell death as well as longer-term neuroinflammatory events (Jayaraj et al. 2019). Within hours of the event, resident microglia and astrocytes are activated, leading to the production of various cytokines and chemokines that recruit peripheral immune cells and compromise the integrity of the blood brain barrier (BBB) (Jassam et al. 2017; Jayaraj et al. 2019). Phagocytic immune cells attempt to clear cell debris and produce additional cytokines to promote a highly inflammatory environment (Jayaraj et al. 2019).

Neutrophil extracellular traps (NETs) are chromatin-based structures released by neutrophils that trap pathogens and promote pro-inflammatory cytokine production (Papayannopoulos 2017). NETs have been shown to activate cGAS (Apel et al. 2021) and impact vascular remodeling following stroke (Kang et al. 2020). In a murine middle cerebral artery occlusion (MCAO) model, cGAS was shown to be upregulated after injury, but this elevation was somewhat reduced when NETs were degraded with DNase, suggesting cGAS may be activated by binding NETs after stroke (R. Wang et al. 2021). Loss of cGAS also reduced hemorrhage and motor deficit after MCAO, whereas activation of STING with cGAMP administration worsened outcome (R. Wang et al. 2021). Similarly, pharmaceutical inhibition of cGAS with RU.512 in a rat model of neonatal hypoxia-ischemia reduced infarct size (Gamdzyk et al. 2020), and neuronal apoptosis and cognitive deficit were reduced when STING was inhibited with C-176 in subarachnoid hemorrhage (SAH) (Peng et al. 2020). While microglia shifted towards an inflammatory M1-phenotype after MCAO, administration of C-176 resulted in reduced M1 microglia and increased anti-inflammatory M2 microglia (Kong et al. 2022), suggesting that the brain's inflammatory state is altered by the loss of STING signaling. Of note, mice lacking cGAS specifically in microglia showed a significant reduction in lesion size after MCAO (Q. Li et al. 2020), suggesting that microglia are an important cell type for cGAS-STING signaling after stroke.

cGAS-STING signaling also appears influence the cell populations that infiltrate the brain parenchyma after stroke. In cerebral venous sinus thrombosis (CVST), a less common form of stroke that results from impaired venous drainage, mice showed a significant reduction in the infiltration of neutrophils when cGAS was inhibited with RU.521 (R. Ding et al. 2022). Despite having a significant decrease in monocyte chemoattractant protein 1 (MCP1), the number of infiltrating monocytes/macrophages was not significantly altered by the inhibition of cGAS (R. Ding et al. 2022). Regardless, inhibition of cGAS-STING signaling may alter the recruitment of peripheral immune cells, influencing more chronic aspects of neuroinflammation. Interestingly, pre-treatment with the STING agonist, DMXAA, reduced infarct size in a murine MCAO model, leading the authors to conclude that activation of STING may be useful as a prophylactic treatment (Kundu et al. 2022). Consistently, administration of IFN β before a rabbit model of ischemic stroke reduces infarct size (H. Liu et al. 2002). Together, these data strongly support a role for cGAS-STING signaling in both ischemic and hemorrhagic brain events, but show that the effect of Type I IFNs in stroke may be temporally-dependent.

cGAS-STING signaling can be activated by cytosolic mitochondrial DNA (mtDNA) (West et al. 2015; Rongvaux et al. 2014; White et al. 2014) and nuclear DNA (Yang et al. 2017; Volkman et al. 2019); therefore, several groups have sought to determine if self-DNA may be responsible for triggering cGAS-STING activation in stroke. Double stranded DNA (dsDNA) has been found in the cytosol of brain cells after MCAO (Kong et al. 2022; Q. Li et al. 2020) and CVST (R. Ding et al. 2022). While it is challenging to prove whether this DNA is nuclear or mitochondrial in origin, these studies suggest that the aberrant accumulation of self-DNA following stroke may prompt activation of cGAS-STING-mediated inflammation to facilitate clearance of damaged tissue. Taken together, there is substantial evidence for a role of cGAS-STING signaling in stroke and related ischemic CNS events. While some evidence suggests that there may be a benefit to

Model	Treatment/ Manipulation	Outcome Measure (Timepoint)	Result compared to control	References
MCAO	cGAS KO	Hemorrhage (1d)	Reduced	(Wang et al. 2021)
		Motor deficit (1d)	Reduced	
MCAO	STING antagonism (C- 176)	Neuronal apoptosis (1d)	Reduced	(Kong et al. 2022)
		Neurological deficit (1-21d)	Reduced at 7-21d	
SAH	STING antagonism (C- 176)	Neuronal apoptosis (1d, 28d)	Reduced (both)	(Peng et al. 2020)
		Cognitive impairment (28d)	Reduced	
		Neurological severity (1d, 3d)	Reduced (both)	
SAH	STING agonism (CMA)	Neuronal apoptosis (1d, 28d)	Increased (both)	(Peng et al. 2020)
		Cognitive impairment (28d)	Increased	
		Neurological severity (1d, 3d)	Increased (both)	
Neonatal HI (rat)	cGAS inhibition (RU.512)	Infarct size (2d)	Reduced	(Gamdzyk et al. 2020)
		Neuronal apoptosis (2d)	Reduced	
CVST	cGAS antagonism (RU.521)	Neutrophil infiltration (3d)	Reduced	(Ding et al. 2022)
		Monocyte/macrophage infiltration (3d)	No change	
		Apoptosis (3d)	Reduced	
MCAO	Microglial cGAS KO	Infarct volume (3d)	Reduced	(Li et al. 2020)
MCAO	cGAS antagonism (A151)	Neutrophil infiltration (3d)	Reduced	(Li et al. 2020)
		Chemokine production (3d)	Reduced	
		Apoptosis (3d)	Reduced	
		Neurological severity (1-14d)	Reduced	

Table 1: Summary of key findings from studies specifically addressing STING-IFN signaling in stroke. References are specific to each row. Boxes where one reference is given indicates all information is from that reference. Timepoint is given as days (d) after injury. Studies were performed in mice unless otherwise noted. MCAO = middle cerebral artery occlusion; SAH = subarachnoid hemorrhage; HI = hypoxia-ischemia; CVST = cerebral venous sinus thrombosis.

prophylactically activating STING in these contexts (Kundu et al. 2022), the majority of the studies have found that impairing cGAS-STING signaling by genetic or pharmaceutical measures improves outcome in rodent models of stroke (**Table 1**).

Traumatic Brain Injury

Traumatic brain injuries (TBIs) are a leading cause of death and disability across the globe, affecting 69 million people annually (Dewan et al. 2018). TBI occurs in two phases: primary mechanical injury that causes immediate damage to the brain, and secondary injury that occurs over the minutes to weeks after injury resulting in exacerbated tissue damage. As a key aspect of secondary injury, neuroinflammation is currently one of the most promising targets for therapeutic intervention (McKee and Lukens 2016; Jassam et al. 2017). The neuroinflammatory events that occur after TBI are remarkably similar to those discussed in the stroke section above.

There is substantial evidence the role of interferons in TBI and spinal cord injury (SCI). While most studies on human patient samples have focused on Type II IFNs (reviewed in (Roselli, Chandrasekar, and Morganti-Kossmann 2018)), some evidence does suggest that IFN β is upregulated several hours after TBI (Karve et al. 2016). In addition, numerous studies have shown an upregulation of Type I IFNs in rodent models of TBI (Karve et al. 2016; Fritsch et al. 2022; Barrett et al. 2020).

Karve et al. showed that male IFNAR KO mice have significantly reduced lesion size acutely after a controlled cortical impact (CCI) model of moderate TBI (Karve et al. 2016). These animals also showed reduced pro-inflammatory and increased anti-inflammatory cytokine production, as well as increased microglial expression of CD206, a marker for the anti-inflammatory M2 phenotype. In line with this, another group showed that treatment of primary microglia with IFN β caused a shift towards the inflammatory M1 phenotype (Sen et al. 2020). However, IFNAR KO mice also showed increased Iba1 and GFAP expression, indicative of microgliosis and astrogliosis, respectively (Ito, Fukazawa, and Yoshida 2007; Eng and Ghirnikar 1994). Despite this, mice that were treated with MAR1, an antibody that blocks IFNAR, had smaller lesion sizes up to one-week post-TBI, as well as reduced motor deficit (Karve et al. 2016), suggesting that inhibiting Type I IFN signaling is broadly protective. Of note, data from bone marrow chimeras utilized in this study also suggest that hematopoietic IFNAR is also responsible for driving detrimental IFN signaling in TBI (Karve et al. 2016). In contrast to the benefits of IFNAR KO, another group using female IFNAR KO mice showed that these mice had increased macrophage infiltration after axonal injury, which they attributed to increased matrix metalloproteinase 9 (MMP9) in IFNAR KO animals (Khoroshii and Owens 2010). MMP9 facilitates leukocyte recruitment by degrading extracellular matrix (Cabral-Pacheco et al. 2020), and has previously been shown to be negatively regulated by Type I IFNs (Stuve et al. 1997); therefore, it is possible that uninhibited MMP9 results in increased BBB permeability, allowing increased immune cell infiltration.

In a moderate CCI injury, IFN β KO animals had reduced motor impairment and cognitive deficit up to one month after injury, as well as blunted pro-inflammatory cytokine production three

days after injury (Barrett et al. 2020). In confirmation of the study by Karve et al., this study also utilized an anti-IFNAR antibody (Barrett et al. 2020). Motor skills were improved up to two weeks after injury when mice received the antibody, but no difference was seen after one month (Barrett et al. 2020). Of note, mRNA for STING and other interferon stimulated genes (ISGs), but not cGAS, remained upregulated 60 days after injury (Barrett et al. 2020), suggesting that STING-mediated neuroinflammation plays a chronic role in TBI. This same group later showed that when compared to young animals, aged animals showed elevated cGAS and IFN mRNA expression after TBI (Barrett et al. 2021).

The majority of the studies assessing IFNAR and IFN β in TBI show that loss or inhibition of Type I IFN signaling is beneficial; however, other mechanisms for producing Type I IFNs exist. Two studies have specifically addressed the effects of loss of cGAS or STING in TBI (Abdullah et al. 2018; Fritsch et al. 2022). STING mRNA is elevated in post-mortem human TBI patients, which was also seen in a mouse controlled cortical impact (CCI) model (Abdullah et al. 2018). STING KO mice showed significant reduction in lesion size and pro-inflammatory cytokines one day after injury (Abdullah et al. 2018). In contrast to earlier work showing that IFNAR KO mice had increased GFAP and Iba1 reactivity (Karve et al. 2016), STING KO animals had reduced GFAP and Iba1 immunofluorescence (Abdullah et al. 2018). Using the same model, we showed that cGAS KO and STING KO mice both showed reduced lesion size, cell death, and cytokine production one day after injury (Fritsch et al. 2022). Of note, levels of chemokines including MCP1 and CXCL10, were reduced in KO animals compared to WT, suggesting peripheral immune cell infiltration could possibly be reduced (Fritsch et al. 2022). cGAS KO mice showed decreased motor deficit, and both cGAS KO and STING KO mice had reduced lesion sizes two weeks after injury. Importantly, we also demonstrated that the immune modulator NLRX1 appears to negatively regulate STING after brain trauma (Fritsch et al. 2022), a finding that previously had only been demonstrated *in vitro* (H. Guo et al. 2016) and provides an additional potential target for therapeutics. Furthermore, we showed that mtDNA is present in the cytosol of injured cells acutely after TBI (Fritsch et al. 2022), indicating that cGAS may be activated by the release of mtDNA in brain trauma. Given that STING may be activated through cGAS-independent mechanisms (Dunphy et al. 2018; Unterholzner and Dunphy 2019), these findings suggest that STING signaling in brain injury at least in part depends upon cGAS. cGAS STING double knockout mice would be needed to elucidate whether cGAS-independent signaling STING occurred during brain injury.

Taken together, these studies show that STING-IFN signaling is an important aspect of neuroinflammation after brain injury. Based on the current literature, a working model of STING-IFN signaling in stroke and brain injury is proposed (**Figure 1**). The majority of this work demonstrates a detrimental role for the Type I IFN response in TBI (**Table 2**).

STING Signaling in Neurodegenerative Disease

Though the exact mechanisms remain unclear, brain trauma is a known risk factor for the development of neurodegenerative disease (Graham and Sharp 2019; Smith, Johnson, and Stewart

Model	Treatment/ Manipulation	Outcome Measure (Timepoint)	Result compared to control	References
CCI	STING KO (STING ^{gt/gt})	Lesion size (1d)	Reduced	(Abdullah et al. 2018; Fritsch et al. 2022)
		Lesion size (14d)	Reduced	(Fritsch et al. 2022)
		Cell death (1d)	Reduced	(Fritsch et al. 2022)
		Cytokine production (2-24h)	Reduced	(Abdullah et al. 2018; Fritsch et al. 2022)
		Microgliosis/astrogliosis	Reduced	(Abdullah et al. 2018)
CCI	cGAS KO	Lesion size (1d, 14d)	Reduced	(Fritsch et al. 2022)
		Cell death (1d)	Reduced	
		Cytokine production (1d, 14d)	Reduced (both)	
		Motor deficit (4-14d)	Reduced 4d	
CCI	IFNB KO	Lesion size (28d)	Reduced	(Barrett et al. 2020)
		Cytokine production (3d)	Reduced	
		Motor deficit (1-28d)	No change 1-3d, reduced 7-28d	
		Memory deficit (18d)	Reduced	
CCI	IFNAR KO	Lesion size (1d)	Reduced	(Karve et al. 2016)
		Cytokine production (2-24h)	Reduced pro-, increased anti- inflammatory	
		Microgliosis/astrogliosis (1d)	Increased	
Axonal injury	INFAR KO	Immune cell infiltration (1d)	Increased	(Khorrooshi and Owens 2010)
		MMP9 expression (1d)	Increased	
CCI	Anti-IFNAR monoclonal antibody	Lesion size (1d, 7d)	Reduced	(Karve et al. 2016)
		Lesion size (28d)	No change	(Barrett et al. 2020)
		IFN production (2-4h)	Reduced	(Karve et al. 2016)
		Motor deficit (1-28d)	Reduced 14d	(Barrett et al. 2020)

Table 2: Summary of key findings from studies specifically addressing STING-IFN signaling in brain injury. References are specific to each row. Boxes where one reference is given indicates all information is from that reference. Timepoint is given as hours (h) or days (d) after injury. Studies were performed in mice unless otherwise noted. CCI = controlled cortical impact; STING^{gt/gt} = STING goldenticket KO.

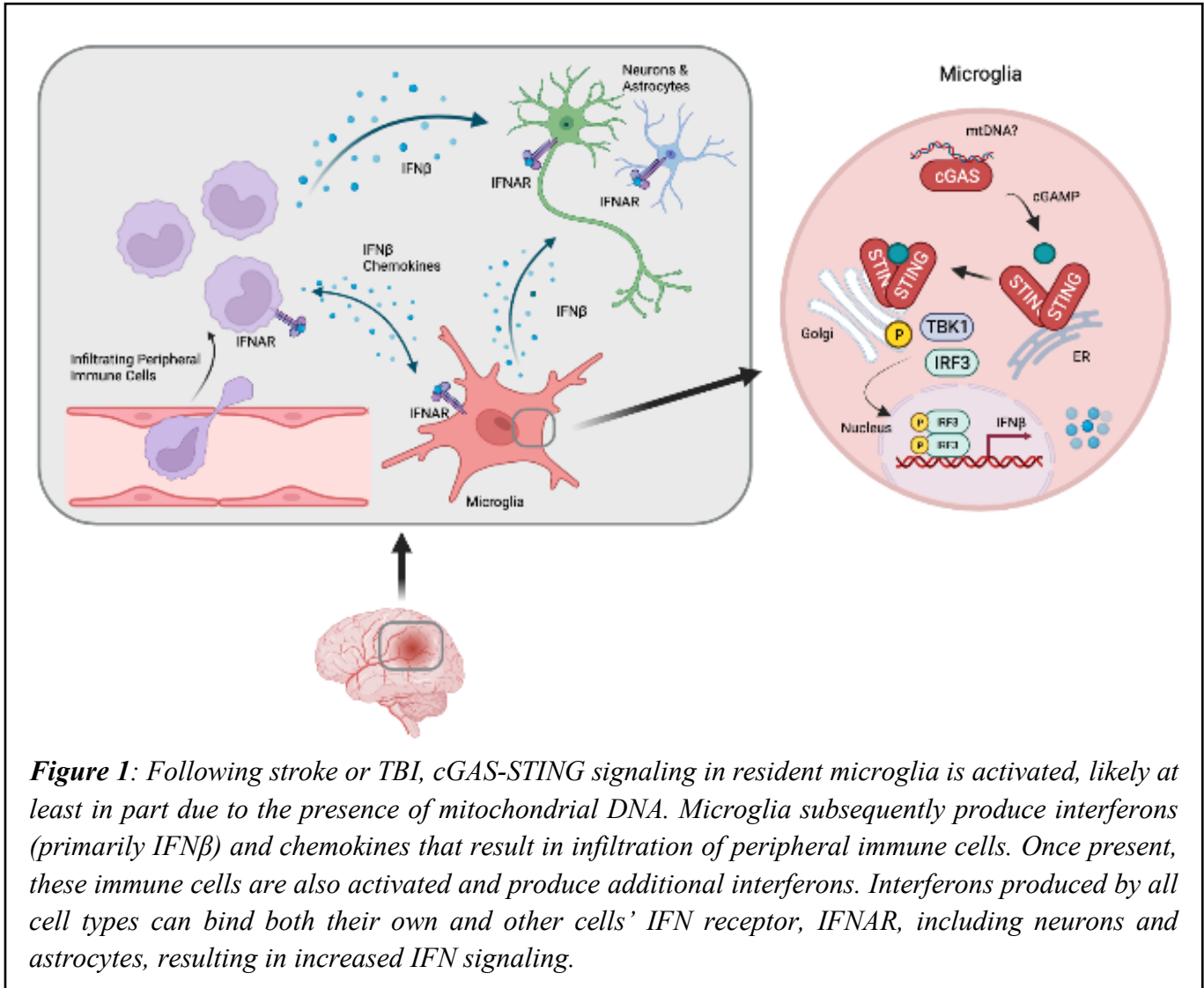
2013). Increasingly, mitochondrial DNA (mtDNA) is being recognized as a DAMP in traumatic brain injury (Fritsch et al. 2022; Walko et al. 2014; Kilbaugh et al. 2015). Mitochondrial damage and dysfunction are also hallmarks of aging (Jang et al. 2018), and serum mtDNA levels have been shown to increase with age, as well as correlate with systemic inflammation (Pinti et al. 2014).

Furthermore, mitochondrial mutations and alterations in mtDNA are associated with a number of different neurodegenerative diseases (Keogh and Chinnery 2015). cGAS, STING, and Type I IFN signaling have been implicated in a number of neurodegenerative and aging-related disorders. Several recent reviews have covered these relationships (Paul, Snyder, and Bohr 2021; Decout et al. 2021; Fryer et al. 2021); therefore, this review will focus on the role of mitochondrial DNA in neurodegeneration-related STING-IFN signaling.

Huntington's disease (HD) is a relatively rare, progressive, genetic disease that results from CAG repeats in the huntingtin (HTT) gene causing polyglutamine expansions. Chorea is the primary symptom for diagnosis, and the basal ganglia is the primary brain region affected (Waldvogel et al. 2015). As the disease progresses, other brain regions such as the cerebral cortex are affected, and symptoms expand to include more advanced cognitive and behavioral changes (Bates et al. 2015; Waldvogel et al. 2015). Unfortunately, most HD patients die within 15-20 years of symptom onset (Bates et al. 2015). Mitochondrial dysfunction is prevalent in HD (Y. Wang et al. 2021), and a study in a small cohort of patients showed that mtDNA copy number is elevated in leukocytes compared to healthy controls, particularly for female patients (Jędrak et al. 2017). Recently, two groups have shown that cGAS and STING are upregulated in the brains of HD patients (Sharma et al. 2020; Jauhari et al. 2020). One study also showed that HD mutant cell lines had increased cytosolic mtDNA, leading the authors to conclude that cGAS-STING signaling may be activated by the release of mtDNA (Jauhari et al. 2020). Depletion of cGAS in HD mutant cells also showed a reduction in inflammation and autophagy (Sharma et al. 2020).

Alzheimer's disease (AD) is the most common cause of dementia, affecting millions of patients each year. While the cause of AD is unknown, neuroinflammation, including IFN signaling (Gorlé and Vandenbroucke 2019), is believed to play a key role in AD pathology. While memory deficit is the most common symptom of AD, the disease is characterized by widespread distribution of intracellular neurofibrillary tangles and extracellular amyloid beta plaques, and ultimately affects many brain functions (Deture and Dickson 2019). Mitochondria are known to be impacted by AD. Next generation sequencing of hippocampal neurons showed a significant increase in mtDNA mutations in patients with early stage AD (Hoekstra et al. 2016). Furthermore, brain tissue from AD patients have higher levels of oxidized nucleic acids than healthy controls (J. Wang et al. 2005), suggesting that mtDNA mutations may be due to increased oxidation in the AD brain. The supplementation of the precursor for nicotinamide adenine dinucleotide (NAD⁺), an important coenzyme for mitophagy and DNA repair, has been shown to alter cGAS-STING-mediated neuroinflammation in AD (Hou et al. 2021). Fibroblasts from AD patients showed that mtDNA was released into the cytosol, and increased cortical expression of cGAS and STING was noted in a transgenic mouse model of AD (Hou et al. 2021). When an NAD⁺ precursor was given to transgenic AD mice, cGAS and STING expression was reduced (Hou et al. 2021). While the alterations in mtDNA appears to be subsequent to other changes in AD, understanding how mtDNA is impacted may offer insight into the early neuroinflammatory events in AD.

Amyotrophic lateral sclerosis (ALS) is a progressive neuromuscular disorder that affects motor neurons. Degeneration of motor neurons in the brain and spinal cord results in muscle



atrophy that eventually becomes fatal, with a median survival of three years (Grad et al. 2017). Transactive response DNA binding protein 43 (TDP-43) is known to be a pathological protein in ALS (Mackenzie and Rademakers 2008). TDP-43 induces mtDNA release into the cytosol by opening the mitochondrial permeability transition pore (mPTP) (Yu et al. 2020). This cytosolic mtDNA prompts inflammation in a STING dependent manner, which was verified by both genetic KO mouse models and pharmaceutical STING inhibition (Yu et al. 2020). Of note, cGAMP is elevated in the spinal cords of ALS patients and in a mouse model of ALS (Yu et al. 2020), indicating that cGAS is activated in ALS. Further study is needed to confirm the role of cGAS-STING signaling in ALS to determine if targeting this pathway may help improve motor function.

Parkinson's disease (PD) is a highly prevalent neurodegenerative disease that primarily involves motor dysfunction due to the loss of dopaminergic neurons in the substantia nigra (Pickrell and Youle 2015). The hallmark pathology of surviving neurons is the accumulation of α -synuclein, which is thought to be harmful to neuronal health (Stefanis 2012). While the cause of PD is unknown, mitochondrial dysfunction and mutations in PINK1 and Parkin, proteins involved

in mitophagy, are associated with PD (Valente et al. 2004; Kitada et al. 1998). Mitophagy is the selective removal of damaged or unneeded mitochondria, and is known to be altered in aging and neurodegenerative disease (Chen, Kroemer, and Kepp 2020). When PINK1 or Parkin KO mice were subjected to acute mitochondrial stress via exhaustive exercise, mitophagy was impaired and serum concentrations of inflammatory cytokines were elevated (Sliter et al. 2018). Crossing these mice with STING KO animals completely abolished the inflammatory phenotype seen after mitochondrial stress, indicating that this inflammation is STING-mediated (Sliter et al. 2018). The authors hypothesized that the accumulation of damaged mitochondria resulted in mtDNA release that activated cGAS-STING signaling to promote inflammation (Sliter et al. 2018). While this study utilized an intensive exercise paradigm to induce mitochondrial stress, it remains plausible that mtDNA-STING signaling occurs when mitophagy is impaired in PD.

Conclusions and Future Directions

STING signaling has been implicated in dozens of protective and pathological functions across nearly every organ system. In particular, the role of STING in disease and injury conditions has become a growing area of interest, highlighting not just the importance of the STING-IFN response, but also the paucity of treatments for countless diseases. Current evidence suggests targeting STING signaling either via agonism or inhibition may be beneficial for a number of diseases. In fact, several clinical trials have sought to address whether STING agonism is beneficial in cancer (Jiang et al. 2020). Unfortunately, the results available so far are disappointing, with STING agonists showing little anti-tumor effect in human patients, despite promising pre-clinical results (Gogoi, Mansouri, and Jin 2020). While any number of factors can cause failures in clinical trials, this may in part be due to the newly discovered pro-cancer functions of STING (discussed in (Jiang et al. 2020)), underscoring how much remains to be understood in STING signaling. Several important questions must be addressed before considering STING-based therapeutics in neuroinflammatory disease.

The time course of STING activation and subsequent window for therapeutics must be thoroughly characterized for each disease or injury paradigm. For most neurological disorders, the aim is to limit inflammatory events that are harmful. However, it is likely that there are critical timepoints in which STING activation may be beneficial. Moreover, clarifying whether targeting cGAS, STING, IFNs, or another protein involved in this response, such as NLRX1, may be of therapeutic benefit will require better understanding of the timing of activation. For example, in mouse models of TBI, STING and many ISGs remain upregulated two months post-injury while cGAS is unaltered from sham levels (Barrett et al. 2020). At an acute timepoint after TBI, cGAS expression was particularly elevated in aged animals compared to young animals (Barrett et al. 2021), indicating that the magnitude and/or timing of cGAS-STING-IFN signaling may also be age-dependent. Though it is clear that STING signaling occurs after TBI and other neuroinflammatory events, the temporal dynamics of this response remain unclear.

There is not yet a consensus on which cell types in the brain express STING. As discussed previously, even when using the same reagents, studies by separate groups have concluded

different cell types express STING. Additionally, STING is itself has been described as an ISG (Ma et al. 2015), therefore it is possible that any cell with even low basal expression has the ability to upregulate STING in disease contexts. As we move towards targeted therapeutics, clarifying which cell types pharmaceutical interventions must reach is critical.

The translatability of pharmaceuticals must be properly assessed before considering specific drugs for progression to human patients. There are considerable molecular differences between murine STING and human STING (Shih, Damm-Ganamet, and Mirzadegan 2018), and some agonists for murine STING are ineffective in activating human STING. DMXAA, for example, showed excellent promise preclinically but failed in clinical trials due to its specificity for murine, but not human STING (Conlon et al. 2013). Rat STING, however, appears to more closely mimic how human STING responds to agonists (H. Zhang et al. 2015). Use of multiple animal models may be beneficial for assessing STING as a therapeutic target due to these known differences. Alternatively, targeting other proteins in the STING pathway, such as NLRX1 (H. Guo et al. 2016; Fritsch et al. 2022), may be an option. While there remains much to learn about STING, particularly in the context of neuroinflammation, answering these questions will pave the way for the use of STING-based therapeutics.

Author Contributions

Conceptualization, LEF and AMP; writing-original draft preparation, LEF; writing-review and editing, LEF and AMP. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This work was supported by the National Institutes of Health (R35GM142368) and Commonwealth Health Research Board (#208-05-20) (AMP).

Conflicts of Interest

The authors declare no conflicts of interest.

References

- Abdullah, Amar, Moses Zhang, Tony Frugier, Sammy Bedoui, Juliet M. Taylor, and Peter J. Crack. 2018. "STING-Mediated Type-I Interferons Contribute to the Neuroinflammatory Process and Detrimental Effects Following Traumatic Brain Injury." *Journal of Neuroinflammation* 15 (1). <https://doi.org/10.1186/S12974-018-1354-7>.
- Ablasser, Andrea, Marion Goldeck, Taner Cavlar, Tobias Deimling, Gregor Witte, Ingo Röhl, Karl Peter Hopfner, Janos Ludwig, and Veit Hornung. 2013. "CGAS Produces a 2'-5'-Linked Cyclic Dinucleotide Second Messenger That Activates STING." *Nature* 498 (7454): 380–84. <https://doi.org/10.1038/NATURE12306>.
- Alexopoulou, Alexandra, Larisa Vasilieva, and Peter Karayiannis. 2020. "New Approaches to the Treatment of Chronic Hepatitis B." *Journal of Clinical Medicine* 2020, Vol. 9, Page 3187 9 (10): 3187. <https://doi.org/10.3390/JCM9103187>.
- Almeida, Leonardo A. de, Natalia B. Carvalho, Fernanda S. Oliveira, Thais L.S. Lacerda, Anilton C. Vasconcelos, Lucas Nogueira, Andre Bafica, Aristócolo M. Silva, and Sergio C. Oliveira. 2011. "MyD88 and STING Signaling Pathways Are Required for IRF3-Mediated IFN- β Induction in Response to Brucella Abortus Infection." *PloS One* 6 (8). <https://doi.org/10.1371/JOURNAL.PONE.0023135>.
- Apel, Falko, Liudmila Andreeva, Lorenz Sebastian Knackstedt, Robert Streeck, Christian Karl Frese, Christian Goosmann, Karl Peter Hopfner, and Arturo Zychlinsky. 2021. "The Cytosolic DNA Sensor CGAS Recognizes Neutrophil Extracellular Traps." *Science Signaling* 14 (673).

- https://doi.org/10.1126/SCISIGNAL.AAX7942/SUPPL_FILE/AAX7942_SM.PDF.
- Archer, Kristina A., Juliana Durack, and Daniel A. Portnoy. 2014. "STING-Dependent Type I IFN Production Inhibits Cell-Mediated Immunity to *Listeria Monocytogenes*." *PLoS Pathogens* 10 (1). <https://doi.org/10.1371/JOURNAL.PPAT.1003861>.
- Baral, Pankaj, Swalpa Udit, and Isaac M. Chiu. 2019. "Pain and Immunity: Implications for Host Defence." *Nature Reviews. Immunology* 19 (7): 433. <https://doi.org/10.1038/S41577-019-0147-2>.
- Barrett, James P., Rebecca J. Henry, Kari Ann Shirey, Sarah J. Doran, Oleg D. Makarevich, Rodney R. Ritzel, Victoria A. Meadows, et al. 2020. "Interferon- β Plays a Detrimental Role in Experimental Traumatic Brain Injury by Enhancing Neuroinflammation That Drives Chronic Neurodegeneration." *The Journal of Neuroscience*, February, 2516–19. <https://doi.org/10.1523/jneurosci.2516-19.2020>.
- Barrett, James P., Susan M. Knoblach, Surajit Bhattacharya, Heather Gordish-Dressman, Bogdan A. Stoica, and David J. Loane. 2021. "Traumatic Brain Injury Induces CGAS Activation and Type I Interferon Signaling in Aged Mice." *Frontiers in Immunology* 12 (August): 3396. <https://doi.org/10.3389/FIMMU.2021.710608/BIBTEX>.
- Basbaum, Allan I., Diana M. Bautista, Grégory Scherrer, and David Julius. 2009. "Cellular and Molecular Mechanisms of Pain." *Cell* 139 (2): 267. <https://doi.org/10.1016/J.CELL.2009.09.028>.
- Bates, Gillian P., Ray Dorsey, James F. Gusella, Michael R. Hayden, Chris Kay, Blair R. Leavitt, Martha Nance, et al. 2015. "Huntington Disease." *Nature Reviews Disease Primers* 1 (April). <https://doi.org/10.1038/NRDP.2015.5>.
- Bodda, Chiranjeevi, Line S. Reinert, Stefanie Fruhwürth, Timmy Richardo, Chenglong Sun, Bao Cun Zhang, Maria Kalamvoki, et al. 2020. "HSV1 VP1-2 Deubiquitinates STING to Block Type I Interferon Expression and Promote Brain Infection." *The Journal of Experimental Medicine* 217 (7). <https://doi.org/10.1084/JEM.20191422>.
- Burdette, Dara L., Kathryn M. Monroe, Katia Sotelo-Troha, Jeff S. Iwig, Barbara Eckert, Mamoru Hyodo, Yoshihiro Hayakawa, and Russell E. Vance. 2011. "STING Is a Direct Innate Immune Sensor of Cyclic Di-GMP." *Nature* 2011 478:7370 478 (7370): 515–18. <https://doi.org/10.1038/nature10429>.
- Burdette, Dara L., and Russell E. Vance. 2013. "STING and the Innate Immune Response to Nucleic Acids in the Cytosol." *Nature Immunology* 14 (1): 19–26. <https://doi.org/10.1038/NI.2491>.
- Cabral-Pacheco, Griselda A., Idalia Garza-Veloz, Claudia Castruita De La Rosa, Jesus M. Ramirez-Acuña, Braulio A. Perez-Romero, Jesus F. Guerrero-Rodriguez, Nadia Martinez-Avila, and Margarita L. Martinez-Fierro. 2020. "The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases." *International Journal of Molecular Sciences* 21 (24): 1–53. <https://doi.org/10.3390/IJMS21249739>.
- Chen, Guo, Guido Kroemer, and Oliver Kepp. 2020. "Mitophagy: An Emerging Role in Aging and Age-Associated Diseases." *Frontiers in Cell and Developmental Biology* 8 (March): 200. <https://doi.org/10.3389/FCELL.2020.00200/BIBTEX>.
- Chopy, Damien, Claudia N. Detje, Mireille Lafage, Ulrich Kalinke, and Monique Lafon. 2011. "The Type I Interferon Response Bridles Rabies Virus Infection and Reduces Pathogenicity." *Journal of Neurovirology* 17 (4): 353–67. <https://doi.org/10.1007/S13365-011-0041-6>.
- Chu, Ting-Ting, Xintao Tu, Kun Yang, Jianjun Wu, Joyce J. Repa, and Nan Yan. 2021. "Tonic Prime-Boost of STING Signalling Mediates Niemann–Pick Disease Type C." *Nature* 2021, July, 1–6. <https://doi.org/10.1038/s41586-021-03762-2>.
- Civril, Filiz, Tobias Deimling, Carina C. De Oliveira Mann, Andrea Ablasser, Manuela Moldt, Gregor Witte, Veit Hornung, and Karl Peter Hopfner. 2013. "Structural Mechanism of Cytosolic DNA Sensing by CGAS." *Nature* 498 (7454): 332. <https://doi.org/10.1038/NATURE12305>.
- Conlon, Joseph, Dara L. Burdette, Shruti Sharma, Numana Bhat, Mikayla Thompson, Zhaozhao Jiang, Vijay A. K. Rathinam, et al. 2013. "Mouse, but Not Human STING, Binds and Signals in Response to the Vascular Disrupting Agent DMXAA." *Journal of Immunology (Baltimore, Md. : 1950)* 190 (10): 5216. <https://doi.org/10.4049/JIMMUNOL.1300097>.
- Decout, Alexiane, Jason D. Katz, Shankar Venkatraman, and Andrea Ablasser. 2021. "The CGAS–STING Pathway as a Therapeutic Target in Inflammatory Diseases." *Nature Reviews Immunology* 2021 21:9 21 (9): 548–69. <https://doi.org/10.1038/s41577-021-00524-z>.
- Deschamps, Thibaut, and Maria Kalamvoki. 2017. "Evasion of the STING DNA-Sensing Pathway by VP11/12 of Herpes Simplex Virus 1." *Journal of Virology* 91 (16). <https://doi.org/10.1128/JVI.00535-17>.
- Deture, Michael A., and Dennis W. Dickson. 2019. "The Neuropathological Diagnosis of Alzheimer's Disease." *Molecular Neurodegeneration* 2019 14:1 14 (1): 1–18. <https://doi.org/10.1186/S13024-019-0333-5>.
- Dewan, Michael C., Abbas Rattani, Saksham Gupta, Ronnie E. Baticulon, Ya Ching Hung, Maria Punchak, Amit

- Agrawal, et al. 2018. "Estimating the Global Incidence of Traumatic Brain Injury." *Journal of Neurosurgery* 130 (4): 1080–97. <https://doi.org/10.3171/2017.10.JNS17352>.
- Ding, Qiang, Jenna M. Gaska, Florian Douam, Lei Wei, David Kim, Metodi Balev, Brigitte Heller, and Alexander Ploss. 2018. "Species-Specific Disruption of STING-Dependent Antiviral Cellular Defenses by the Zika Virus NS2B3 Protease." *Proceedings of the National Academy of Sciences of the United States of America* 115 (27): E6310–18. <https://doi.org/10.1073/PNAS.1803406115>.
- Ding, Rui, Haiyan Li, Yaqi Liu, Weiyang Ou, Xifang Zhang, Huihui Chai, Xiaofei Huang, Weijie Yang, and Qiujiang Wang. 2022. "Activating CGAS-STING Axis Contributes to Neuroinflammation in CVST Mouse Model and Induces Inflammasome Activation and Microglia Pyroptosis." *Journal of Neuroinflammation* 19 (1): 137. <https://doi.org/10.1186/S12974-022-02511-0/FIGURES/9>.
- Dobbs, Nicole, Nikolay Burnaevskiy, Didi Chen, Vijay K. Gonugunta, Neal M. Alto, and Nan Yan. 2015. "STING Activation by Translocation from the ER Is Associated with Infection and Autoinflammatory Disease." *Cell Host & Microbe* 18 (2): 157–68. <https://doi.org/10.1016/J.CHOM.2015.07.001>.
- Donnelly, Christopher R., Ouyang Chen, and Ru Rong Ji. 2020. "How Do Sensory Neurons Sense Danger Signals?" *Trends in Neurosciences* 43 (10): 822–38. <https://doi.org/10.1016/J.TINS.2020.07.008>.
- Donnelly, Christopher R., Changyu Jiang, Amanda S. Andriessen, Kaiyuan Wang, Zilong Wang, Huiping Ding, Junli Zhao, et al. 2021. "STING Controls Nociception via Type I Interferon Signalling in Sensory Neurons." *Nature* 591 (7849): 275–80. <https://doi.org/10.1038/S41586-020-03151-1>.
- Duarte, Luisa F., Mónica A. Fariás, Diana M. Álvarez, Susan M. Bueno, Claudia A. Riedel, and Pablo A. González. 2019. "Herpes Simplex Virus Type 1 Infection of the Central Nervous System: Insights Into Proposed Interrelationships With Neurodegenerative Disorders." *Frontiers in Cellular Neuroscience* 13 (January): 1–23. <https://doi.org/10.3389/FNCEL.2019.00046>.
- Dunphy, Gillian, Sinéad M Flannery, Jessica F Almine, Dymphna J Connolly, Christina Paulus, Kasper L Jønsson, Martin R Jakobsen, Michael M Nevels, Andrew G Bowie, and Leonie Unterholzner. 2018. "Non-Canonical Activation of the DNA Sensing Adaptor STING by ATM and IFI16 Mediates NF-KB Signaling after Nuclear DNA Damage." *Molecular Cell* 71 (5): 745-760.e5. <https://doi.org/10.1016/j.molcel.2018.07.034>.
- Eng, Lawrence F., and Roopa S. Ghirnikar. 1994. "GFAP and Astroglisis." *Brain Pathology (Zurich, Switzerland)* 4 (3): 229–37. <https://doi.org/10.1111/J.1750-3639.1994.TB00838.X>.
- Fritsch, Lauren E., Jing Ju, Erwin Kristobal Gudenschwager Basso, Eman Soliman, Swagatika Paul, Jiang Chen, Alexandra M. Kaloss, et al. 2022. "Type I Interferon Response Is Mediated by NLRX1-CGAS-STING Signaling in Brain Injury." *Frontiers in Molecular Neuroscience* 0 (February): 53. <https://doi.org/10.3389/FNMOL.2022.852243>.
- Fryer, Amelia L., Amar Abdullah, Juliet M. Taylor, and Peter J. Crack. 2021. "The Complexity of the CGAS-STING Pathway in CNS Pathologies." *Frontiers in Neuroscience* 15 (February): 82. <https://doi.org/10.3389/FNINS.2021.621501/BIBTEX>.
- Gamdzik, Marcin, Desislava Met Doycheva, Camila Araujo, Umut Ocak, Yujie Luo, Jiping Tang, and John H. Zhang. 2020. "CGAS/STING Pathway Activation Contributes to Delayed Neurodegeneration in Neonatal Hypoxia-Ischemia Rat Model: Possible Involvement of LINE-1." *Molecular Neurobiology* 57 (6): 2600–2619. <https://doi.org/10.1007/S12035-020-01904-7>.
- Gao, Daxing, Jiaxi Wu, You Tong Wu, Fenghe Du, Chukwuemika Aroh, Nan Yan, Lijun Sun, and Zhijian J. Chen. 2013. "Cyclic GMP-AMP Synthase Is an Innate Immune Sensor of HIV and Other Retroviruses." *Science (New York, N.Y.)* 341 (6148): 903–6. <https://doi.org/10.1126/SCIENCE.1240933>.
- Gao, Pu, Manuel Ascano, Thomas Zillinger, Weiyl Wang, Peihong Dai, Artem A. Serganov, Barbara L. Gaffney, et al. 2013. "Structure-Function Analysis of STING Activation by c[G(2',5')PA(3',5')p] and Targeting by Antiviral DMXAA." *Cell* 154 (4). <https://doi.org/10.1016/J.CELL.2013.07.023>.
- Gogoi, Himanshu, Samira Mansouri, and Lei Jin. 2020. "The Age of Cyclic Dinucleotide Vaccine Adjuvants." *Vaccines* 2020, Vol. 8, Page 453 8 (3): 453. <https://doi.org/10.3390/VACCINES8030453>.
- Gorlé, N., and R. E. Vandenbroucke. 2019. "Interferons: A Molecular Switch between Damage and Repair in Ageing and Alzheimer's Disease." *Mechanisms of Ageing and Development* 183 (October): 111148. <https://doi.org/10.1016/J.MAD.2019.111148>.
- Grad, Leslie I., Guy A. Rouleau, John Ravits, and Neil R. Cashman. 2017. "Clinical Spectrum of Amyotrophic Lateral Sclerosis (ALS)." *Cold Spring Harbor Perspectives in Medicine* 7 (8). <https://doi.org/10.1101/CSHPERSPECT.A024117>.
- Graham, Neil S.N., and David J. Sharp. 2019. "Understanding Neurodegeneration after Traumatic Brain Injury: From Mechanisms to Clinical Trials in Dementia." *Journal of Neurology, Neurosurgery & Psychiatry* 90 (11): 1221–33. <https://doi.org/10.1136/JNNP-2017-317557>.

- Guimarães, Erika S., Fabio V. Marinho, Nina M.G.P. de Queiroz, Máisa M. Antunes, and Sergio C. Oliveira. 2021. "Impact of STING Inflammatory Signaling during Intracellular Bacterial Infections." *Cells* 11 (1). <https://doi.org/10.3390/CELLS11010074>.
- Guo, Fang, Yanxing Han, Xuesen Zhao, Jianghua Wang, Fei Liu, Chunxiao Xu, Lai Wei, et al. 2015. "Sting Agonists Induce an Innate Antiviral Immune Response against Hepatitis B Virus." *Antimicrobial Agents and Chemotherapy* 59 (2): 1273–81. <https://doi.org/10.1128/AAC.04321-14/ASSET/7C0D8A85-3AFC-4F36-923D-CC0761EEC150/ASSETS/GRAPHIC/ZAC0021537300008.JPEG>.
- Guo, Haitao, Renate König, Meng Deng, Maximilian Riess, Jinyao Mo, Lu Zhang, Alex Petrucelli, et al. 2016. "NLRX1 Sequesters STING to Negatively Regulate the Interferon Response, Thereby Facilitating the Replication of HIV-1 and DNA Viruses." *Cell Host and Microbe*. <https://doi.org/10.1016/j.chom.2016.03.001>.
- He, Jing, Ruidong Hao, Dan Liu, Xing Liu, Shaoshuai Wu, Shuting Guo, Yuan Wang, Po Tien, and Deyin Guo. 2016. "Inhibition of Hepatitis B Virus Replication by Activation of the CGAS-STING Pathway." *The Journal of General Virology* 97 (12): 3368–78. <https://doi.org/10.1099/JGV.0.000647>.
- Hoekstra, Jake G., Michael J. Hipp, Thomas J. Montine, and Scott R. Kennedy. 2016. "Mitochondrial DNA Mutations Increase in Early Stage Alzheimer's Disease and Are Inconsistent with Oxidative Damage." *Annals of Neurology* 80 (2): 301. <https://doi.org/10.1002/ANA.24709>.
- Hou, Yujun, Yong Wei, Sofie Lautrup, Beimeng Yang, Yue Wang, Stephanie Cordonnier, Mark P. Mattson, Deborah L. Croteau, and Vilhelm A. Bohr. 2021. "NAD+ Supplementation Reduces Neuroinflammation and Cell Senescence in a Transgenic Mouse Model of Alzheimer's Disease via CGAS-STING." *Proceedings of the National Academy of Sciences of the United States of America* 118 (37). <https://doi.org/10.1073/PNAS.2011226118/-/DCSUPPLEMENTAL>.
- Huang, Jian, Hongjuan You, Chenhe Su, Yangxin Li, Shunhua Chen, and Chunfu Zheng. 2018. "Herpes Simplex Virus 1 Tegument Protein VP22 Abrogates CGAS/STING-Mediated Antiviral Innate Immunity." *Journal of Virology* 92 (15). <https://doi.org/10.1128/JVI.00841-18/ASSET/CB6A5295-FC14-468F-9F78-FFEDCCB31479/ASSETS/GRAPHIC/ZJV0151837310004.JPEG>.
- Isaacs, A, and J Lindenmann. 1957. "Virus Interference. I. The Interferon." *Proceedings of the Royal Society of London. Series B, Biological Sciences* 147 (927): 258–67. <https://doi.org/10.1098/RSPB.1957.0048>.
- Ishikawa, Hiroki, and Glen N. Barber. 2008. "STING Is an Endoplasmic Reticulum Adaptor That Facilitates Innate Immune Signalling." *Nature* 455 (7213): 674–78. <https://doi.org/10.1038/nature07317>.
- Ishikawa, Hiroki, Zhe Ma, and Glen N. Barber. 2009. "STING Regulates Intracellular DNA-Mediated, Type I Interferon-Dependent Innate Immunity." *Nature* 2009 461:7265 461 (7265): 788–92. <https://doi.org/10.1038/nature08476>.
- Ito, Ichiaki, Jutarou Fukazawa, and Michiteru Yoshida. 2007. "Post-Translational Methylation of High Mobility Group Box 1 (HMGB1) Causes Its Cytoplasmic Localization in Neutrophils." *Journal of Biological Chemistry* 282 (22): 16336–44. <https://doi.org/10.1074/jbc.M608467200>.
- James, Charlotte, Manale Harfouche, Nicky J. Welton, Katherine M.E. Turner, Laith J. Abu-Raddad, Sami L. Gottlieb, and Katharine J. Looker. 2020. "Herpes Simplex Virus: Global Infection Prevalence and Incidence Estimates, 2016." *Bulletin of the World Health Organization* 98 (5): 315. <https://doi.org/10.2471/BLT.19.237149>.
- Jang, Ji Yong, Arnon Blum, Jie Liu, and Toren Finkel. 2018. "The Role of Mitochondria in Aging." *The Journal of Clinical Investigation* 128 (9): 3662–70. <https://doi.org/10.1172/JCI120842>.
- Jassam, Yasir N., Saef Izzy, Michael Whalen, Dorian B. McGavern, and Joseph El Khoury. 2017. "Neuroimmunology of Traumatic Brain Injury: Time for a Paradigm Shift." *Neuron*. Cell Press. <https://doi.org/10.1016/j.neuron.2017.07.010>.
- Jauhari, Abhishek, Sergei V. Baranov, Yalikul Suofu, Jinho Kim, Tanisha Singh, Svitlana Yablonska, Fang Li, et al. 2020. "Melatonin Inhibits Cytosolic Mitochondrial DNA-Induced Neuroinflammatory Signaling in Accelerated Aging and Neurodegeneration." *The Journal of Clinical Investigation* 130 (6): 3124–36. <https://doi.org/10.1172/JCI135026>.
- Jayaraj, Richard L., Sheikh Azimullah, Rami Beiram, Fakhreya Y. Jalal, and Gary A. Rosenberg. 2019. "Neuroinflammation: Friend and Foe for Ischemic Stroke." *Journal of Neuroinflammation* 2019 16:1 16 (1): 1–24. <https://doi.org/10.1186/S12974-019-1516-2>.
- Jędrak, Paulina, Magdalena Krygier, Katarzyna Tońska, Małgorzata Drozd, Magdalena Kaliszewska, Ewa Bartnik, Witold Sołtan, et al. 2017. "Mitochondrial DNA Levels in Huntington Disease Leukocytes and Dermal Fibroblasts." *Metabolic Brain Disease* 32 (4): 1237. <https://doi.org/10.1007/S11011-017-0026-0>.
- Jeffries, Austin M., and Ian Marriott. 2017. "Human Microglia and Astrocytes Express CGAS-STING Viral Sensing Components." *Neuroscience Letters* 658 (September): 53–56.

- <https://doi.org/10.1016/J.NEULET.2017.08.039>.
- Jiang, Minlin, Minlin Jiang, Peixin Chen, Peixin Chen, Lei Wang, Wei Li, Bin Chen, et al. 2020. "CGAS-STING, an Important Pathway in Cancer Immunotherapy." *Journal of Hematology and Oncology* 13 (1): 1–11. <https://doi.org/10.1186/S13045-020-00916-Z/FIGURES/4>.
- Jin, Lei, Andrew Getahun, Heather M. Knowles, Jennifer Mogan, Linda J. Akerlund, Thomas A. Packard, Anne-Laure Perraud, and John C. Cambier. 2013. "STING/MPYS Mediates Host Defense against *Listeria Monocytogenes* Infection by Regulating Ly6Chi Monocyte Migration." *The Journal of Immunology* 190 (6): 2835–43. <https://doi.org/10.4049/JIMMUNOL.1201788>.
- Jin, Lei, Paul M. Waterman, Karen R. Jonscher, Cindy M. Short, Nichole A. Reisdorph, and John C. Cambier. 2008. "MPYS, a Novel Membrane Tetraspanner, Is Associated with Major Histocompatibility Complex Class II and Mediates Transduction of Apoptotic Signals." *Molecular and Cellular Biology* 28 (16): 5014–26. https://doi.org/10.1128/MCB.00640-08/SUPPL_FILE/MPYS_SUPPL_DATA_4_18_08.PDF.
- Johnson, Catherine Owens, Minh Nguyen, Gregory A. Roth, Emma Nichols, Tahiya Alam, Degu Abate, Foad Abd-Allah, et al. 2019. "Global, Regional, and National Burden of Stroke, 1990-2016: A Systematic Analysis for the Global Burden of Disease Study 2016." *The Lancet. Neurology* 18 (5): 439–58. [https://doi.org/10.1016/S1474-4422\(19\)30034-1](https://doi.org/10.1016/S1474-4422(19)30034-1).
- Kalamvoki, Maria, Te Du, and Bernard Roizman. 2014. "Cells Infected with Herpes Simplex Virus 1 Export to Uninfected Cells Exosomes Containing STING, Viral MRNAs, and MicroRNAs." *Proceedings of the National Academy of Sciences of the United States of America* 111 (46): E4991–96. <https://doi.org/10.1073/PNAS.1419338111/ASSET/41494EEF-3E96-4F14-B6D2-DD54A4F3540B/ASSETS/GRAPHIC/PNAS.1419338111FIG07.JPEG>.
- Kalamvoki, Maria, and Bernard Roizman. 2014. "HSV-1 Degrades, Stabilizes, Requires, or Is Stung by STING Depending on ICP0, the US3 Protein Kinase, and Cell Derivation." *Proceedings of the National Academy of Sciences of the United States of America* 111 (5). <https://doi.org/10.1073/PNAS.1323414111>.
- Kang, Lijing, Huilin Yu, Xing Yang, Yuanbo Zhu, Xiaofei Bai, Ranran Wang, Yongliang Cao, et al. 2020. "Neutrophil Extracellular Traps Released by Neutrophils Impair Revascularization and Vascular Remodeling after Stroke." *Nature Communications* 11 (1). <https://doi.org/10.1038/S41467-020-16191-Y>.
- Karve, Ila P., Moses Zhang, Mark Habgood, Tony Frugier, Kate M. Brody, Maithili Sashindranath, C. Joakim Ek, et al. 2016. "Ablation of Type-1 IFN Signaling in Hematopoietic Cells Confers Protection Following Traumatic Brain Injury." *ENeuro* 3 (1): 89–93. <https://doi.org/10.1523/ENEURO.0128-15.2016>.
- Katzilieris-Petras, Georgios, Xin Lai, Ahmad S. Rashidi, Georges M. G. M. Verjans, Line S. Reinert, and Søren R. Paludan. 2022. "Microglia Activate Early Antiviral Responses upon Herpes Simplex Virus 1 Entry into the Brain to Counteract Development of Encephalitis-Like Disease in Mice." *Journal of Virology* 96 (6). <https://doi.org/10.1128/JVI.01311-21/ASSET/73DB7756-6384-4815-B69F-48962E2D5019/ASSETS/IMAGES/LARGE/JVI.01311-21-F008.JPG>.
- Keogh, Michael J., and Patrick F. Chinnery. 2015. "Mitochondrial DNA Mutations in Neurodegeneration." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1847 (11): 1401–11. <https://doi.org/10.1016/J.BBABIO.2015.05.015>.
- Khorooshi, Reza, and Trevor Owens. 2010. "Injury-Induced Type I IFN Signaling Regulates Inflammatory Responses in the Central Nervous System." *The Journal of Immunology* 185 (2): 1258–64. <https://doi.org/10.4049/JIMMUNOL.0901753>.
- Kilbaugh, Todd J., Maria Lvova, Michael Karlsson, Zhe Zhang, Jeremy Leipzig, Douglas C. Wallace, and Susan S. Margulies. 2015. "Peripheral Blood Mitochondrial DNA as a Biomarker of Cerebral Mitochondrial Dysfunction Following Traumatic Brain Injury in a Porcine Model." *PLoS ONE* 10 (6). <https://doi.org/10.1371/journal.pone.0130927>.
- Kitada, Tohru, Shuichi Asakawa, Nobutaka Hattori, Hiroto Matsumine, Yasuhiro Yamamura, Shinsei Minoshima, Masayuki Yokochi, Yoshikuni Mizuno, and Nobuyoshi Shimizu. 1998. "Mutations in the Parkin Gene Cause Autosomal Recessive Juvenile Parkinsonism." *Nature* 1998 392:6676 392 (6676): 605–8. <https://doi.org/10.1038/33416>.
- Kong, Lingqi, Wenyu Li, E. Chang, Wuxuan Wang, Nan Shen, Xiang Xu, Xinyue Wang, et al. 2022. "MtDNA-STING Axis Mediates Microglial Polarization via IRF3/NF-KB Signaling After Ischemic Stroke." *Frontiers in Immunology* 13 (April): 1538. <https://doi.org/10.3389/FIMMU.2022.860977/BIBTEX>.
- Kundu, Nandini, Amit Kumar, Carlo Corona, Yingxin Chen, Sonia Seth, Saravanan S. Karuppagounder, and Rajiv R. Ratan. 2022. "A STING Agonist Preconditions against Ischaemic Stroke via an Adaptive Antiviral Type 1 Interferon Response." *Brain Communications* 4 (3). <https://doi.org/10.1093/BRAINCOMMS/FCAC133>.
- Lam, Eric, and Erik Falck-Pedersen. 2014. "Unabated Adenovirus Replication Following Activation of the

- CGAS/STING-Dependent Antiviral Response in Human Cells.” *Journal of Virology* 88 (24): 14426. <https://doi.org/10.1128/JVI.02608-14>.
- Lam, Eric, Saskia Stein, and Erik Falck-Pedersen. 2014. “Adenovirus Detection by the CGAS/STING/TBK1 DNA Sensing Cascade.” *Journal of Virology* 88 (2): 974–81. <https://doi.org/10.1128/JVI.02702-13>.
- Lauterbach-Rivière, Lise, Maïwenn Bergez, Saskia Mönch, Bingqian Qu, Maximilian Riess, Florian W.R. Vondran, Juliane Liese, Veit Hornung, Stephan Urban, and Renate König. 2020. “Hepatitis B Virus DNA Is a Substrate for the CGAS/STING Pathway but Is Not Sensed in Infected Hepatocytes.” *Viruses* 12 (6). <https://doi.org/10.3390/V12060592>.
- Lee, Amanda J., and Ali A. Ashkar. 2018. “The Dual Nature of Type I and Type II Interferons.” *Frontiers in Immunology* 9 (SEP): 2061. <https://doi.org/10.3389/FIMMU.2018.02061/BIBTEX>.
- Li, Qian, Yuze Cao, Chun Dang, Bin Han, Ranran Han, Heping Ma, Junwei Hao, and Lihua Wang. 2020. “Inhibition of Double-strand DNA-sensing CGAS Ameliorates Brain Injury after Ischemic Stroke.” *EMBO Molecular Medicine*. <https://doi.org/10.15252/emmm.201911002>.
- Li, Xiao Dong, Jiaxi Wu, Daxing Gao, Hua Wang, Lijun Sun, and Zhijian J. Chen. 2013. “Pivotal Roles of CGAS-CGAMP Signaling in Antiviral Defense and Immune Adjuvant Effects.” *Science* 341 (6152): 1390–94. https://doi.org/10.1126/SCIENCE.1244040/SUPPL_FILE/LI.SM.PDF.
- Li, Xin, Chang Shu, Guanghui Yi, Catherine T. Chaton, Catherine L. Shelton, Jiasheng Diao, Xiaobing Zuo, C. Cheng Kao, Andrew B. Herr, and Pingwei Li. 2013. “Cyclic GMP-AMP Synthase Is Activated by Double-Stranded DNA-Induced Oligomerization.” *Immunity* 39 (6): 1019–31. <https://doi.org/10.1016/J.IMMUNI.2013.10.019>.
- Liu, Haiping, Lixia Xin, Bernard P.L. Chan, Robert Teoh, Bor Luen Tang, and Y. H. Tan. 2002. “Interferon- β Administration Confers a Beneficial Outcome in a Rabbit Model of Thromboembolic Cerebral Ischemia.” *Neuroscience Letters* 327 (2): 146–48. [https://doi.org/10.1016/S0304-3940\(02\)00371-3](https://doi.org/10.1016/S0304-3940(02)00371-3).
- Liu, Nanxin, Xiaoxiao Pang, Hua Zhang, and Ping Ji. 2022. “The CGAS-STING Pathway in Bacterial Infection and Bacterial Immunity.” *Frontiers in Immunology* 12 (January): 5860. <https://doi.org/10.3389/FIMMU.2021.814709/BIBTEX>.
- Liu, Yiliu, Marie-Line Goulet, Alexandre Sze, Samar Bel Hadj, Sidi Mehdi Belgnaoui, Rassin R. Lababidi, Chunfu Zheng, Jörg Hermann Fritz, David Olgner, and Rongtuan Lin. 2016. “RIG-I-Mediated STING Upregulation Restricts Herpes Simplex Virus 1 Infection.” *Journal of Virology* 90 (20): 9406–19. <https://doi.org/10.1128/JVI.00748-16>.
- Liu, Yinghui, Jianhua Li, Jieliang Chen, Yaming Li, Weixia Wang, Xiaoting Du, Wuhui Song, Wen Zhang, Li Lin, and Zhenghong Yuan. 2015. “Hepatitis B Virus Polymerase Disrupts K63-Linked Ubiquitination of STING To Block Innate Cytosolic DNA-Sensing Pathways.” *Journal of Virology* 89 (4): 2287–2300. <https://doi.org/10.1128/JVI.02760-14/ASSET/2D733823-E812-45EA-B75A-D93B0A1B5F23/ASSETS/GRAPHIC/ZJV9990900560006.JPEG>.
- Liu, Yuan, Beth Gordesky-Gold, Michael Leney-Greene, Nathan L. Weinbren, Matthew Tudor, and Sara Cherry. 2018. “Inflammation-Induced, STING-Dependent Autophagy Restricts Zika Virus Infection in the Drosophila Brain.” *Cell Host and Microbe*. <https://doi.org/10.1016/j.chom.2018.05.022>.
- Low-Calle, Ana Maria, Jeanette Prada-Arismendi, and Jaime E. Castellanos. 2014. “Study of Interferon- β Antiviral Activity against Herpes Simplex Virus Type 1 in Neuron-Enriched Trigeminal Ganglia Cultures.” *Virus Research* 180 (February): 49–58. <https://doi.org/10.1016/J.VIRUSRES.2013.12.022>.
- Ma, Feng, Bing Li, Yongxin Yu, Shankar S Iyer, Mingyu Sun, and Genhong Cheng. 2015. “Positive Feedback Regulation of Type I Interferon by the Interferon-Stimulated Gene STING.” *EMBO Reports*. <https://doi.org/10.15252/embr.201439366>.
- Mackenzie, Ian R.A., and Rosa Rademakers. 2008. “The Role of TDP-43 in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia.” *Current Opinion in Neurology* 21 (6): 693. <https://doi.org/10.1097/WCO.0B013E3283168D1D>.
- Marinho, Fabio V., Sulayman Benmerzoug, Sergio C. Oliveira, Bernhard Ryffel, and V. F.J. Quesniaux. 2017. “The Emerging Roles of STING in Bacterial Infections.” *Trends in Microbiology* 25 (11): 906. <https://doi.org/10.1016/J.TIM.2017.05.008>.
- McKee, Celia A., and John R. Lukens. 2016. “Emerging Roles for the Immune System in Traumatic Brain Injury.” *Frontiers in Immunology*. Frontiers Media S.A. <https://doi.org/10.3389/fimmu.2016.00556>.
- McNab, Finlay, Katrin Mayer-Barber, Alan Sher, Andreas Wack, and Anne O’Garra. 2015. “Type I Interferons in Infectious Disease.” *Nature Reviews Immunology* 15:2 15 (2): 87–103. <https://doi.org/10.1038/nri3787>.
- Mukai, Kojiro, Hiroyasu Konno, Tatsuya Akiba, Takefumi Uemura, Satoshi Waguri, Toshihide Kobayashi, Glen N. Barber, Hiroyuki Arai, and Tomohiko Taguchi. 2016. “Activation of STING Requires Palmitoylation at the

- Golgi.” *Nature Communications* 2016 7:1 7 (1): 1–10. <https://doi.org/10.1038/ncomms11932>.
- Nazmi, Arshed, Rupanjan Mukhopadhyay, Kallol Dutta, and Anirban Basu. 2012. “STING Mediates Neuronal Innate Immune Response Following Japanese Encephalitis Virus Infection.” *Scientific Reports* 2012 2:1 2 (1): 1–10. <https://doi.org/10.1038/srep00347>.
- Papayannopoulos, Venizelos. 2017. “Neutrophil Extracellular Traps in Immunity and Disease.” *Nature Reviews Immunology* 2017 18:2 18 (2): 134–47. <https://doi.org/10.1038/nri.2017.105>.
- Parker, Zachary M., Aisling A. Murphy, and David A. Leib. 2015. “Role of the DNA Sensor STING in Protection from Lethal Infection Following Corneal and Intracerebral Challenge with Herpes Simplex Virus 1.” *Journal of Virology* 89 (21): 11080–91. <https://doi.org/10.1128/JVI.00954-15/ASSET/5645425D-5F1A-450F-ACCD-C1703C821C49/ASSETS/GRAPHIC/ZJV9990909380007.JPEG>.
- Paul, Bindu D., Solomon H. Snyder, and Vilhelm A. Bohr. 2021. “Signaling by CGAS-STING in Neurodegeneration, Neuroinflammation and Aging.” *Trends in Neurosciences* 44 (2): 83. <https://doi.org/10.1016/J.TINS.2020.10.008>.
- Peng, Yucong, Jianfeng Zhuang, Guangyu Ying, Hanhai Zeng, Hang Zhou, Yang Cao, Huaijun Chen, et al. 2020. “Stimulator of IFN Genes Mediates Neuroinflammatory Injury by Suppressing AMPK Signal in Experimental Subarachnoid Hemorrhage.” *Journal of Neuroinflammation* 17 (1): 1–20. <https://doi.org/10.1186/S12974-020-01830-4/FIGURES/10>.
- Pickrell, Alicia M., and Richard J. Youle. 2015. “The Roles of PINK1, Parkin and Mitochondrial Fidelity in Parkinson’s Disease.” *Neuron* 85 (2): 257. <https://doi.org/10.1016/J.NEURON.2014.12.007>.
- Pinti, Marcello, Elisa Cevenini, Milena Nasi, Sara De Biasi, Stefano Salvioli, Daniela Monti, Stefania Benatti, et al. 2014. “Circulating Mitochondrial DNA Increases with Age and Is a Familiar Trait: Implications for ‘Inflamm-Aging.’” *European Journal of Immunology* 44 (5): 1552–62. <https://doi.org/10.1002/EJL.201343921>.
- Prantner, Daniel, Darren J. Perkins, Wendy Lai, Mark S. Williams, Shruti Sharma, Katherine A. Fitzgerald, and Stefanie N. Vogel. 2012. “5,6-Dimethylxanthenone-4-Acetic Acid (DMXAA) Activates Stimulator of Interferon Gene (STING)-Dependent Innate Immune Pathways and Is Regulated by Mitochondrial Membrane Potential.” *The Journal of Biological Chemistry* 287 (47): 39776–88. <https://doi.org/10.1074/JBC.M112.382986>.
- Reinert, Line S., Katarína Lopusná, Henriette Winther, Chenglong Sun, Martin K. Thomsen, Ramya Nandakumar, Trine H. Mogensen, et al. 2016. “Sensing of HSV-1 by the CGAS-STING Pathway in Microglia Orchestrates Antiviral Defence in the CNS.” *Nature Communications* 7 (1): 1–12. <https://doi.org/10.1038/ncomms13348>.
- Reinert, Line S., Ahmad S. Rashidi, Diana N. Tran, Georgios Katzilieris-Petras, Astrid K. Hvidt, Mette Gohr, Stefanie Fruhwürth, et al. 2021. “Brain Immune Cells Undergo CGAS/STING-Dependent Apoptosis during Herpes Simplex Virus Type 1 Infection to Limit Type I IFN Production.” *Journal of Clinical Investigation* 131 (1). <https://doi.org/10.1172/JCI136824>.
- Rongvaux, Anthony, Ruaidhrí Jackson, Christian C.D. C.D. Harman, Tuo Li, A. Phillip Phillip West, Marcel R. de Zoete, Youtong Wu, et al. 2014. “Apoptotic Caspases Prevent the Induction of Type I Interferons by Mitochondrial DNA.” *Cell* 159 (7): 1563–77. <https://doi.org/10.1016/j.cell.2014.11.037>.
- Rosato, Pamela C., and David A. Leib. 2015. “Neuronal Interferon Signaling Is Required for Protection against Herpes Simplex Virus Replication and Pathogenesis.” *PLOS Pathogens* 11 (7): e1005028. <https://doi.org/10.1371/JOURNAL.PPAT.1005028>.
- Roselli, Francesco, Akila Chandrasekar, and Maria C. Morganti-Kossmann. 2018. “Interferons in Traumatic Brain and Spinal Cord Injury: Current Evidence for Translational Application.” *Frontiers in Neurology* 9 (JUN): 458. <https://doi.org/10.3389/FNEUR.2018.00458/BIBTEX>.
- Sato, Mitsuharu, Hirofumi Suemori, Naoki Hata, Masataka Asagiri, Kouetsu Ogasawara, Kazuki Nakao, Takeo Nakaya, et al. 2000. “Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for IFN- α/β Gene Induction.” *Immunity* 13 (4): 539–48. [https://doi.org/10.1016/S1074-7613\(00\)00053-4](https://doi.org/10.1016/S1074-7613(00)00053-4).
- Sauer, John Demian, Katia Sotelo-Troha, Jakob Von Moltke, Kathryn M. Monroe, Chris S. Rae, Sky W. Brubaker, Mamoru Hyodo, et al. 2011. “The N-Ethyl-N-Nitrosourea-Induced Goldenticket Mouse Mutant Reveals an Essential Function of Sting in the in Vivo Interferon Response to *Listeria Monocytogenes* and Cyclic Dinucleotides.” *Infection and Immunity* 79 (2): 688–94. <https://doi.org/10.1128/IAI.00999-10>.
- Sen, Tanusree, Pampa Saha, Rajaneesh Gupta, Lesley M. Foley, Tong Jiang, Olena S. Abakumova, T. Kevin Hitchens, and Nilkantha Sen. 2020. “Aberrant ER Stress Induced Neuronal-IFN β Elicits White Matter Injury Due to Microglial Activation and T-Cell Infiltration after TBI.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 40 (2): 424–46. <https://doi.org/10.1523/JNEUROSCI.0718-19.2019>.
- Shang, Guijun, Conggang Zhang, Zhijian J. Chen, Xiao chen Bai, and Xuewu Zhang. 2019. “Cryo-EM Structures of

- STING Reveal Its Mechanism of Activation by Cyclic GMP–AMP.” *Nature*. <https://doi.org/10.1038/s41586-019-0998-5>.
- Sharma, Manish, Sumitha Rajendrarao, Neelam Shahani, Uri Nimrod Ramírez-Jarquín, and Srinivasa Subramaniam. 2020. “Cyclic GMP-AMP Synthase Promotes the Inflammatory and Autophagy Responses in Huntington Disease.” *Proceedings of the National Academy of Sciences of the United States of America* 117 (27): 15989–99. https://doi.org/10.1073/PNAS.2002144117/SUPPL_FILE/PNAS.2002144117.SAPP.PDF.
- Shih, Amy Y., Kelly L. Damm-Ganamet, and Taraneh Mirzadegan. 2018. “Dynamic Structural Differences between Human and Mouse STING Lead to Differing Sensitivity to DMXAA.” *Biophysical Journal* 114 (1): 32–39. <https://doi.org/10.1016/J.BPJ.2017.10.027/ATTACHMENT/A86F37BC-2B91-41D2-AC62-0D9CB8AD6113/MMC3.MP4>.
- Sliter, Danielle A., Jennifer Martinez, Ling Hao, Xi Chen, Nuo Sun, Tara D. Fischer, Jonathon L. Burman, et al. 2018. “Parkin and PINK1 Mitigate STING-Induced Inflammation.” *Nature* 2018 561:7722 561 (7722): 258–62. <https://doi.org/10.1038/s41586-018-0448-9>.
- Smith, Douglas H., Victoria E. Johnson, and William Stewart. 2013. “Chronic Neuropathologies of Single and Repetitive TBI: Substrates of Dementia?” *Nature Reviews. Neurology* 9 (4): 211–21. <https://doi.org/10.1038/NRNEUROL.2013.29>.
- Stefanis, Leonidas. 2012. “ α -Synuclein in Parkinson’s Disease.” *Cold Spring Harbor Perspectives in Medicine* 2 (2). <https://doi.org/10.1101/CSHPERSPECT.A009399>.
- Stetson, Daniel B., and Ruslan Medzhitov. 2006. “Recognition of Cytosolic DNA Activates an IRF3-Dependent Innate Immune Response.” *Immunity*. <https://doi.org/10.1016/j.immuni.2005.12.003>.
- Stuve, Olaf, Sophie Chabot, Sonia S. Jung, Gary Williams, and Voon Wee Yong. 1997. “Chemokine-Enhanced Migration of Human Peripheral Blood Mononuclear Cells Is Antagonized by Interferon Beta-1b through an Effect on Matrix Metalloproteinase-9.” *Journal of Neuroimmunology* 80 (1–2): 38–46. [https://doi.org/10.1016/S0165-5728\(97\)00134-3](https://doi.org/10.1016/S0165-5728(97)00134-3).
- Su, Chenhe, and Chunfu Zheng. 2017. “Herpes Simplex Virus 1 Abrogates the CGAS/STING-Mediated Cytosolic DNA-Sensing Pathway via Its Virion Host Shutoff Protein, UL41.” *Journal of Virology* 91 (6). <https://doi.org/10.1128/JVI.02414-16>.
- Sun, Lijun, Jiaxi Wu, Fenghe Du, Xiang Chen, and Zhijian J. Chen. 2013. “Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway.” *Science (New York, N.Y.)* 339 (6121): 786–91. <https://doi.org/10.1126/science.1232458>.
- Sun, W., Y. Li, L. Chen, H. Chen, F. You, X. Zhou, Y. Zhou, Z. Zhai, D. Chen, and Z. Jiang. 2009. “ERIS, an Endoplasmic Reticulum IFN Stimulator, Activates Innate Immune Signaling through Dimerization.” *Proceedings of the National Academy of Sciences* 106 (21): 8653–58. <https://doi.org/10.1073/pnas.0900850106>.
- Tanaka, Yasuo, and Zhijian J. Chen. 2012. “STING Specifies IRF3 Phosphorylation by TBK1 in the Cytosolic DNA Signaling Pathway.” *Science Signaling*. <https://doi.org/10.1126/scisignal.2002521>.
- Todd, Brittany P., Michael S. Chimenti, Zili Luo, Polly J. Ferguson, Alexander G. Bassuk, and Elizabeth A. Newell. 2021. “Traumatic Brain Injury Results in Unique Microglial and Astrocyte Transcriptomes Enriched for Type I Interferon Response.” *Journal of Neuroinflammation* 18 (1): 1–15. <https://doi.org/10.1186/S12974-021-02197-W/FIGURES/9>.
- Trottier, Mark D., Beth M. Palian, and Carol Shoshkes Reiss. 2005. “VSV Replication in Neurons Is Inhibited by Type I IFN at Multiple Stages of Infection.” *Virology* 333 (2): 215–25. <https://doi.org/10.1016/J.VIROL.2005.01.009>.
- Tsuchida, Tetsuo, Jian Zou, Tatsuya Saitoh, Himanshu Kumar, Takayuki Abe, Yoshiharu Matsuura, Taro Kawai, and Shizuo Akira. 2010. “The Ubiquitin Ligase TRIM56 Regulates Innate Immune Responses to Intracellular Double-Stranded DNA.” *Immunity* 33 (5): 765–76. <https://doi.org/10.1016/J.IMMUNI.2010.10.013>.
- Unterholzner, Leonie, and Gillian Dunphy. 2019. “CGAS-Independent STING Activation in Response to DNA Damage.” *Molecular & Cellular Oncology* 6 (4): 1558682. <https://doi.org/10.1080/23723556.2018.1558682>.
- Valente, Enza Maria, Sergio Salvi, Tamara Ialongo, Roberta Marongiu, Antonio Emanuele Elia, Viviana Caputo, Luigi Romito, Alberto Albanese, Bruno Dallapiccola, and Anna Rita Bentivoglio. 2004. “PINK1 Mutations Are Associated with Sporadic Early-Onset Parkinsonism.” *Annals of Neurology* 56 (3): 336–41. <https://doi.org/10.1002/ANA.20256>.
- Verrier, Eloi R., Seung Ae Yim, Laura Heydmann, Houssein El Saghire, Charlotte Bach, Vincent Turon-Lagot, Laurent Mailly, et al. 2018. “Hepatitis B Virus Evasion From Cyclic Guanosine Monophosphate–Adenosine Monophosphate Synthase Sensing in Human Hepatocytes.” *Hepatology* 68 (5): 1695–1709. <https://doi.org/10.1002/HEP.30054>.

- Verzosa, Amanda L., Lea A. McGeever, Shun Je Bhark, Tracie Delgado, Nicole Salazar, and Erica L. Sanchez. 2021. "Herpes Simplex Virus 1 Infection of Neuronal and Non-Neuronal Cells Elicits Specific Innate Immune Responses and Immune Evasion Mechanisms." *Frontiers in Immunology* 12 (May): 1955. <https://doi.org/10.3389/FIMMU.2021.644664/BIBTEX>.
- Vilcek, Jan. 2006. "Fifty Years of Interferon Research: Aiming at a Moving Target." *Immunity* 25 (3): 343–48. <https://doi.org/10.1016/J.IMMUNI.2006.08.008>.
- Volkman, Hannah E., Stephanie Cambier, Elizabeth E. Gray, and Daniel B. Stetson. 2019. "Tight Nuclear Tethering of CGAS Is Essential for Preventing Autoreactivity." *ELife* 8 (December). <https://doi.org/10.7554/ELIFE.47491>.
- Waldvogel, Henry J., Eric H. Kim, Lynette J. Tippett, Jean Paul G. Vonsattel, and Richard L.M. Faull. 2015. "The Neuropathology of Huntington's Disease." *Current Topics in Behavioral Neurosciences* 22 (January): 33–80. https://doi.org/10.1007/7854_2014_354.
- Walko, Thomas D., R. Aaron Bola, John D. Hong, Alicia K. Au, Michael J. Bell, Patrick M. Kochanek, Robert S.B. Clark, and Rajesh K. Aneja. 2014. "Cerebrospinal Fluid Mitochondrial DNA: A Novel DAMP in Pediatric Traumatic Brain Injury." *Shock* 41 (6): 499–503. <https://doi.org/10.1097/SHK.0000000000000160>.
- Wang, J., S. Xiong, C. Xie, W. R. Markesbery, and M. A. Lovell. 2005. "Increased Oxidative Damage in Nuclear and Mitochondrial DNA in Alzheimer's Disease." *Journal of Neurochemistry* 93 (4): 953–62. <https://doi.org/10.1111/J.1471-4159.2005.03053.X>.
- Wang, Kaiyuan, Christopher R. Donnelly, Changyu Jiang, Yihan Liao, Xin Luo, Xueshu Tao, Sangsu Bang, et al. 2021. "STING Suppresses Bone Cancer Pain via Immune and Neuronal Modulation." *Nature Communications* 12 (1). <https://doi.org/10.1038/S41467-021-24867-2>.
- Wang, Ranran, Yuanbo Zhu, Zhongwang Liu, Luping Chang, Xiaofei Bai, Lijing Kang, Yongliang Cao, et al. 2021. "Neutrophil Extracellular Traps Promote TPA-Induced Brain Hemorrhage via CGAS in Mice with Stroke." *Blood* 138 (1): 91. <https://doi.org/10.1182/BLOOD.2020008913>.
- Wang, Wenbiao, Dingwen Hu, Caifeng Wu, Yuqian Feng, Aixin Li, Weiyong Liu, Yingchong Wang, et al. 2020. "STING Promotes NLRP3 Localization in ER and Facilitates NLRP3 Deubiquitination to Activate the Inflammasome upon HSV-1 Infection." *PLoS Pathogens* 16 (3). <https://doi.org/10.1371/JOURNAL.PPAT.1008335>.
- Wang, Yiqin, Xiaoxian Guo, Kaixiong Ye, Michael Orth, and Zhenglong Gu. 2021. "Accelerated Expansion of Pathogenic Mitochondrial DNA Heteroplasmies in Huntington's Disease." *Proceedings of the National Academy of Sciences of the United States of America* 118 (30). https://doi.org/10.1073/PNAS.2014610118/SUPPL_FILE/PNAS.2014610118.SD02.XLSX.
- West, A. Phillip, William Khoury-Hanold, Matthew Staron, Michal C. Tal, Cristiana M. Pineda, Sabine M. Lang, Megan Bestwick, et al. 2015. "Mitochondrial DNA Stress Primes the Antiviral Innate Immune Response." *Nature* 520 (7548): 553–57. <https://doi.org/10.1038/NATURE14156>.
- White, Michael J. J., Kate McArthur, Donald Metcalf, Rachael M. M. Lane, John C. C. Cambier, Marco J. J. Herold, Mark F. van Delft, et al. 2014. "Apoptotic Caspases Suppress MtDNA-Induced STING-Mediated Type I IFN Production." *Cell* 159 (7): 1549–62. <https://doi.org/10.1016/j.cell.2014.11.036>.
- Wu, Jiayi, Lijun Sun, Xiang Chen, Fenghe Du, Heping Shi, Chuo Chen, and Zhijian J. Chen. 2013. "Cyclic-GMP-AMP Is An Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA." *Science (New York, N.Y.)* 339 (6121): 826–30. <https://doi.org/10.1126/SCIENCE.1229963>.
- Wuertz, Kathryn Mc Guckin, Piper M. Treuting, Emily A. Hemann, Katharina Esser-Nobis, Annelise G. Snyder, Jessica B. Graham, Brian P. Daniels, et al. 2019. "STING Is Required for Host Defense against Neuropathological West Nile Virus Infection." *PLOS Pathogens* 15 (8): e1007899. <https://doi.org/10.1371/JOURNAL.PPAT.1007899>.
- Yang, Hui, Hanze Wang, Unyao Ren, Qi Chen, and Zhijian J. Chena. 2017. "CGAS Is Essential for Cellular Senescence." *Proceedings of the National Academy of Sciences of the United States of America* 114 (23): E4612–20. <https://doi.org/10.1073/pnas.1705499114>.
- You, Hongjuan, Sisilia Zheng, Zhiming Huang, Yingying Lin, Qingtang Shen, and Chunfu Zheng. 2019. "Herpes Simplex Virus 1 Tegument Protein UL46 Inhibits Tank-Binding Kinase 1-Mediated Signaling." *MBio* 10 (3). <https://doi.org/10.1128/MBIO.00919-19/ASSET/A9E7A26A-BCCF-415F-A325-F5666F7388AD/ASSETS/GRAPHIC/MBIO.00919-19-F0004.JPEG>.
- Yu, Chien Hsiung, Sophia Davidson, Cassandra R. Harapas, James B. Hilton, Michael J. Mlodzikowski, Pawat Laohamonthonkul, Cynthia Louis, et al. 2020. "TDP-43 Triggers Mitochondrial DNA Release via MPTP to Activate CGAS/STING in ALS." *Cell* 183 (3): 636–649.e18. <https://doi.org/10.1016/j.cell.2020.09.020>.
- Zhang, Conggang, Guijun Shang, Xiang Gui, Xuewu Zhang, Xiao-chen Bai, and Zhijian J. Chen. 2019. "Structural

- Basis of STING Binding with and Phosphorylation by TBK1.” *Nature* 567 (7748): 394–98.
<https://doi.org/10.1038/s41586-019-1000-2>.
- Zhang, Heng, Min Jie Han, Jianli Tao, Zhao Yang Ye, Xiao Xia Du, Ming Jing Deng, Xiao Yan Zhang, Lan Fen Li, Zheng Fan Jiang, and Xiao Dong Su. 2015. “Rat and Human STINGs Profile Similarly towards Anticancer/Antiviral Compounds.” *Scientific Reports* 2015 5:1 5 (1): 1–12. <https://doi.org/10.1038/srep18035>.
- Zhang, Xu, Heping Shi, Jiayi Wu, Xuewu Zhang, Lijun Sun, Chuo Chen, and Zhijian J. Chen. 2013. “Cyclic GMP-AMP Containing Mixed Phosphodiester Linkages Is an Endogenous High-Affinity Ligand for STING.” *Molecular Cell*. <https://doi.org/10.1016/j.molcel.2013.05.022>.
- Zhang, Xu, Jiayi Wu, Fenghe Du, Hui Xu, Lijun Sun, Zhe Chen, Chad A. Brautigam, Xuewu Zhang, and Zhijian J. Chen. 2014. “The Cytosolic DNA Sensor CGAS Forms an Oligomeric Complex with DNA and Undergoes Switch-like Conformational Changes in the Activation Loop.” *Cell Reports* 6 (3): 421–30.
<https://doi.org/10.1016/J.CELREP.2014.01.003>.
- Zhang, Ye, Kenian Chen, Steven A. Sloan, Mariko L. Bennett, Anja R. Scholze, Sean O’Keeffe, Hemali P. Phatnani, et al. 2014. “An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 34 (36): 11929–47. <https://doi.org/10.1523/JNEUROSCI.1860-14.2014>.
- Zhang, Ye, Steven A. Sloan, Laura E. Clarke, Christine Caneda, Colton A. Plaza, Paul D. Blumenthal, Hannes Vogel, et al. 2016. “Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse.” *Neuron* 89 (1): 37–53.
<https://doi.org/10.1016/J.NEURON.2015.11.013/ATTACHMENT/52207ACF-50BF-40FC-B0B3-B547B21C06FE/MMC5.XLSX>.
- Zhong, Bo, Yan Yang, Shu Li, Yan Yi Wang, Ying Li, Feici Diao, Caoqi Lei, et al. 2008. “The Adaptor Protein MITA Links Virus-Sensing Receptors to IRF3 Transcription Factor Activation.” *Immunity*.
<https://doi.org/10.1016/j.immuni.2008.09.003>.
- Zhou, Jian Hua, Yi Ning Wang, Qiu Yan Chang, Peng Ma, Yonghao Hu, and Xin Cao. 2018. “Type III Interferons in Viral Infection and Antiviral Immunity.” *Cellular Physiology and Biochemistry* 51 (1): 173–85.
<https://doi.org/10.1159/000495172>.
- Zhou, Qian, Heng Lin, Suyun Wang, Shuai Wang, Yong Ran, Ying Liu, Wen Ye, et al. 2014. “The ER-Associated Protein ZDHHC1 Is a Positive Regulator of DNA Virus-Triggered, MITA/STING-Dependent Innate Immune Signaling.” *Cell Host & Microbe* 16 (4): 450–61. <https://doi.org/10.1016/J.CHOM.2014.09.006>.

Chapter 3

Type I Interferon Response is Mediated by NLRX1-cGAS-STING Signaling in Brain Injury

This work has been published in *Frontiers in Molecular Neuroscience*.



Type I Interferon Response Is Mediated by NLRX1-cGAS-STING Signaling in Brain Injury

Lauren E. Fritsch¹, Jing Ju², Erwin Kristobal Gudenschwager Basso³, Eman Soliman³, Swagatika Paul⁴, Jiang Chen^{2†}, Alexandra M. Kaloss³, Elizabeth A. Kowalski³, Taylor C. Tuhy⁵, Rachana Deven Somaiya¹, Xia Wang³, Irving Coy Allen³, Michelle H. Theus^{3,5*} and Alicia M. Pickrell^{5*}

OPEN ACCESS

Edited by:

Andrei Surguchov,
University of Kansas Medical Center,
United States

Reviewed by:

Irina G. Sourgoutcheva,
University of Kansas Medical Center,
United States
Herman Sintim,
Purdue University, United States
Christian Zierhut,
Institute of Cancer Research (ICR),
United Kingdom

*Correspondence:

Alicia M. Pickrell
alicia.pickrell@vt.edu
Michelle H. Theus
mtheus@vt.edu

†Present address:

Jiang Chen,
Sorrento Therapeutics,
Inc.,
San Diego, CA, United States

Specialty section:

This article was submitted to
Brain Disease Mechanisms,
a section of the journal
Frontiers in Molecular Neuroscience

Received: 11 January 2022

Accepted: 02 February 2022

Published: 25 February 2022

Citation:

Fritsch LE, Ju J, Gudenschwager Basso EK, Soliman E, Paul S, Chen J, Kaloss AM, Kowalski EA, Tuhy TC, Somaiya RD, Wang X, Allen IC, Theus MH and Pickrell AM (2022) Type I Interferon Response Is Mediated by NLRX1-cGAS-STING Signaling in Brain Injury. *Front. Mol. Neurosci.* 15:852243. doi: 10.3389/fnmol.2022.852243

¹Translational Biology, Medicine, and Health Graduate Program, Virginia Polytechnic Institute and State University, Roanoke, VA, United States, ²Molecular and Cellular Biology Graduate Program, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States, ³Department of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States, ⁴Biomedical and Veterinary Sciences Graduate Program, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States, ⁵School of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States

Background: Inflammation is a significant contributor to neuronal death and dysfunction following traumatic brain injury (TBI). Recent evidence suggests that interferons may be a key regulator of this response. Our studies evaluated the role of the Cyclic GMP-AMP Synthase-Stimulator of Interferon Genes (cGAS-STING) signaling pathway in a murine model of TBI.

Methods: Male, 8-week old wildtype, *STING* knockout ($-/-$), *cGAS* $-/-$, and *NLRX1* $-/-$ mice were subjected to controlled cortical impact (CCI) or sham injury. Histopathological evaluation of tissue damage was assessed using non-biased stereology, which was complemented by analysis at the mRNA and protein level using qPCR and western blot analysis, respectively.

Results: We found that *STING* and Type I interferon-stimulated genes were upregulated after CCI injury in a bi-phasic manner and that loss of *cGAS* or *STING* conferred neuroprotection concomitant with a blunted inflammatory response at 24 h post-injury. *cGAS* $-/-$ animals showed reduced motor deficits 4 days after injury (dpi), and amelioration of tissue damage was seen in both groups of mice up to 14 dpi. Given

Abbreviations: BBB, blood brain barrier; CCI, controlled cortical impact; CDN, cyclic dinucleotide; cGAMP, cyclic guanosine monophosphate-adenosine monophosphate; cGAS, cyclic GMP-AMP synthase; CNS, central nervous system; CXCL10, C-X-C motif chemokine ligand 10; DAMP, damage-associated molecular pattern; Drp1, dynamin-related protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFIH, interferon-induced helicase C domain-containing protein; IFIT, interferon-induced proteins with tetratricopeptide repeats; IFN, interferon; IFNAR, interferon alpha/beta receptor; IL, interleukin; IRF, interferon response factor; ISG, interferon-stimulated gene; JAK-STAT, Janus kinase-signal transducer and activator of transcription; KO, knock out; MCP-1, monocyte chemoattractant protein 1; MMP, matrix metalloproteinase; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRX1, NOD-like receptor containing X1; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; qPCR, quantitative polymerase chain reaction; RIG-I, retinoic acid-inducible gene I; ROS, reactive oxygen species; SAVI, *STING*-associated vasculopathy with onset in infancy; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription; *STING*, stimulator of interferon genes; TBI, traumatic brain injury; TBK1, tank-binding kinase 1; TFAM, transcription factor A, mitochondrial; TLR, toll-like receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VEGF, vascular endothelial growth factor; WT, wildtype.

that cGAS requires a cytosolic damage- or pathogen-associated molecular pattern (DAMP/PAMP) to prompt downstream STING signaling, we further demonstrate that mitochondrial DNA is present in the cytosol after TBI as one possible trigger for this pathway. Recent reports suggest that the immune modulator NLR containing X1 (NLRX1) may sequester STING during viral infection. Our findings show that NLRX1 may be an additional regulator that functions upstream to regulate the cGAS-STING pathway in the brain.

Conclusions: These findings suggest that the canonical cGAS-STING-mediated Type I interferon signaling axis is a critical component of neural tissue damage following TBI and that mtDNA may be a possible trigger in this response.

Keywords: brain injury, inflammation, STING, cGAS, innate immunity

INTRODUCTION

Traumatic brain injury (TBI) is a complex neurological condition that is a leading cause of death and disability in children and adults (Roozenbeek et al., 2013; Surgucheva et al., 2014). The injury occurs in two phases: an initial, acute mechanical injury resulting from the external force, and secondary injury/cell death due to complications such as hypoxia, ischemia, and inflammation (Werner and Engelhard, 2007; Greve and Zink, 2009). While the use of improved safety measures has helped minimize the severity of the initial impact, little progress has been made in understanding or treating secondary injuries.

Neuroinflammation is a key mediator of secondary brain injury; however, anti-inflammatory pharmacological approaches largely fail in clinical trials (Simon et al., 2017). Interferons (IFNs) are elevated in post-mortem human TBI samples (IFN- γ ; Frugier et al., 2010; Karve et al., 2016) and in experimental TBI murine models (IFN- α , IFN- β , IFN- γ ; Lagraoui et al., 2012; Karve et al., 2016), but their functional role has been understudied in TBI. Interferons are produced in response to the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs; Abe et al., 2019). Upon detection of pathogenic nucleic acids, PRRs trigger the production of Type I IFNs to prime both the affected and adjacent cells to neutralize the pathogen. While a number of Type I IFN subtypes have been identified, IFN- α and IFN- β are the most well-studied (Trinchieri, 2010). These IFNs act *via* binding to the cell surface complex known as IFN- α/β receptor (IFNAR), resulting in the expression of IFN-stimulated genes (ISGs) *via* the JAK-STAT pathway (Ivashkiv and Donlin, 2014).

The endoplasmic reticulum protein, Stimulator of Interferon Genes (STING), is known to trigger Type I IFN responses after being activated by cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), a second messenger produced by the DNA sensor, cyclic GMP-AMP synthase (cGAS; Shang et al., 2019; Zhang et al., 2019). cGAS is able to bind nuclear and mitochondrial DNA (Sun et al., 2013; West et al., 2015) to promote STING activation and subsequent translocation of transcription factors (Seth et al., 2005; Ishikawa and Barber, 2008), resulting in the production of innate immune genes, including IFNs and ISGs (Barber, 2014).

Previous studies have demonstrated that STING mRNA is elevated in post-mortem human TBI brain samples, and genetic loss of STING or IFNAR in murine models of TBI reduces lesion size and autophagy markers (Karve et al., 2016; Abdullah et al., 2018). Pharmaceutical inhibition of cGAS, the upstream mediator of STING, in a murine stroke model reduced microglial activation and peripheral immune cell infiltration (Li et al., 2020). Interferon signaling is gaining increasing attention for its role in mediating progressive damage in TBI (Barrett et al., 2020; Sen et al., 2020). Taken together, this suggests that cGAS-STING signaling may represent a novel mechanism for controlling post-traumatic neuroinflammation; however, there is evidence of non-canonical, cGAS-independent STING activation, particularly in response to DNA damage (Dunphy et al., 2018; Unterholzner and Dunphy, 2019). Because upstream STING signaling is undefined in the brain, clarifying the mechanisms of STING activation in the context of inflammation without a known pathogen is critical for identifying targets for therapeutic intervention.

In this study, we utilized genetic knockout mouse models to elucidate the role of the cGAS-STING signaling pathway after TBI in a preclinical model of controlled cortical impact (CCI) injury. We report that the ISG response is immediately upregulated after injury and provide evidence that cytoplasmic mtDNA is available for cGAS binding in the injured cortex. In addition to confirming that loss of endogenous STING is protective (Abdullah et al., 2018), our data suggests that canonical cGAS-STING signaling is a critical component of trauma-induced neuroinflammation and tissue damage. We also uncover *in vivo* evidence for the first time that nucleotide-binding oligomerization domain, leucine-rich repeat containing X1 (NLRX1) abrogates this pathway in the brain. Taken together, we conclude canonical cGAS-STING signaling plays a significant role in influencing TBI outcome.

MATERIALS AND METHODS

Animals

All mice were housed in a pathogen-free facility on a 12 h light/dark cycle at Virginia Tech and provided the standard rodent diet and water *ad libitum*. Male CD-1, C57BL/6J

(wildtype), C57/BL/6J-TMEM173^{gt/J} (STING^{-/-}; Sauer et al., 2011), and B6(C)-*Cgas*^{tm1d(EUCOMM)Hmgu/J} (cGAS^{-/-}) mice were purchased from Jackson Laboratories (Ellsworth, ME, USA). *NLRX1*^{-/-} mice were previously described (Allen et al., 2011). *STING*^{-/-}, *cGAS*^{-/-}, and *NLRX1*^{-/-} mice were genotyped according to protocols provided by Jackson Laboratories. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under the approval of the Virginia Tech Institutional Animal Care and Use Committee.

Controlled Cortical Impact (CCI) Injury

Animals were prepared for surgery as previously described (Brickler et al., 2016). Male CD-1, wildtype, *STING*^{-/-}, *cGAS*^{-/-}, and *NLRX1*^{-/-} mice aged 8–10 weeks were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), then positioned in a stereotaxic frame. Body temperature was continually monitored *via* a rectal probe and maintained at 37°C with an autoregulated heating pad. A 4 mm craniotomy was made with a portable drill over the right parietal-temporal cortex (−2.5 mm A/P and 2.0 mm lateral from bregma). Moderate CCI was induced with an eCCI-6.3 device (Custom Design and Fabrication, Richmond, VA, USA) using a 3 mm impact tip at an angle of 70°, 5.0 m/s velocity, 2.0 mm impact depth, and 100 ms dwell period (Theus et al., 2010). The incision was closed with Vetbond tissue adhesive (3M, St. Paul, MN, USA), and post-surgery animals received Buprenorphine SR (1 mg/kg, ZooPharm, Windsor, CO, USA) subcutaneously. Sham animals received a craniotomy only.

Histology and TUNEL Staining

At the indicated times post-CCI injury, mice were anesthetized by isoflurane (IsoFlo®, Zoetis, Parsippany-Troy Hills, NJ, USA) and euthanized by cervical dislocation. Brains were fresh frozen on dry ice while embedded in O.C.T. (Tissue-Plus™ O.C.T. Compound, Fisher HealthCare, Houston, TX, USA). Brains were coronally sectioned (30 μm thickness) using a cryostat (CryoStar NX50, Thermo Scientific, Waltham, MA, USA) through the lesion site (−1.1 to −2.6 mm posterior to bregma). Serial sections 300 μm apart were stained with Cresyl violet (Electron Microscopy Sciences, Hatfield, PA, USA).

To identify cells undergoing apoptosis, slides were fixed in 10% formalin (Fisher Chemicals, Pittsburgh, PA) for 5 min, washed with 1× PBS, permeabilized in 2:1 ethanol:acetic acid at −20°C for 10 min and 0.4% Triton for 5 min, then washed with 1× PBS and TUNEL stained according to the manufacturer's suggestions (DeadEnd™ Fluorometric TUNEL System, Promega, Madison, WI). Slides were then fixed for 5 min in 10% formalin, blocked for 30 min in 0.2% Triton, 2% cold water fish gelatin (Sigma, St. Louis, MO, USA), and stained for Nissl (1:100, NeuroTrace™ 530/615 Red Fluorescence Nissl, Invitrogen, Carlsbad, CA, USA). Slides were mounted with DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Representative confocal images were taken on a Nikon C2 at 20× magnification using the recommended z-step size. Maximum intensity projections were created in Nikon NIS-Elements.

Estimating Lesion Size and TUNEL⁺/Nissl⁺ Cells

Lesion volume (mm³) was assessed by a blinded investigator using StereoInvestigator's Cavalieri estimator (MicroBrightField, Williston, VT, USA) and an Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA, USA), as previously described (Theus et al., 2017). Five coronal serial sections for each animal were spaced 300 μm apart surrounding the epicenter of injury were stained for Nissl (described above) and viewed at 4× magnification under brightfield illumination. A grid (100 μm spacing) was set over the ipsilateral lesion site and markers were placed over the contused tissue, as identified by diminished Nissl staining intensity, morphology, and pyknotic neurons. The contoured area with the section thickness, section interval, and the number of sections were used by the Cavalieri program to estimate the volume of contused tissue.

Apoptotic cells (TUNEL⁺) were counted by a blinded investigator using five adjacent coronal serial sections (spaced 300 μm apart) with the StereoInvestigator Optical Fractionator (MicroBrightField, Williston, VT, USA) probe. Approximately 100 randomized sites per animal (grid size: 500 × 500 μm, counting frame size: 100 × 100 μm) were assessed to identify TUNEL⁺ and TUNEL⁺/Nissl⁺ cells (apoptotic neurons), and section thickness was estimated every five sites to improve the accuracy of the cell count estimation. The number of cells per contour, average estimated section thickness, section interval, and the number of sections were used to estimate the number of cells within the lesion volume.

Real Time qPCR

A 4 × 4 mm section of the injured cortex tissue was micro-dissected from each animal and immediately submerged in TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Either sham surgery animals' parietal cortices or the contralateral parietal cortex from injured animals were extracted to serve as the control. Cortical tissue was mechanically homogenized, lysed, and extracted with TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Reactions containing SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA), 10–50 ng of cDNA, and 0.4 mM of each primer set were run on the CFX96 System (Bio-Rad, Hercules, CA, USA). qPCRs were performed in technical triplicates for each gene/primer set (Table 1). Expression levels were normalized to GAPDH and fold change was determined by the comparative C_T method (Schmittgen and Livak, 2008). Primer efficiency was determined using a 4-point log concentration curve (Bio-Rad CFX Maestro software, Hercules, CA, USA).

Western Blot

A 4 × 4 mm section of injured cortex tissue was micro-dissected from each animal, snap-frozen in liquid nitrogen, and stored at −80°C until use. Extracts were homogenized with a hand-held mortar/pestle (VWR, Radnor, PA, USA) on ice in RIPA buffer (Thermo Scientific Pierce Protein Biology, Waltham, MA, USA) containing proteinase and phosphatase inhibitors

TABLE 1 | qPCR primers used in experiments.

Gene	Forward Seq. (5' - 3')	Reverse Seq. (5' - 3')
IRF7	CAA TTC AGG GGA TCC AGT TG	AGC ATT GCT GAG GCT CAC TT
IFIT1	ACC ATG GGA GAG AAT GCT GAT G	TGT GCA TCC CCA ATG GGT TC
STAT1	GCG GCA TGC AAC TGG CAT ATA ACT	ATG CTT CCG TTC CCA CGT AGA CTT
STAT2	TGA TCT CTA ACA GAC AGG TGG	CTG CAT TCA CTT CTA AAG ACT C
IFIT3	ATC ATG ATG GAG GTC AAC CG	TTG CAC ACC CTG TCT TCC AT
IFNA4	CTT TCC TCA TGA TCC TGG TAA TGA T	AAT CCA AAA TCC TTC CTG TCC TCC
IFNB1	AAC TCC ACC AGC AGA CAG TG	GGT ACC TTT GCA CCC TCC AG
RIG-I	GAG TAC CAC TTA AAG CCA GAG	AAT CCA TTT CTT CAG AGC ATC C
IFIH1	CGG AAG TTG GAG TCA AAG C	TTT GTT CAG TCT GAG TCA TGG
IL-10	AGA CCAAGGTGTCTACAAGGC	TCA TCA TGT ATG CTT CTA TGC AGT
IL-6	CTA GCT CAG GCT CGT CAG TTC	CTA GCT CAG GCT CGT CAG TTC
MCP1	CTA GCT CAG GCT CGT CAG TTC	CTA GCT CAG GCT CGT CAG TTC
CXCL10	ATA ACC CCT TGG GAA GAT GGT G	CTA GCT CAG GCT CGT CAG TTC
GAPDH	ATT GTG TCC GTC GTG GAT CTG A	AGA TGC CTG CTT CAC CAC CTT CTT
STING	GCC TTC AGA GCT TGA CTC CA	GTA CAG TCT TCG GCT CCC TG
ND1	CAG CCT GAC CCA TAG CCA TA	ATT CTC CTT CTG TCA GGT CGA A
COX1	AGG CTT CAC CCT AGA TGA CAC	GTA GCG TCG TGG TAT TCC TGA A

List of forward and reverse sequences used for qPCR.

(Thermo Scientific Pierce Protein Biology, Waltham, MA, USA). Homogenates were spun at 4°C at 15,000× *g* for 15 min and the supernatant was stored at −80°C until use. Protein quantification was determined using the DC protein assay kit with BSA standards (Bio-Rad, Hercules, CA, USA). Fifty milligram of protein was run on a 4–12 percent NuPage Bis-Tris Gel (Thermo Fisher Scientific, Waltham, MA, USA) and transferred onto a PVDF membrane (MilliporeSigma, Burlington, MA, USA). Primary antibodies were incubated overnight. Primary antibodies used were p-STING S365, STING, cGAS, histone H3 (Cell Signaling Technology, Danvers, MA, USA), α-tubulin (MilliporeSigma, Burlington, MA, USA), Mfn2 (was a kind gift from Richard Youle's laboratory), and HMGB1 (R&D Systems, Minneapolis, MN, USA). Membranes were washed in 1× TBST, and secondary HRP conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were incubated at RT for 1 h. Chemiluminescent detection (Thermo Scientific Pierce Protein Biology, Waltham, MA, USA) was used to detect a signal with the Bio-Rad ChemiDoc system (Bio-Rad,

Hercules, CA, USA). Relative optical density was determined with ImageLab software (Bio-Rad, Hercules, CA, USA).

Evans Blue

Twenty-four hours after the CCI injury, the animals received an intravenous injection of 300 μl Evans blue. After 3 h, animals were sacrificed, and ipsilateral and contralateral hemispheres were collected. The distribution of Evans Blue was verified by opening the thoracic and abdominal cavities. The tissue was incubated in 500 μl 10% formamide at 55°C for 24 h, then centrifuged for 4 min at 210× *g* to pellet the tissue. Absorbance for each hemisphere was measured in triplicate at 610 nm.

Rotarod

Gross motor function was evaluated by Rotarod (Columbus Instruments, Columbus, OH, USA) testing from 4 to 14 days post-TBI. The initial velocity was 5 rpm, with an acceleration of 0.1 rpm/s. Each animal underwent three trials per day with a 2 min rest between each trial. The average time of the three trials was used for analysis. Eight-week-old animals were trained for four consecutive days with a baseline measurement taken on the 5th day. Animals underwent sham or CCI surgery, then rotarod performance was evaluated at 4-, 7-, and 14-days post-surgery. Each animal's performance was compared to its baseline measurement, and average performance for all animals was reported. After the final day of testing, animals were euthanized for histology, qPCR, or western blotting as described above.

Cytosolic Fractionation

The cytosolic fraction was extracted as previously reported (West et al., 2015). Cortical tissue was homogenized in PBS plus protease and phosphatase inhibitors (Thermo Scientific Pierce Protein Biology, Waltham, MA, USA). Dissociated tissues were incubated in the cytosolic extraction buffer containing 150 mM NaCl, 50 mM HEPES, pH 7.4, and 15–25 μg/ml digitonin (Gold Biotechnology, St Louis, MO, USA). The homogenates were incubated end over end for 10 min to allow selective plasma membrane permeabilization, then centrifuged at 980 *g* for 3 min three times to pellet intact cells. Pellets were retained for western blotting. The supernatant was centrifuged at 17,000 *g* for 10 min to pellet any remaining cellular debris. DNA was extracted the Zymo DNA extraction kit.

MtDNA detection and quantification of cytosolic extracts were performed by real time PCR as previously published (West et al., 2015). Ct values obtained for mtDNA abundance for whole cell extracts served as normalization controls for the mtDNA values obtained from the cytosolic fractions. It was also used to ensure there were no hemispheric differences in mtDNA levels in an individual animal. To prepare extracts, injured and contralateral tissues were weighed, and 25 mg of tissue was used for downstream processing. Ten percent of the homogenized tissue was sampled prior to cytosolic fractionation for whole cell extracts. Ct values from the ipsilateral cortex were normalized to that individual animal's contralateral hemisphere using the comparative Ct method as whole mtDNA copy number as the reference.

Cell Isolations

Murine cells were isolated using the Worthington Dissociation Kit (Worthington Biochemical Corporation, Lakewood, NJ, USA) and slight modifications to published protocols (Holt and Olsen, 2016; Holt et al., 2019). Briefly, WT animals were deeply anesthetized with a ketamine (500 mg/kg)/xylazine (10 mg/kg) cocktail and hand perfused with cold PBS to remove blood. The brain was removed, cortices dissected, and finely minced in warmed papain with DNase. Tissue was digested in papain at 37°C for 15 min for astrocytes and endothelial cells or 45 min for microglia with gentle inversions every 5 min. For astrocytes and endothelial cells, the solution was triturated, centrifuged at 300 g for 5 min 4°C, and the pellet was resuspended in resuspension buffer per the Worthington protocol to stop the digestion. The dissociated cells were spun down again, filtered through a 70 µm cell strained with 10 ml 0.5% BSA PBS, then resuspended in 200 µl 0.5% BSA PBS and microbeads. Oligodendrocytes were removed with anti-myelin beads, then endothelial cells and astrocytes were isolated with CD31 and ACSA-2 beads (all microbeads from Miltenyi Biotec, Auburn, CA, USA), respectively, per published protocols (Holt and Olsen, 2016; Holt et al., 2019). **Microglia:** Microglia were isolated by plating the cells collected following the Worthington Papain Dissociation System protocol. Cells were incubated for one hat 37°C and non-adherent cells were washed off, leaving microglia adherent to the plate. **Primary Neurons:** Primary neurons were isolated from P0 mouse pups per the Worthington Papain Dissociation System protocol and cultured on poly-d-lysine-coated plates in Neurobasal Medium with B27 supplement (Gibco, Waltham, MA, USA). Primary neurons were collected 14 days after plating for RNA isolation.

Statistical Analysis

Data were analyzed with GraphPad Prism 9 (GraphPad, San Diego, CA, USA). A Student's two-tailed *t*-test was used for comparison of two experimental groups. One-way or two-way ANOVA with Tukey's multiple comparison test were used for comparison of more than two experimental groups as appropriate. Differences were considered statistically significant at *p* < 0.05. Data reported as mean ± SEM. *n* values are reported in the figure legends.

RESULTS

CCI Injury Induces a Biphasic ISG Response in the Damaged Cortex

To provide further insight into how TBI alters inflammatory gene transcription in a temporal manner, we first sought to broadly profile changes in cytokines, PRRs, ISGs, IFNs, and transcription factors that are known to be upregulated by the innate immune system (Schneider et al., 2014). Cortices from male 8-week injured mice showed a temporally biphasic increase in mRNA expression for most (10 of 13) genes tested compared to shams (**Figures 1A–C**). Expression of *Il-10*, *MCP-1*, *RIG-I*, *CXCL10*, *IFIT1*, *IFIT3*, *IFNA4*, *IFNB1*, *IRF7*, and *STAT1* was significantly increased at 2- and 24-h (h) post-injury, which was blunted at

4 h. *IFIH1* (also known as MDA5), and *STAT2* expression were unchanged. Furthermore, *Il10*, *MCP1*, and *Il-6* did not show a biphasic expression pattern. Of note, the Type I IFNs *IFNA4* and *IFNB1* showed a biphasic upregulation after injury.

Previous reports demonstrate neuroprotection in *STING*^{−/−} mice after CCI injury (Abdullah et al., 2018). To gain a more in-depth understanding of the expression pattern of STING, we assessed mRNA levels at 2, 4, and 24 h in the ipsilateral parietal cortex (**Figure 1D**). We find *STING* is upregulated at all time points tested but shows the greatest change in expression at 2 and 4 post-injury (**Figure 1D**). Interestingly, *STING* itself is an ISG and is positively regulated by its own transcription upon activation (Ma et al., 2015). Taken together, these data demonstrate a strong innate immune response occurring within hours after TBI.

CCI Injury Induces the Presence of Cytosolic Mitochondrial DNA in Damaged Cortex

Loss of *STING* (Abdullah et al., 2018), *IFNAR* (Karve et al., 2016), or *IFNβ* (Barrett et al., 2020; Sen et al., 2020) function has been shown to be beneficial in TBI outcome; however, the mechanism regulating their induction remains unclear. The canonical *STING*-cGAS pathway is activated by the binding of viral nucleic acids found in the cytoplasm (Sun et al., 2013), resulting in the production of the second messenger cGAMP which binds and activates *STING* (Shang et al., 2019; Zhang et al., 2019). In addition, mitochondrial DNA (mtDNA) can activate *STING* in models where mtDNA packaging proteins and mitochondrial permeability proteins are disrupted genetically (West et al., 2015; McArthur et al., 2018), and mtDNA is present in cerebral spinal fluid and serum following TBI (Walko et al., 2014; Kilbaugh et al., 2015).

To determine whether mtDNA is present in the cytoplasm, we isolated the cytoplasmic fraction of cells isolated from the ipsilateral and contralateral cortex. We used primers that targeted two different locations on the mitochondrial genome corresponding to the coding region for COX1 and ND1 (**Figure 2A**). To ensure that our cytosolic fractions were enriched, western blotting detected the presence of the cytosolic protein α-tubulin but showed that the cytosolic fraction was devoid of the nuclear protein histone H3 and the outer mitochondrial membrane protein Mfn2 (**Figure 2B**). Interestingly, we saw a significant elevation in mtDNA at 2 h (**Figure 2C**), and 4 h (**Figure 2D**) post-injury, which was resolved by 24 h (**Figure 2E**), indicating that mtDNA is present in the cytoplasm of the injured cells. These data correlated with ISG induction at 2 h post-injury (**Figure 1A**).

To determine whether cytoplasmic nuclear DNA was also present, we performed western blotting on cytoplasmic extracts at 2 h to evaluate the expression of the chromosome-associated protein nuclear protein high mobility group box protein 1 (HMGB1), whose expression is increased when nuclear DNA is present in the cytosol (Urbanaviciute et al., 2008; Pisetsky, 2014). cGAS is also more easily bound to and activated by

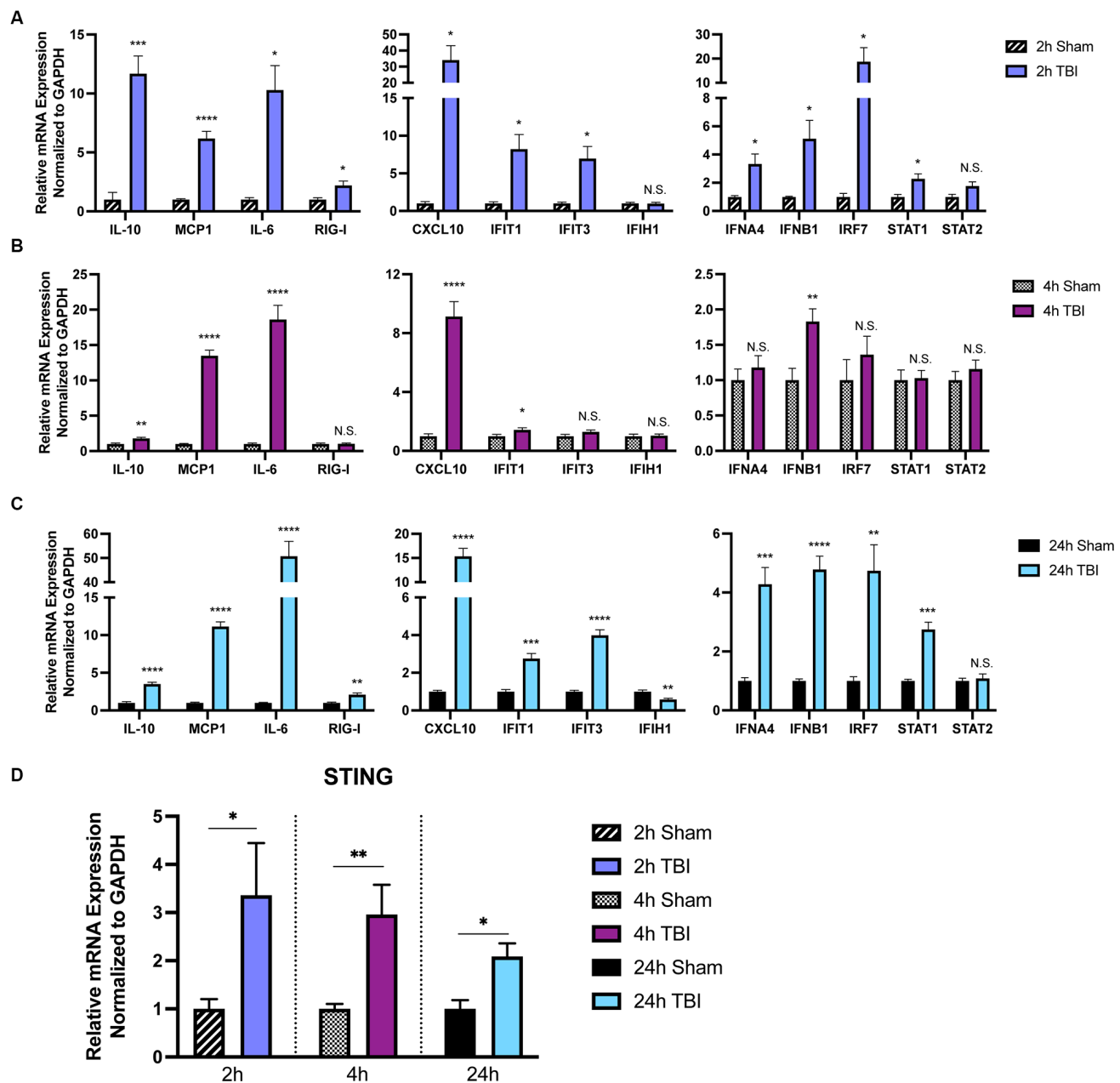


FIGURE 1 | The immune response to TBI is biphasic. Cytokine and interferon-stimulated gene (ISG) expression profiled at 2 h (A), 4 h (B), and 24 h (C) after injury or sham surgery in male CD-1 mice. (D) mRNA expression of STING in the ipsilateral cortex 2, 4, and 24 h after injury or sham surgery. Gene expression was normalized to GAPDH. $n = 5-7$ per group. Data presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. N.S. = not significant.

HMGB1-coated nuclear DNA than DNA in its free form (Andreeva et al., 2017). We found HMGB1 protein was present in cytosolic fractions isolated from both contralateral and ipsilateral hemispheres (Figure 2F); however, ipsilateral cytoplasmic HMGB1 expression was not increased compared to contralateral (Figure 2G). This suggests that mtDNA is available to drive cGAS activation in the damaged cortex after CCI injury, though alternative DNA sources (including nuclear DNA) cannot be fully ruled out.

Loss of cGAS-STING Confers Neuroprotection After CCI Injury

cGAS is necessary for canonical STING activation (Sun et al., 2013; Wu et al., 2013). To verify this pathway's involvement in TBI, we utilized cGAS KO mice (cGAS^{-/-}; Supplementary Figure 1A) and STING KO (STING^{-/-}; Supplementary Figure 1B) mice. STING^{-/-} mice displayed a significant reduction in lesion volume compared to WT at 1 day post-injury (dpi; Figures 3A,B), confirming prior

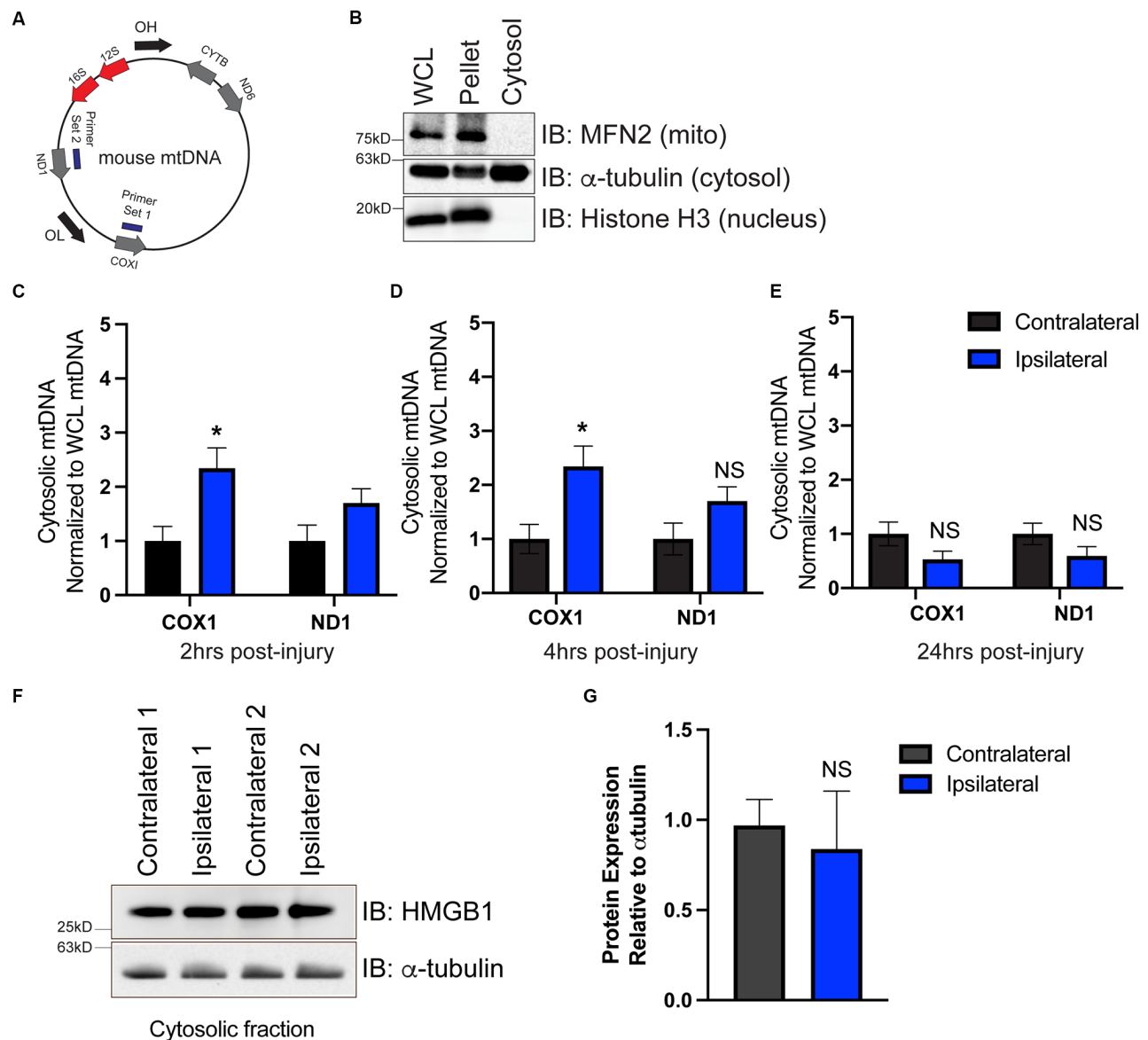
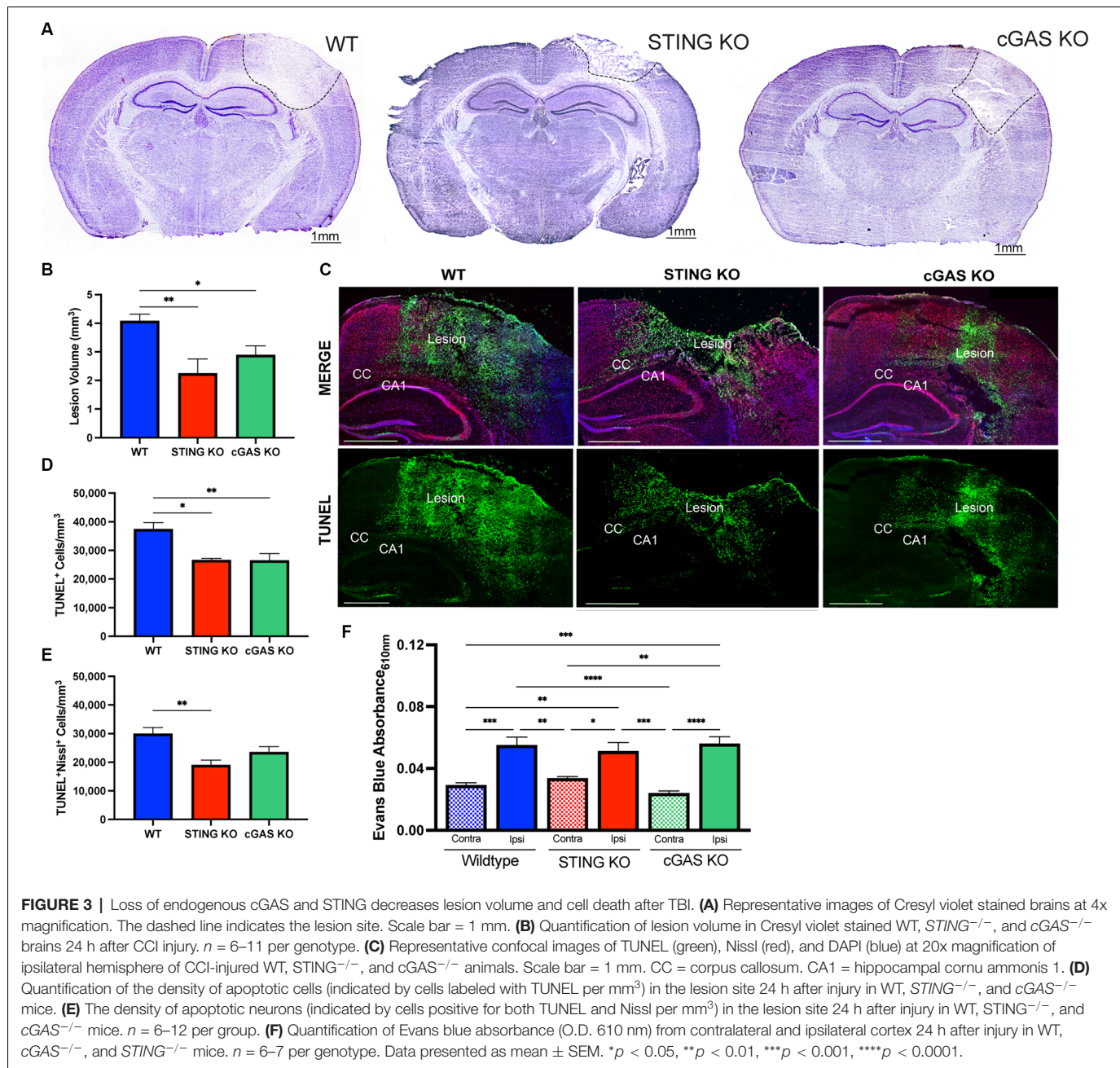


FIGURE 2 | mtDNA is present in the cytosol as a possible trigger for cGAS-STING activation after TBI. **(A)** Diagram of mtDNA showing primer sets for ND1 and COX1. **(B)** Western blot of whole cell lysate (WCL), cell pellet, and isolated cytosol to confirm cytosolic purity. Mitofusin 2 (MFN2) was used to identify mitochondria, alpha-tubulin was used for detecting cytosol, and histone H3 indicated the nuclear fraction. **(C–E)** mtDNA was directly detected in the cytosol via qPCR at 2, 4, and 24 h post-TBI. Each animal was normalized to the respective hemisphere's WCL. **(F)** Representative western blotting of the DNA-binding protein high mobility group box protein 1 (HMGB1) in the cytosol 2 h post-TBI. **(G)** Quantification of HMGB1 western blot normalized to alpha-tubulin. $n = 5$ per group for all experiments. Data presented as mean $\pm$ SEM. * $p < 0.05$. NS = not significant.

work (Abdullah et al., 2018). Moreover, $cGAS^{-/-}$ mice also showed significant neuroprotection compared to WT mice (Figures 3A,B). To determine whether the reduction in lesion volume was due to increased neuronal survival, we performed immunodetection of apoptotic neurons by TUNEL staining (Figure 3C). TUNEL detects nuclear DNA fragmentation, a hallmark of apoptosis and necrosis (Grasl-Kraupp et al., 1995). A significant reduction of TUNEL⁺ cells was detected 24 h after injury in both $cGAS^{-/-}$ and $STING^{-/-}$ mice (Figure 3D). Co-labeling with Nissl, an unspecific neuronal marker, showed the number of apoptotic neurons was significantly reduced in

the ipsilateral cortex of $STING^{-/-}$ mice after injury and trending toward a significant reduction in $cGAS^{-/-}$ mice (Figure 3E). Although cGAS/STING deficiency is neuroprotective, no difference was observed in blood-brain barrier function as seen by quantifying Evans Blue infiltration in the damaged cortex compared to contralateral (Figure 3F). Our results suggest that the cGAS-STING pathway contributes to the neurotoxic effects induced by CCI injury.

Behavioral impairments have been previously assessed in $IFN\beta^{-/-}$ mice after TBI (Barrett et al., 2020), therefore we sought to provide further confirmation that canonical



cGAS-STING signaling is critical in TBI outcome. Using rotarod assessment, we found no difference in motor function between sham-injured *cGAS*^{-/-} and WT mice (Supplementary Figure 2A). However, *cGAS*^{-/-} mice showed a significant reduction in motor deficit at 4 dpi compared to WT (Supplementary Figure 2B) but no difference at 7 and 14 dpi (Supplementary Figure 2B). *cGAS*^{-/-} mice also showed a significant reduction in lesion volume at 14 dpi relative to WT (Supplementary Figures 2C,D), despite their comparable motor performance (Supplementary Figure 2B). Similarly, *STING*^{-/-} mice also showed reduced lesion volume at 14 dpi (Supplementary Figures 2C,D). We also assessed mRNA levels of *IFNA4*, *IFNB1*, and *IL6* at 14 days post-injury.

Interestingly, all three genes were downregulated at this chronic timepoint relative to WT sham animals (Supplementary Figure 2E).

Loss of cGAS-STING Ameliorates Pro-inflammatory Gene Expression After CCI Injury

In addition to histological and functional changes, we profiled changes in gene expression in the cortex at 24 h post-injury in WT, *STING*^{-/-}, and *cGAS*^{-/-} mice. We found no difference in the contralateral cortex when compared to sham (Supplementary Figure 3), therefore we used contralateral tissue when performing our relative analysis. Both *STING*^{-/-},

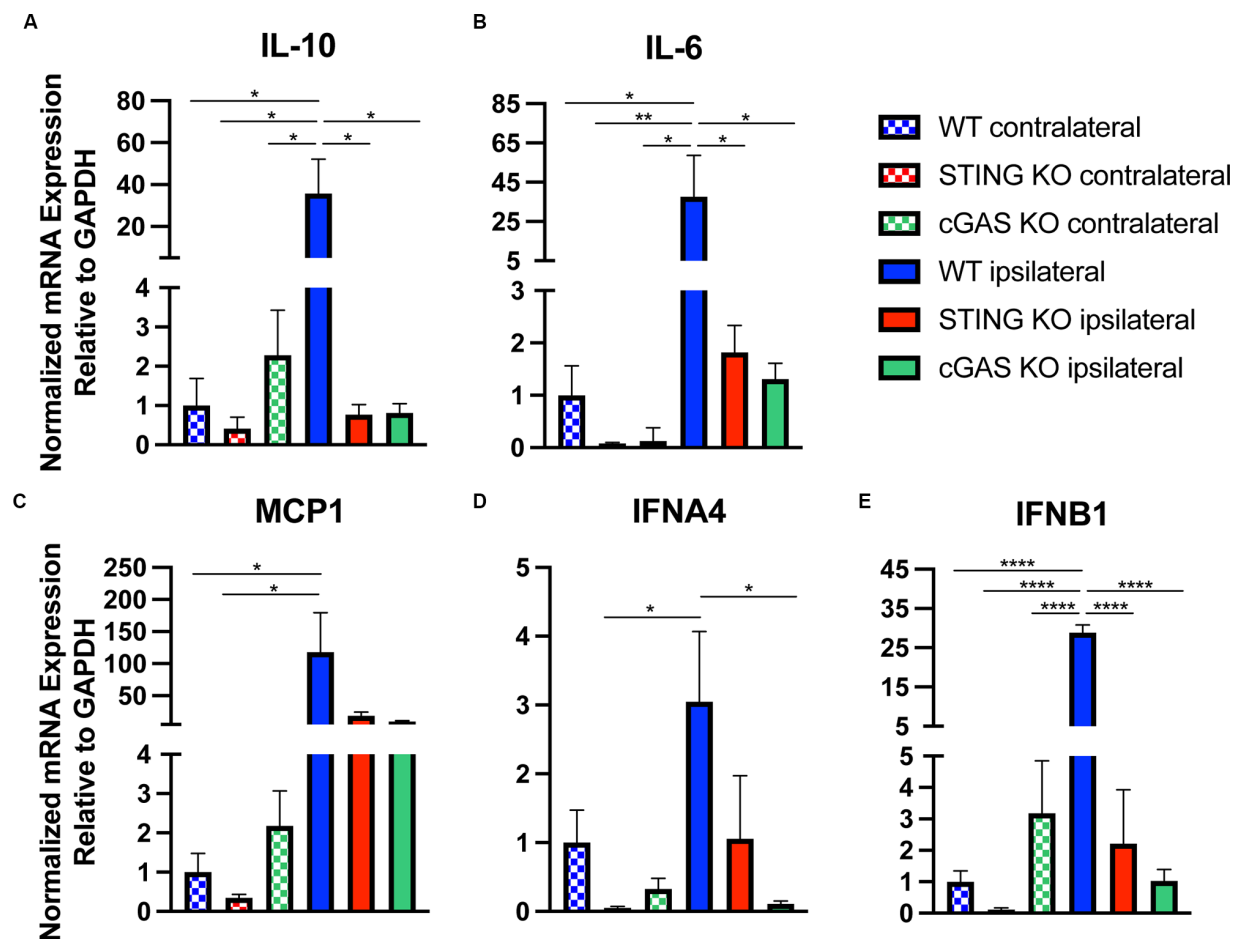


FIGURE 4 | Loss of cGAS-STING blunts the innate immune response to TBI. Quantified mRNA expression of (A) *Il10*, (B) *Il6*, (C) *Mcp1*, (D) *Ifna4*, and (E) *Ifnb1* assessed by qPCR at 24 h post-injury in wildtype, *STING*^{-/-}, and *cGAS*^{-/-} cortices. Gene expression normalized to GAPDH. *n* = 5–6 per group. Data presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001.

and *cGAS*^{-/-} mice showed a significant reduction in mRNA expression of *Il10*, *Il6*, *Mcp1*, *Ifna4*, and *Ifnb1* (Figures 4A–E) in the ipsilateral cortex when compared to WT. To provide further insight into the transcriptional changes, we assessed the complete panel of genes described in Figure 1. We found all genes tested were significantly altered in *STING*^{-/-} mice compared to WT (Supplementary Figure 4). These findings suggest that cGAS-STING signaling plays a key role in regulating innate immune gene expression in the damaged cortex after CCI injury.

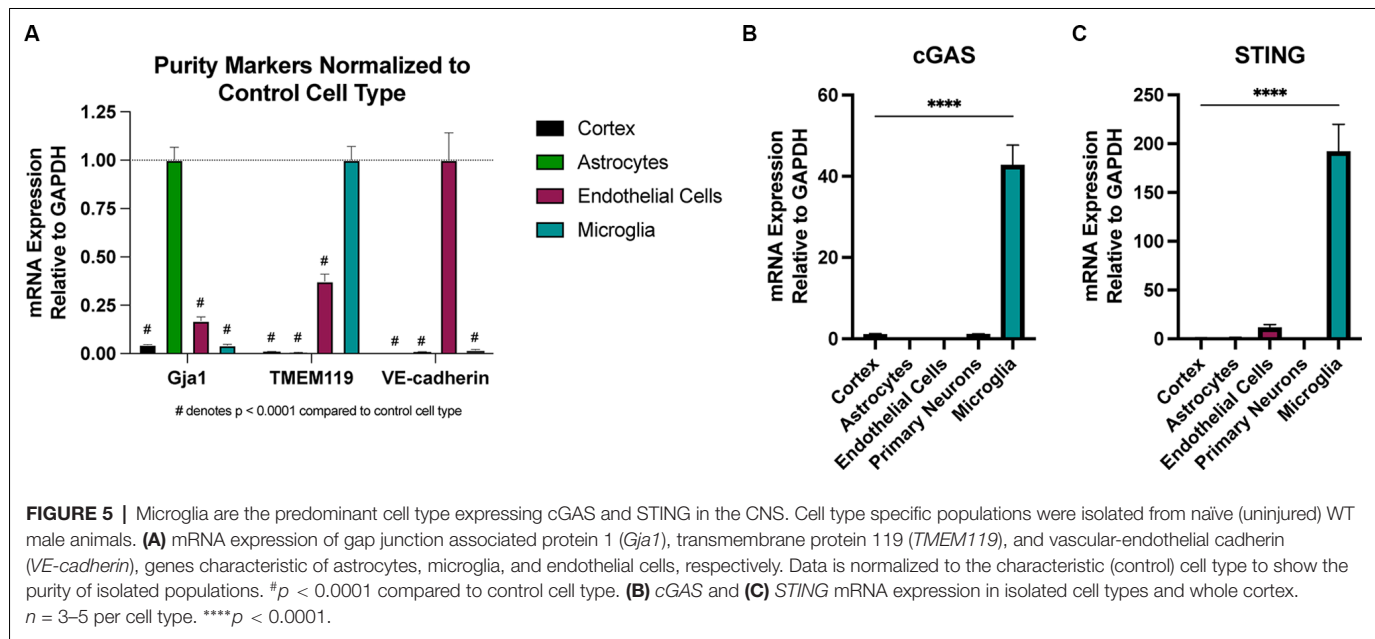
Microglia Are the Predominant Cell Type Expressing cGAS and STING in the Brain

There is conflicting evidence regarding which CNS cell types express cGAS and STING (Nazmi et al., 2012; Abdullah et al., 2018; Li et al., 2020; Zhang et al., 2020). To test this, we employed several techniques for isolating pure CNS cell populations for qPCR assessment. Naïve (uninjured) astrocytes and endothelial cells were extracted using magnetic bead sorting (Holt and Olsen, 2016; Holt et al., 2019), while the remaining cells were plated for isolating microglia and primary neuronal cultures to assess

expression in these cell types. Real-time qPCR analysis of cell-type-specific genes was used to verify the purity of the isolated cell populations (Figure 5A). We observed that naïve microglia showed the greatest enrichment of transcripts for both cGAS and STING when compared to all other cell types (Figures 5B,C). In line with the biphasic cytokine response we noted from the whole cortex after injury, microglia showed upregulation of cGAS at 24 h post-injury and a trending increase of STING at 2- and 24 h after CCI (Supplementary Figures 5A,B). Non-adherent cells (CNS cells remaining after the removal of microglia) did not show increased expression of STING in response to injury. cGAS expression in these cells was undetectable in the sham groups making it difficult to quantify (data not shown). This suggests that microglia are a main cell type influencing the Type I interferon response *via* the cGAS-STING pathway in TBI.

NLRX1 Negatively Regulates cGAS-STING Activation After CCI Injury

We recently showed that loss of NLRX1 exacerbates tissue damage after CCI injury, in part by increasing NF-κB activity



in microglial and/or peripheral-derived immune cells (Theus et al., 2017). It is also well-established that NLRX1 may sequester STING to prevent the interferon response (Guo et al., 2016); however, this association has not been evaluated in the brain. To test whether NLRX1 represents a novel upstream regulator of STING in the cortex after injury, we evaluated activated STING expression and the ISG response. Interestingly, *NLRX1*^{-/-} mice showed a significant increase in activated (phosphorylated) *p*-STING (S365) compared to WT at 3 dpi (Figures 6A,B). We also assessed mRNA expression of *IL10*, *IL6*, *MCPI*, *IFNA4*, and *IFNB1* 24 h post-injury. Relative to WT, *NLRX1*^{-/-} mice showed a significant increase in cortical expression of all genes tested, including the interferons *IFNA4* and *IFNB1* (Figures 6C–G). These data suggest that NLRX1 plays a central role in suppressing the Type I interferon response by limiting STING activation following CCI injury.

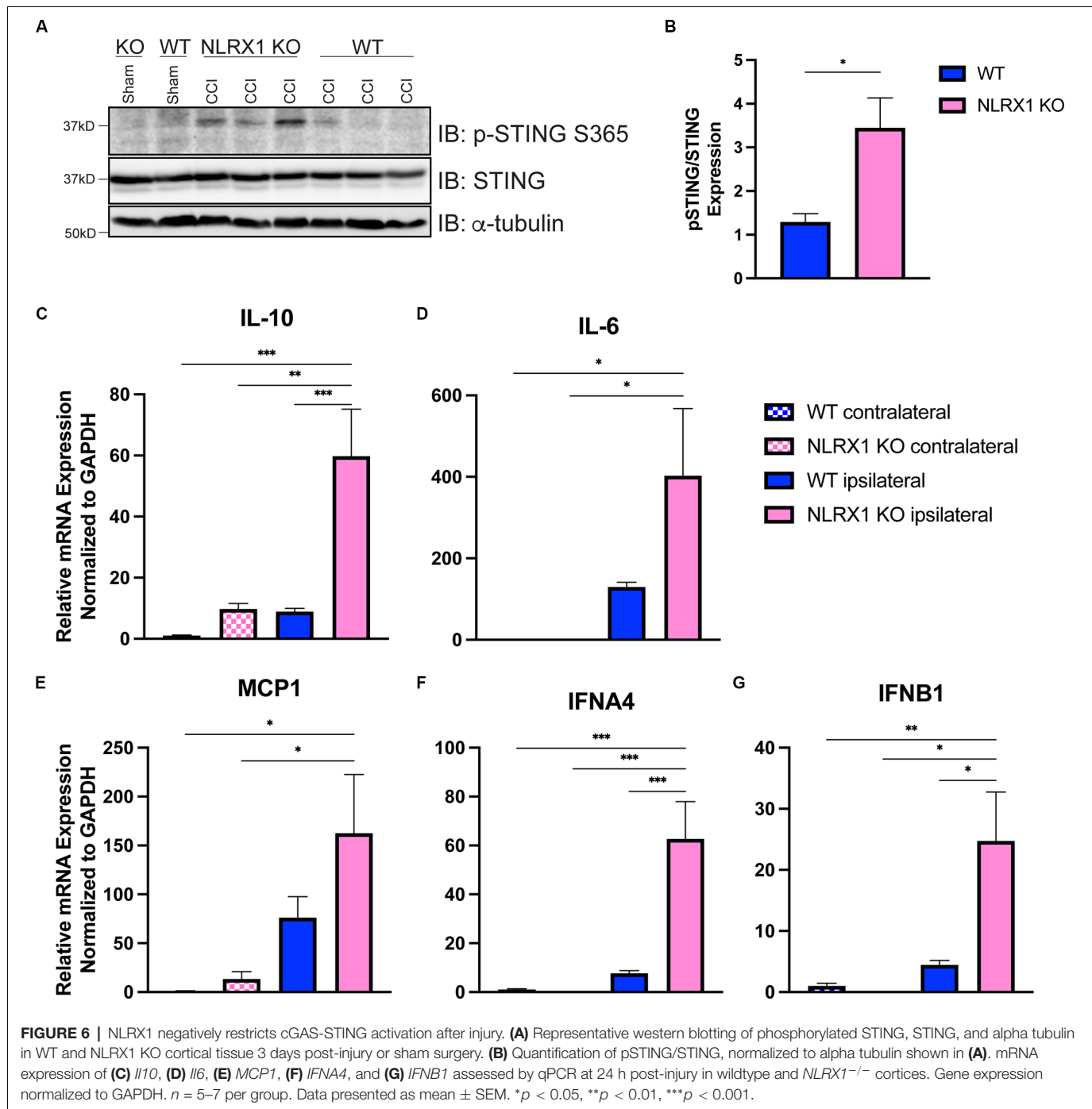
DISCUSSION

Our data suggest that the antiviral interferon pathway mediated by cGAS-STING contributes to the secondary injury after TBI (Figure 7). The activation of STING in the nervous system has recently been brought to the attention of those studying CNS viral infections. STING is highly conserved among organisms (Goto et al., 2018; Martin et al., 2018) and restricts Zika infection in the *Drosophila* brain (Liu et al., 2018). Microglial expression and activation of STING also restricts herpes simplex virus-1 (HSV-1) infection in neurons or promotes apoptosis, depending on viral load (Reinert et al., 2016, 2021). However, the classical viral/microbe-induced innate immune pathways in the brain may not necessarily need viral-induced stimulation for activation. In mouse models of multiple sclerosis,

a demyelinating neurodegenerative disease, STING may control microglial reactivity (Mathur et al., 2017).

The present work demonstrates that STING is upregulated in the ipsilateral cortex of CCI-injured mice, which correlates with a biphasic increase in a variety of cytokines, including *IFNA4* and *IFNB1*. We show that cGAS and STING are highly expressed in microglia (Figure 5) and that microglia show increases in expression of cGAS 24 h after injury (Supplementary Figure 5), suggesting they may be the primary cell type responsible for this response. Of note, it is unclear whether neurons contribute to cGAS-STING signaling after TBI. While our data show that STING expression in primary neurons is low (Figure 5), we were unable to isolate healthy neurons from adult animals in a similar manner to the other cell types due to neuronal breakage; therefore, it is possible that mature neurons *in vivo* have higher baseline STING expression. We also collected the non-microglial population of cells for qPCR analysis when isolating microglia. These cells did not show cGAS or STING expression (Supplementary Figure 5C); however, it is possible that very few neurons survived the dissociation process. Regardless, our data suggest that microglia are likely the predominant cell type driving cGAS-STING signaling after TBI.

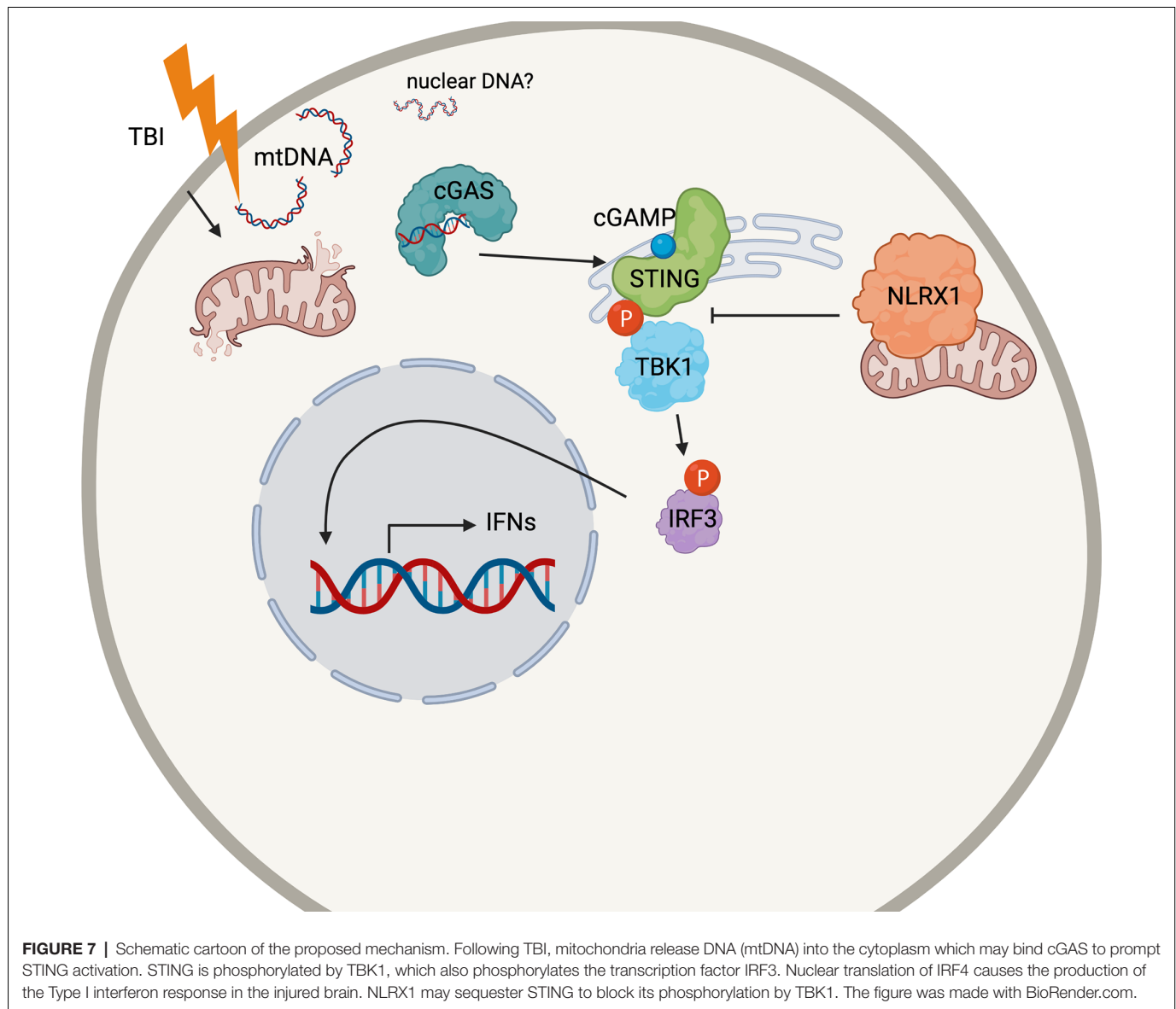
While previous work has shown that loss of endogenous STING reduces lesion size following TBI (Abdullah et al., 2018), recent evidence suggests that STING may be able to function independently of its canonical upstream mediator, cGAS (Almine et al., 2017; Dunphy et al., 2018; Luksch et al., 2019; Chu et al., 2021). Therefore, this study sought to determine the effects of cGAS deficiency and to identify a potential DAMP that may influence the induction of the canonical cGAS-STING pathway in CCI injury. Our data shows that *cGAS*^{-/-} mice display significant neuroprotection and a blunted ISG response, similar to *STING*^{-/-} mice. This correlates with the observation of increased cytoplasmic mtDNA in the damaged



cortex and suggests mtDNA is a possible DAMP that induces the cGAS-STING pathway in microglia leading to Type I interferon-induced tissue damage in TBI. Moreover, we demonstrate that NLRX1 is a novel upstream regulator of STING in this response.

Neuroinflammation is a critical component of the secondary injury response in TBI and offers a number of potential therapeutic targets, but is highly complex and remains poorly understood (Jassam et al., 2017). Our study selected a panel of genes associated with the Type I interferon response, including pro-inflammatory (*Il6*), anti-inflammatory (*Il10*),

and pro-immune migratory cytokines (*MCP-1*), as well as transcription factors (*STAT1*, *STAT2*, and *IRF7*), interferons (*IFNA4* and *IFNB1*), and ISGs (*CXCL10*, *IFIT1*, *IFIT3*, and *IFIH1*). We determined that loss of cGAS or STING resulted in a broadly blunted immune response 24 h after injury. The decreased production of pro-inflammatory cytokines may contribute to the reduction in cell death seen in *cGAS*^{-/-} and *STING*^{-/-} animals (Figure 3). For example, work on stroke has shown that MCP1 deficiency reduces infarct size (Hughes et al., 2002) and BBB breakdown



(Strecker et al., 2013), while MCP1 overexpression exacerbates tissue damage (Chen et al., 2003). Proteins in the JAK-STAT pathway, particularly STAT1, are also known to increase apoptosis both in the brain and periphery (Stephanou et al., 2000; Takagi et al., 2002; Nicolas et al., 2013).

Recent work has suggested that STING simultaneously stimulates the production of pro- and anti-inflammatory cytokines to facilitate the maintenance of gut homeostasis (Ahn et al., 2017), and studies in mouse models of systemic lupus erythematosus (SLE) have indicated STING signaling can be pro- or anti-inflammatory depending on the model (Sharma et al., 2015; Thim-uam et al., 2020; Motwani et al., 2021). Still, the autoimmune syndrome SAVI, caused by gain-of-function mutations in STING, results in excessive inflammation, indicating a primarily pro-inflammatory role for STING (Liu et al., 2014). Our data show altered mRNA expression of

both pro- and anti-inflammatory cytokines in *cGAS*^{-/-} and *STING*^{-/-} mice, suggesting that the effects of cGAS-STING signaling are highly complex and likely context-dependent. Further, the unselective upregulation of mRNAs for proteins with predominantly antiviral roles, such as *IFIT1* and *IFIT3*, suggests that this innate immune pathway is activated aberrantly after injury, unlike its normal role in viral or bacterial clearance. Further investigation is needed to clarify how the balance of pro- and anti-inflammatory cytokines are disrupted or skewed by alterations in cGAS-STING activity.

Recent findings show that mRNA expression of *STING* and key ISGs are elevated up to 60 days after experimental TBI (Barrett et al., 2020), indicating that STING activity may also contribute to chronic neuroinflammation. Consistently, we found that *cGAS*^{-/-} mice showed reduced motor deficits at 4 dpi and reduced lesion volume up to 14 dpi. Interestingly, we found that the Type I interferon ISG response was

significantly reduced by 4 dpi (data not shown), and entirely resolved at 14 days (**Supplementary Figure 2E**). These data suggest that while the cGAS-STING signaling axis is acutely activated after injury, additional subsequent mechanisms may further contribute to the chronic progression of injury after trauma (McKee and Lukens, 2016). Further work is needed to define the temporal dynamics of cGAS-STING signaling after TBI.

It has been well-established that biological sex affects how the immune system responds to insult. Generally, females have a more robust immune response. Females produce more antibodies in response to H1N1 vaccination (Fink et al., 2018) and are at greater risk for autoimmune disease (Angum et al., 2020). However, how biological sex impacts TBI recovery appears highly dependent upon the model, outcome measure, and time points examined after injury (Berry et al., 2009; Späni et al., 2018; Gupte et al., 2019). While the incidence of TBI is significantly higher in males than females (Späni et al., 2018), we did not include female animal data in this study, and future studies will need to determine whether female animals show the same benefit with the loss of cGAS-STING signaling.

Conflicting evidence exists regarding whether NF- κ B signaling is a major pathway activated downstream of STING (Stetson and Medzhitov, 2006; Zhong et al., 2008; Tanaka and Chen, 2012; Abe and Barber, 2014). However, recent work in mice with a point mutation in STING (S365A) that interfered with IRF3 binding elucidated that the switch between NF- κ signaling and Type I interferon signaling was context-dependent (Yum et al., 2021). With the generation of STING point mutation mouse models, future work could further define the contribution of different downstream effects of STING during TBI (Yum et al., 2021). Yet, Type-I IFN receptor (IFNAR1) knockout mice are protected from TBI injury (Karve et al., 2016), indicating that the interferon pathway is still a major contributor to neuroinflammation in TBI. Future work is needed to elucidate cell-type-specific effects mediating the IFN response to TBI.

While our findings demonstrate the presence of cytosolic mtDNA in the injured cortex, it remains unclear how it is released into the cytosol after injury. Recently, the DNA/RNA binding protein TDP-43 has been implicated in the release of mtDNA *via* the mitochondrial permeability transition pore (mPTP) and subsequently caused cGAS-STING activation in a mouse model of ALS (Yu et al., 2020). Other work has shown that BAK/BAX macropores facilitate mitochondrial herniation and mtDNA efflux independent of the mPTP (McArthur et al., 2018; Riley et al., 2018). Mechanical forces have also been shown to promote mitochondrial fission events (Helle et al., 2017) that may allow for mtDNA release; indeed, TBI is associated with increases in mitochondrial fission and the fission-initiating dynamin-related protein 1 (Drp1; Fischer et al., 2016). Clarifying how mtDNA is released following neurotrauma may offer alternative therapeutic targets for reducing cGAS-STING-mediated neuroinflammation. Additionally, it remains possible that nuclear DNA is present in the cytosol. Further study will be required to determine whether nuclear DNA is or is not responsible for the activation of this pathway in neurotrauma.

The development of commercially-available small molecule STING antagonists provides the possibility for *in vivo* assessment of pharmaceutical inhibition of this pathway in the near future. Indeed, a few of these molecules have already shown promise in preventing viral infection tissue damage and autoinflammatory disease in mouse models (Haag et al., 2018; Domizio et al., 2022). Future work should evaluate whether these molecules may be beneficial for TBI, including evaluating their ability to cross the BBB.

Taken together, these data confirm that STING-mediated IFN signaling is detrimental to TBI-induced tissue damage. We have shown that loss of cGAS or STING results in improved histological and functional measures up to 14 days after TBI. Additionally, we provide evidence that NLRX1 negatively regulates STING activation in the brain, offering an additional potential target for therapeutic intervention. Perhaps most significantly, this study is the first to investigate mtDNA as a possible trigger for STING-IFN signaling in neurotrauma. Overall, our findings indicate that the canonical cGAS-STING-mediated ISG response is an early neuroinflammatory event occurring after cortical trauma, which represents a novel therapeutic target for treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Virginia Tech Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LF wrote the first draft, performed experiments, and analyzed data. JJ, EG, ES, SP, AK, EK, JC, TT, RS, and XW performed experiments and analyzed data. IA provided the *Nlrp1*^{-/-} mice and analyzed data. MT designed the project, analyzed data, and edited the manuscript. AP designed the project, performed experiments, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health, R01NS096281 and R01NS121103 (MT), Commonwealth Health Research Board #208-05-20 (AP), and departmental start-up funds (AP).

ACKNOWLEDGMENTS

We would like to thank Dr. Jenny P. Y. Ting (UNC Chapel Hill) for providing *Nlrp1*^{-/-} mice.

SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnmol.2022.852243/full#supplementary-material>.

Supplementary Figure 1 | Western blotting to confirm cGAS and STING KO. **(A,B)** Representative western blot for STING and cGAS protein in brain homogenates from WT, *STING*^{-/-}, and *cGAS*^{-/-} mice. Each lane represents an individual animal.

Supplementary Figure 2 | *cGAS*^{-/-} mice show reduced motor deficit after TBI. Rotarod performance compared to baseline for *cGAS*^{-/-} and WT animals 4–14 days following sham **(A)** or CCI **(B)** surgery. *n* = 5 per genotype for **(A)** and *n* = 15 per genotype for **(B)**. **(C)** Lesion volume of WT, *STING*^{-/-}, and *cGAS*^{-/-} brains 14 dpi. **(D)** Representative Cresyl violet stained WT, *STING*^{-/-}, and *cGAS*^{-/-} brains 14 dpi. Dashed lines indicate lesion site. Scale bar = 1 mm. **(E)** mRNA expression of *IFNA4*, *IFNB1* and *Il6* assessed via qPCR 14 dpi or sham surgery for WT and *cGAS* KO animals. *n* = 5–7 per group. Data presented as mean ± SEM. Two-way ANOVA used for **(A)** and **(B)**, one-way ANOVA for **(C)** and **(E)**. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

REFERENCES

- Abdullah, A., Zhang, M., Frugier, T., Bedoui, S., Taylor, J. M., and Crack, P. J. (2018). STING-mediated type-I interferons contribute to the neuroinflammatory process and detrimental effects following traumatic brain injury. *J. Neuroinflammation* 15:323. doi: 10.1186/s12974-018-1354-7
- Abe, T., and Barber, G. N. (2014). Cytosolic-DNA-mediated, STING-dependent proinflammatory gene induction necessitates canonical NF-κB activation through TBK1. *J. Virol.* 88, 5328–5341. doi: 10.1128/JVI.00037-14
- Abe, T., Marutani, Y., and Shoji, I. (2019). Cytosolic DNA-sensing immune response and viral infection. *Microbiol. Immunol.* 63, 51–64. doi: 10.1111/1348-0421.12669
- Ahn, J., Son, S., Oliveira, S. C., and Barber, G. N. (2017). STING-dependent signaling underlies IL-10 controlled inflammatory colitis. *Cell Rep.* 21, 3873–3884. doi: 10.1016/j.celrep.2017.11.101
- Allen, I. C., Moore, C. B., Schneider, M., Lei, Y., Davis, B. K., Scull, M. A., et al. (2011). NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-κB signaling pathways. *Immunity* 34, 854–865. doi: 10.1016/j.immuni.2011.03.026
- Almine, J. F., O'Hare, C. A. J., Dunphy, G., Haga, I. R., Naik, R. J., Atrih, A., et al. (2017). IFI16 and cGAS cooperate in the activation of STING during DNA sensing in human keratinocytes. *Nat. Commun.* 8:14392. doi: 10.1038/ncomms14392
- Andreeva, L., Hiller, B., Kostrewa, D., Lässig, C., De Oliveira Mann, C. C., Jan Drexler, D., et al. (2017). CGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein-DNA ladders. *Nature* 549, 394–398. doi: 10.1038/nature23890
- Angum, F., Khan, T., Kaler, J., Siddiqui, L., and Hussain, A. (2020). The prevalence of autoimmune disorders in women: a narrative review. *Cureus* 12:e8094. doi: 10.7759/cureus.8094
- Barber, G. N. (2014). STING-dependent cytosolic DNA sensing pathways. *Trends Immunol.* 35, 88–93. doi: 10.1016/j.it.2013.10.010
- Barrett, J. P., Henry, R. J., Ann Shirey, K., Doran, S. J., Makarevich, O. D., Ritzel, R. R., et al. (2020). Interferon-β plays a detrimental role in experimental traumatic brain injury by enhancing neuroinflammation that drives chronic neurodegeneration. *J. Neurosci.* 40, 2516–2519. doi: 10.1523/JNEUROSCI.2516-19.2020
- Berry, C., Ley, E. J., Tillou, A., Cryer, G., Margulies, D. R., and Salim, A. (2009). The effect of gender on patients with moderate to severe head injuries. *J. Trauma* 67, 950–953. doi: 10.1097/TA.0b013e3181ba3354
- Brickler, T., Gresham, K., Meza, A., Coutermarsh-Ott, S., Williams, T. M., Rothschild, D. E., et al. (2016). Nonessential role for the NLRP1 inflammasome complex in a murine model of traumatic brain injury. *Mediators Inflamm.* 2016:6373506. doi: 10.1155/2016/6373506
- Chen, Y., Hallenbeck, J. M., Ruetzler, C., Bol, D., Thomas, K., Berman, N. E. J., et al. (2003). Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells. *J. Cereb. Blood Flow Metab.* 23, 748–755. doi: 10.1097/01.WCB.0000071885.63724.20
- Chu, T.-T., Tu, X., Yang, K., Wu, J., Repa, J. J., and Yan, N. (2021). Tonic prime-boost of STING signalling mediates Niemann-Pick disease type C. *Nature* 596, 570–575. doi: 10.1038/s41586-021-03762-2
- Domizio, J. Di., Gulen, M. F., Saidoune, F., Thacker, V. V., and Yatim, A. (2022). The cGAS-STING pathway drives type I IFN immunopathology in COVID-19. *Nature* doi: 10.1038/s41586-022-04421-w. [Online ahead of print].
- Dunphy, G., Flannery, S. M., Almine, J. F., Connolly, D. J., Paulus, C., Jönsson, K. L., et al. (2018). Non-canonical activation of the DNA sensing adaptor STING by ATM and IFI16 mediates NF-κB signaling after nuclear DNA damage. *Mol. Cell* 71, 745–760.e5. doi: 10.1016/j.molcel.2018.07.034
- Fink, A. L., Engle, K., Ursin, R. L., Tang, W. Y., and Klein, S. L. (2018). Biological sex affects vaccine efficacy and protection against influenza in mice. *Proc. Natl. Acad. Sci. U S A* 115, 12477–12482. doi: 10.1073/pnas.1805268115
- Fischer, T. D., Hylin, M. J., Zhao, J., Moore, A. N., Waxham, M. N., and Dash, P. K. (2016). Altered mitochondrial dynamics and TBI pathophysiology. *Front. Syst. Neurosci.* 10:29. doi: 10.3389/fnsys.2016.00029
- Frugier, T., Morganti-Kossmann, M. C., O'Reilly, D., and McLean, C. A. (2010). *in situ* detection of inflammatory mediators in post mortem human brain tissue after traumatic injury. *J. Neurotrauma* 27, 497–507. doi: 10.1089/neu.2009.1120
- Goto, A., Okado, K., Martins, N., Cai, H., Barbier, V., Lamielle, O., et al. (2018). The Kinase IKKβ regulates a STING- and NF-κB-dependent antiviral response pathway in drosophila. *Immunity* 49, 225–234.e4. doi: 10.1016/j.immuni.2018.07.013
- Grasl-Kraupp, B., Ruttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., and Schulte-Hermann, R. (1995). *In situ* detection of fragmented DNA (tunel assay) fails to discriminate among apoptosis, necrosis and autolytic cell death: a cautionary note. *Hepatology* 21, 1465–1468. doi: 10.1002/hep.1840210534
- Greve, M. W., and Zink, B. J. (2009). Pathophysiology of traumatic brain injury. *Mt. Sinai J. Med.* 76, 97–104. doi: 10.1002/msj.20104
- Guo, H., König, R., Deng, M., Riess, M., Mo, J., Zhang, L., et al. (2016). NLRX1 sequesters STING to negatively regulate the interferon response, thereby facilitating the replication of HIV-1 and DNA viruses. *Cell Host Microbe* 19, 515–528. doi: 10.1016/j.chom.2016.03.001
- Gupte, R., Brooks, W., Vukas, R., Pierce, J., and Harris, J. (2019). Sex differences in traumatic brain injury: what we know and what we should know. *J. Neurotrauma* 36, 3063–3091. doi: 10.1089/neu.2018.6171
- Haag, S. M., Gulen, M. F., Reymond, L., Gibelin, A., Abrami, L., Decout, A., et al. (2018). Targeting STING with covalent small-molecule inhibitors. *Nature* 559, 269–273. doi: 10.1038/s41586-018-0287-8

- Helle, S. C. J., Feng, Q., Aebbersold, M. J., Hirt, L., Grüter, R. R., Vahid, A., et al. (2017). Mechanical force induces mitochondrial fission. *eLife* 6:e30292. doi: 10.7554/eLife.30292
- Holt, L. M., and Olsen, M. L. (2016). Novel applications of magnetic cell sorting to analyze cell-type specific gene and protein expression in the central nervous system. *PLoS One* 11:e0150290. doi: 10.1371/journal.pone.0150290
- Holt, L. M., Stoyanof, S. T., and Olsen, M. L. (2019). Magnetic cell sorting for *in vivo* and *in vitro* astrocyte, neuron and microglia analysis. *Curr. Protoc. Neurosci.* 88:e71. doi: 10.1002/cpns.71
- Hughes, P. M., Allegrini, P. R., Rudin, M., Perry, V. H., Mir, A. K., and Wiessner, C. (2002). Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model. *J. Cereb. Blood Flow Metab.* 22, 308–317. doi: 10.1097/00004647-200203000-00008
- Ishikawa, H., and Barber, G. N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 455, 674–678. doi: 10.1038/nature07317
- Ivashkiv, L. B., and Donlin, L. T. (2014). Regulation of type I interferon responses. *Nat. Rev. Immunol.* 14, 36–49. doi: 10.1038/nri3581
- Jassam, Y. N., Izzy, S., Whalen, M., McGavern, D. B., and El Khoury, J. (2017). Neuroimmunology of traumatic brain injury: time for a paradigm shift. *Neuron* 95, 1246–1265. doi: 10.1016/j.neuron.2017.07.010
- Karve, I. P., Zhang, M., Habgood, M., Frugier, T., Brody, K. M., Sashindranath, M., et al. (2016). Ablation of type-1 IFN signaling in hematopoietic cells confers protection following traumatic brain injury. *eNeuro* 3:ENEURO.0128-15.2016. doi: 10.1523/ENEURO.0128-15.2016
- Kilbaugh, T. J., Lvova, M., Karlsson, M., Zhang, Z., Leipzig, J., Wallace, D. C., et al. (2015). Peripheral blood mitochondrial DNA as a biomarker of cerebral mitochondrial dysfunction following traumatic brain injury in a porcine model. *PLoS One* 10:e0130927. doi: 10.1371/journal.pone.0130927
- Lagraoui, M., Latoche, J. R., Cartwright, N. G., Sukumar, G., Dalgard, C. L., and Schaefer, B. C. (2012). Controlled cortical impact and craniotomy induce strikingly similar profiles of inflammatory gene expression, but with distinct kinetics. *Front. Neurol.* 3:155. doi: 10.3389/fneur.2012.00155
- Li, Q., Cao, Y., Dang, C., Han, B., Han, R., Ma, H., et al. (2020). Inhibition of double-strand DNA-sensing cGAS ameliorates brain injury after ischemic stroke. *EMBO Mol. Med.* 12:e11002. doi: 10.15252/emmm.201911002
- Liu, Y., Gordesky-Gold, B., Leney-Greene, M., Weinbren, N. L., Tudor, M., and Cherry, S. (2018). Inflammation-induced, STING-dependent autophagy restricts Zika virus infection in the drosophila brain. *Cell Host Microbe* 24, 57–68.e3. doi: 10.1016/j.chom.2018.05.022
- Liu, Y., Jesus, A., Marrero, B., Yang, D., Ramsey, S. E., Montealegre Sanchez, G. A., et al. (2014). Activated STING in a vascular and pulmonary syndrome. *N. Engl. J. Med.* 371, 507–518. doi: 10.1056/NEJMoa1312625
- Luksch, H., Stinson, W. A., Platt, D. J., Qian, W., Kalugotla, G., Miner, C. A., et al. (2019). STING-associated lung disease in mice relies on T cells but not type I interferon. *J. Allergy Clin. Immunol.* 144, 254–266.e8. doi: 10.1016/j.jaci.2019.01.044
- Ma, F., Li, B., Yu, Y., Iyer, S. S., Sun, M., and Cheng, G. (2015). Positive feedback regulation of type I interferon by the interferon-stimulated gene STING. *EMBO Rep.* 16, 202–212. doi: 10.15252/embr.201439366
- Martin, M., Hiroyasu, A., Guzman, R. M., Roberts, S. A., and Goodman, A. G. (2018). Analysis of drosophila STING reveals an evolutionarily conserved antimicrobial function. *Cell Rep.* 23, 3537–3550.e6. doi: 10.1016/j.celrep.2018.05.029
- Mathur, V., Burai, R., Vest, R. T., Bonanno, L. N., Lehallier, B., Zardeneta, M. E., et al. (2017). Activation of the STING-dependent type I interferon response reduces microglial reactivity and neuroinflammation. *Neuron* 96, 1290–1302.e6. doi: 10.1016/j.neuron.2017.11.032
- McArthur, K., Whitehead, L. W., Heddleston, J. M., Li, L., Padman, B. S., Oorschot, V., et al. (2018). BAK/BAX macropores facilitate mitochondrial herniation and mtDNA efflux during apoptosis. *Science* 359:ea06047. doi: 10.1126/science.a06047
- McKee, C. A., and Lukens, J. R. (2016). Emerging roles for the immune system in traumatic brain injury. *Front. Immunol.* 7:556. doi: 10.3389/fimmu.2016.00556
- Motwani, M., McGowan, J., Antonovitch, J., Gao, K. M. J., Jiang, Z., Sharma, S., et al. (2021). cGAS-STING pathway does not promote autoimmunity in murine models of SLE. *Front. Immunol.* 12:605930. doi: 10.3389/fimmu.2021.605930
- Nazmi, A., Mukhopadhyay, R., Dutta, K., and Basu, A. (2012). STING mediates neuronal innate immune response following japanese encephalitis virus infection. *Sci. Rep.* 2:347. doi: 10.1038/srep00347
- Nicolas, C. S., Amici, M., Bortolotto, Z. A., Doherty, A., Csaba, Z., Fafouri, A., et al. (2013). The role of JAK-STAT signaling within the CNS. *JAKSTAT* 2:e22925. doi: 10.4161/jkst.22925
- Pisetsky, D. S. (2014). The complex role of DNA, histones and HMGB1 in the pathogenesis of SLE. *Autoimmunity* 47, 487–493. doi: 10.3109/08916934.2014.921811
- Reinert, L. S., Lopusná, K., Winther, H., Sun, C., Thomsen, M. K., Nandakumar, R., et al. (2016). Sensing of HSV-1 by the cGAS-STING pathway in microglia orchestrates antiviral defence in the CNS. *Nat. Commun.* 7:13348. doi: 10.1038/ncomms13348
- Reinert, L. S., Rashidi, A. S., Tran, D. N., Katzilieris-Petras, G., Hvidt, A. K., Gohr, M., et al. (2021). Brain immune cells undergo cGAS-STING-dependent apoptosis during herpes simplex virus type 1 infection to limit type I IFN production. *J. Clin. Invest.* 131:e136824. doi: 10.1172/JCI.136824
- Riley, J. S., Quarato, G., Cloix, C., Lopez, J., O'Prey, J., Pearson, M., et al. (2018). Mitochondrial inner membrane permeabilisation enables mtDNA release during apoptosis. *EMBO J.* 37:e99238. doi: 10.15252/emboj.201899238
- Roozenbeek, B., Maas, A. I. R., and Menon, D. K. (2013). Changing patterns in the epidemiology of traumatic brain injury. *Nat. Rev. Neurol.* 9, 231–236. doi: 10.1038/nrneuro.2013.22
- Sauer, J. D., Sotelo-Troha, K., Von Moltke, J., Monroe, K. M., Rae, C. S., Brubaker, S. W., et al. (2011). The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of sting in the *in vivo* interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect. Immun.* 79, 688–694. doi: 10.1128/IAI.00999-10
- Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73
- Schneider, W. M., Chevillotte, M. D., and Rice, C. M. (2014). Interferon-stimulated genes: a complex web of host defenses. *Annu. Rev. Immunol.* 32, 513–545. doi: 10.1146/annurev-immunol-032713-120231
- Sen, T., Saha, P., Gupta, R., Foley, L. M., Jiang, T., Abakumova, O. S., et al. (2020). Aberrant ER stress induced neuronal-IFN β elicits white matter injury due to microglial activation and T-Cell infiltration after TBI. *J. Neurosci.* 40, 424–446. doi: 10.1523/JNEUROSCI.0718-19.2019
- Seth, R. B., Sun, L., and Ea C-K, Chen, Z. J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF- κ B and IRF3. *Cell* 122, 669–682. doi: 10.1016/j.cell.2005.08.012
- Shang, G., Zhang, C., Chen, Z. J., Bai, X. C., and Zhang, X. (2019). Cryo-EM structures of STING reveal its mechanism of activation by cyclic GMP-AMP. *Nature* 567, 389–393. doi: 10.1038/s41586-019-0998-5
- Sharma, S., Campbell, A. M., Chan, J., Schattgen, S. A., Orlowski, G. M., Nayar, R., et al. (2015). Suppression of systemic autoimmunity by the innate immune adaptor STING. *Proc. Natl. Acad. Sci. U S A* 112, E710–E717. doi: 10.1073/pnas.1420217112
- Simon, D. W., McGeachy, M. J., Bayır, H., Clark, R. S. B., Loane, D. J., and Kochanek, P. M. (2017). The far-reaching scope of neuroinflammation after traumatic brain injury. *Nat. Rev. Neurol.* 13, 171–191. doi: 10.1038/nrneuro.2017.13
- Späni, C. B., Braun, D. J., and Van Eldik, L. J. (2018). Sex-related responses after traumatic brain injury: considerations for preclinical modeling. *Front. Neuroendocrinol.* 50, 52–66. doi: 10.1016/j.yfrne.2018.03.006
- Stephanou, A., Brar, B. K., Scarabelli, T. M., Jonassen, A. K., Yellon, D. M., Marber, M. S., et al. (2000). Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. *J. Biol. Chem.* 275, 10002–10008. doi: 10.1074/jbc.275.14.10002
- Stetson, D. B., and Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* 24, 93–103. doi: 10.1016/j.immuni.2005.12.003
- Strecker, J. K., Minnerup, J., Schütte-Nütgen, K., Gess, B., Schäbitz, W. R., and Schilling, M. (2013). Monocyte chemoattractant protein-1-deficiency results in altered blood-brain barrier breakdown after experimental stroke. *Stroke* 44, 2536–2544. doi: 10.1161/STROKEAHA.111.000528

- Sun, L., Wu, J., Du, F., Chen, X., and Chen, Z. J. (2013). Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 339, 786–791. doi: 10.1126/science.1232458
- Surgucheva, I., He, S., Rich, M. C., Sharma, R., Ninkina, N. N., Stahel, P. F., et al. (2014). Role of synucleins in traumatic brain injury — an experimental *in vitro* and *in vivo* study in mice. *Mol. Cell Neurosci.* 63, 114–123. doi: 10.1016/j.mcn.2014.10.005
- Takagi, Y., Harada, J., Chiarugi, A., and Moskowitz, M. A. (2002). STAT1 is activated in neurons after ischemia and contributes to ischemic brain injury. *J. Cereb. Blood Flow. Metab.* 22, 1311–1318. doi: 10.1097/01.WCB.0000034148.72481.F4
- Tanaka, Y., and Chen, Z. J. (2012). STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci. Signal* 5:ra20. doi: 10.1126/scisignal.2002521
- Theus, M. H., Brickler, T., Meza, A., Coutermarsh-Ott, S., Hazy, A., Gris, D., et al. (2017). Loss of NLRX1 exacerbates neural tissue damage and NF- κ B signaling following brain injury. *J. Immunol.* 199, 3547–3558. doi: 10.4049/jimmunol.1700251
- Theus, M. H., Ricard, J., Bethea, J. R., and Liebl, D. J. (2010). EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury. *Stem Cells* 28, 1231–1242. doi: 10.1002/stem.449
- Thim-uam, A., Prabakaran, T., Tansakul, M., Makjaroen, J., Wongkongkathap, P., Chantaravisoot, N., et al. (2020). STING mediates lupus *via* the activation of conventional dendritic cell maturation and plasmacytoid dendritic cell differentiation. *iScience* 23:101530. doi: 10.1016/j.isci.2020.101530
- Trinchieri, G. (2010). Type I interferon: friend or foe. *J. Exp. Med.* 207:2053. doi: 10.1084/jem.20101664
- Unterholzner, L., and Dunphy, G. (2019). cGAS-independent STING activation in response to DNA damage. *Mol. Cell Oncol.* 6:1558682. doi: 10.1080/23723556.2018.1558682
- Urbonaviciute, V., F  rnrohr, B. G., Meister, S., Munoz, L., Heyder, P., De Marchis, F., et al. (2008). Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: Implications for the pathogenesis of SLE. *J. Exp. Med.* 205, 3007–3018. doi: 10.1084/jem.20081165
- Walko, T. D., Bola, R. A., Hong, J. D., Au, A. K., Bell, M. J., Kochanek, P. M., et al. (2014). Cerebrospinal fluid mitochondrial DNA: A novel DAMP in pediatric traumatic brain injury. *Shock* 41, 499–503. doi: 10.1097/SHK.0000000000000160
- Werner, C., and Engelhard, K. (2007). Pathophysiology of traumatic brain injury. *Br. J. Anaesth.* 99, 4–9. doi: 10.1093/bja/aem131
- West, A. P., Khoury-Hanold, W., Staron, M., Tal, M. C., Pineda, C. M., Lang, S. M., et al. (2015). Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* 520, 553–557. doi: 10.1038/nature14156
- Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., et al. (2013). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* 339, 826–830. doi: 10.1126/science.1229963
- Yu, C. H., Davidson, S., Harapas, C. R., Hilton, J. B., Mlodzikowski, M. J., Laohamonthonkul, P., et al. (2020). TDP-43 triggers mitochondrial DNA release *via* mPTP to activate cGAS/STING in ALS. *Cell* 183, 636–649.e18. doi: 10.1016/j.cell.2020.09.020
- Yum, S., Li, M., Fang, Y., and Chen, Z. J. (2021). TBK1 recruitment to STING activates both IRF3 and NF- κ B that mediate immune defense against tumors and viral infections. *Proc. Natl. Acad. Sci. U S A* 118:e2100225118. doi: 10.1073/pnas.2100225118
- Zhang, D., Liu, C., Li, H., and Jiao, J. (2020). Deficiency of STING signaling in embryonic cerebral cortex leads to neurogenic abnormalities and autistic-like behaviors. *Adv. Sci.* 7:2002117. doi: 10.1002/adv.202002117
- Zhang, C., Shang, G., Gui, X., Zhang, X., Bai, X., and Chen, Z. J. (2019). Structural basis of STING binding with and phosphorylation by TBK1. *Nature* 567, 394–398. doi: 10.1038/s41586-019-1000-2
- Zhong, B., Yang, Y., Li, S., Wang, Y. Y., Li, Y., Diao, F., et al. (2008). The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 29, 538–550. doi: 10.1016/j.immuni.2008.09.003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright   2022 Fritsch, Ju, Gudenschwager Basso, Soliman, Paul, Chen, Kaloss, Kowalski, Tuhy, Somaiya, Wang, Allen, Theus and Pickrell. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Chapter 4

STING Signaling in Microglia and Peripheral Immune Cells Contributes to Neuroinflammation in Traumatic Brain Injury

This manuscript is in preparation for journal submission.

STING Signaling in Microglia and Peripheral Immune Cells Contributes to Neuroinflammation in Traumatic Brain Injury

Lauren E. Fritsch¹, Nicole DeFoor², Colin Kelly¹, Swagatika Paul⁴, Hannah O'Malley², Jing Ju³, Michelle H. Theus⁵, Alicia M. Pickrell^{2#}

¹Graduate Program in Translational Biology, Medicine, and Health, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

²School of Neuroscience, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

³Molecular and Cellular Biology Graduate Program, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

⁴Biomedical and Veterinary Sciences Graduate Program, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

⁵Department of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Correspondence should be addressed to:

Alicia M. Pickrell, 970 Washington Street SW, Life Science I Rm 217, Blacksburg, VA 24061

Tel: 540-232-8465; Fax: 540-231-1475; Email: alicia.pickrell@vt.edu

Keywords: Interferons, microglia, neuroinflammation, traumatic brain injury

Abbreviations: BBB (blood brain barrier); BMC (bone marrow cells); BMDM (bone-marrow derived macrophage); CBC (complete blood count); CCI (controlled cortical impact); cGAMP (cyclic GMP-AMP); cGAS (cGAMP synthase); CNS (central nervous system); DAMP (danger-associated molecular pattern); HSV-1 (herpes simplex virus 1); ICH (intracerebral hemorrhage); IFN (interferon); IFNAR (interferon α/β receptor); ISG (interferon stimulated gene); KO (knockout); mtDNA (mitochondrial DNA); STING (stimulator of interferon genes); TBI (traumatic brain injury); TBK1 (tank binding kinase 1); TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), VSV (vesicular stomatitis virus), WT (wildtype)

Abstract

While originally identified as an antiviral pathway, recent work has implicated cyclic GMP-AMP-Stimulator of Interferon Genes (cGAS-STING) signaling as playing a critical role in the neuroinflammatory response to traumatic brain injury (TBI). STING activation results in a robust inflammatory response characterized by the production of inflammatory cytokines called interferons, as well as hundreds of interferon stimulated genes (ISGs). In the brain, cytokine and chemokine production promotes substantial immune cell recruitment and can cause exacerbated tissue damage. In this study, we sought to characterize which cell types are responsible for detrimental STING-mediated

neuroinflammation in a murine controlled cortical impact (CCI) model of TBI. Using bone marrow chimeric mice, we show that loss of STING in the brain or hematopoietic cells reduces lesion size and drastically blunts the influx of peripheral immune cells to the injury site. To further investigate which brain cell type(s) contribute to this response, we conditionally knocked out STING in microglia. Microglial-specific STING knockout animals had substantial neuroprotection and reduced motor deficits compared to wildtype controls. Taken together, we find that both microglia and peripheral immune cells contribute to the neuroinflammatory response mediated by STING, and that loss of STING in either microglia or the peripheral immune system confers neuroprotection. This work supports a complex cross-talk between brain-resident and peripheral immune cells to coordinate the inflammatory response to TBI.

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability across the globe (Dewan et al. 2018; Roozenbeek, Maas, and Menon 2013). Following initial mechanical damage to cells, various cellular processes termed secondary injury occur in the hours to weeks after TBI that result in exacerbated tissue damage. A key part of this secondary injury is neuroinflammation (Jassam et al. 2017); however, the neuroinflammatory response to TBI is highly complex and is not fully understood. Immediately following TBI, damage associated molecular patterns (DAMPs) are released from injured cells, resulting in cytokine and chemokine production, activation of resident microglia, and recruitment of peripheral immune cells (Simon et al. 2017; Gyoneva and Ransohoff 2015).

Recent studies have shown that interferons (IFNs), inflammatory cytokines typically produced in response to viral infection, are upregulated in the brain following TBI in human patients (Frugier et al. 2010). The cyclic GMP-AMP Synthase-Stimulator of Interferon Genes (cGAS-STING) pathway is one of the primary mechanisms for producing Type I interferons; therefore, the role of this pathway in neurotrauma has become of interest. While STING activity was originally identified as a critical component of the antiviral response (Ishikawa and Barber 2008; Ishikawa, Ma, and Barber 2009), an increasing number of studies have demonstrated that STING is activated in a number of contexts, including cancer (Ahn et al. 2014; Harding et al. 2017), injury (Maekawa et al. 2019), pain (Donnelly et al. 2021), and autoimmune disease (Liu et al. 2014; Mathur et al. 2017; Thim-uam et al. 2020). In canonical STING signaling, the pattern recognition receptor (PRR) cGAS binds cytosolic DAMPs, including mitochondrial DNA (mtDNA) (West et al. 2015; White et al. 2014; Rongvaux et al. 2014), to promote the production of the second messenger, cyclic GMP-AMP (cGAMP) (Sun et al. 2013; Civril et al. 2013; Ablasser et al. 2013). Upon binding cGAMP and recruiting Tank Binding Kinase 1 (TBK1), activated STING prompts a robust inflammatory response characterized by the production of Type I IFNs and interferon stimulated genes (ISGs) (Ishikawa and Barber 2008; X. Zhang et al. 2013; Wu et al. 2013).

We and others have shown that cGAS-STING-IFN signaling plays an important role in the neuroinflammatory response to TBI (Fritsch et al. 2022; Abdullah et al. 2018; Karve et al. 2016; Barrett et al. 2020; Sen et al. 2020). Though inflammation is important for clearance of damaged tissue after brain trauma, these studies have shown that cGAS-STING-mediated neuroinflammation is largely deleterious. Global knockout (KO) of cGAS (Fritsch et al. 2022), STING (Fritsch et al. 2022; Abdullah et al. 2018), IFN β (Barrett et al. 2020), or the Type I IFN receptor, IFNAR (Karve et al. 2016) largely resulted in

neuroprotection after TBI. However, it remains unclear which cell type(s) are responsible for generating detrimental STING signaling in TBI. While it is clear that many peripheral immune cells express STING, studies in the central nervous system (CNS) are conflicting. Data from CNS infection (Reinert et al. 2016; Katzilieris-Petras et al. 2022), TBI (Fritsch et al. 2022), and stroke (Peng et al. 2020), as well as RNA sequencing databases (Y. Zhang et al. 2014, 2016) suggest that STING is predominately expressed in microglia. In contrast, others have provided evidence that neurons and astrocytes express STING (Sen et al. 2020; Donnelly et al. 2021; Gamdzyk et al. 2020; Abdullah et al. 2018). Regardless, infiltration of peripheral immune cells, including neutrophils and monocytes/macrophages, is a key aspect of the neuroinflammatory response to TBI (Gyoneva and Ransohoff 2015). Given the high level of STING expression in monocytes, it is plausible that STING signaling in infiltrating cells contributes to the overall STING-IFN response. One study in chimeric IFNAR KO animals has shown that peripheral immune cells are important for Type I IFN signaling in TBI (Karve et al. 2016), but there are STING-independent mechanisms for producing IFNs (Ivashkiv and Donlin 2014). The use of global KO animals has made it challenging to determine which cell population(s) drive STING-mediated neuroinflammation in TBI. Furthermore, distinguishing between infiltrating macrophages and microglia is difficult due to the substantial overlap in markers (Grassivaro et al. 2020); therefore, the use of more specific KO models is important for delineating the contributions of different cell types to STING-IFN signaling in the brain.

The present work sought to clarify whether peripheral immune cells or brain-resident microglia are responsible for harmful STING-mediated neuroinflammation following neurotrauma. Utilizing a murine controlled cortical impact (CCI) injury paradigm in bone marrow chimeric and conditional microglial-specific STING knockout (KO) animals, we show that both peripheral immune cells and microglia contribute to this response. While prior studies have shown a benefit to eliminating STING globally, our findings indicate that multiple cell types contribute to STING signaling in neurotrauma. These data suggest that this signaling pathway regulates many aspects of neuroinflammation that are associated with poor outcomes in brain injury, including immune cell infiltration and blood brain barrier permeability, which are dictated by both the peripheral and resident CNS cells.

Results

Loss of STING in brain or peripheral immune cells confers neuroprotection

Our previous work demonstrated that global loss of STING results in reduced cytokine production, reduced cell death, and smaller lesion size up to two weeks after injury (Fritsch et al. 2022). To clarify whether brain-resident cell or infiltrating peripheral immune cells are responsible for detrimental STING signaling, we generated bone marrow chimeric animals (Figure 1a). Irradiated recipient STING KO and wildtype (WT) animals received bone marrow cells (BMCs) from GFP⁺ STING KO or GFP⁺ WT donors, generating four groups (WT^{WTBMC}, KO^{KOBMC}, WT^{KOBMC}, and KO^{WTBMC}). To verify successful reconstitution of the peripheral immune system, white blood cells from chimeric animals were removed and sorted via flow cytometry 28 days after receiving GFP⁺ donor bone marrow. Approximately 94.78% ($\pm 0.88\%$ SD) of the isolated WBCs were GFP⁺, confirming reconstitution of the recipient animals' peripheral immune systems (Figure 1b). To determine whether brain cells were replaced during the procedure, naïve chimeric animals were euthanized 28 days after reconstitution to check for GFP⁺ cells in

the brain. When assessed by flow cytometry, only 0.22% ($\pm 0.037\%$ SD) of brain cells were GFP⁺ (Supplemental Figure 1a), which was verified with confocal microscopy of sectioned brain tissue (Supplemental Figure 1b).

After verifying the model, we assessed lesion size 24 hours after injury. In support of our previous findings (Fritsch et al. 2022), the chimeric KO^{KOBMC} animals had significantly reduced lesion size compared to WT^{WTBMC}. Animals lacking STING in either the brain (KO^{WTBMC}) or hematopoietic cells (WT^{KOBMC}) had smaller lesions than WT^{WTBMC} animals (Figure 1c-d), suggesting that loss of STING in either compartment is beneficial. While the WT^{KOBMC} group did not reach the full level of protection seen

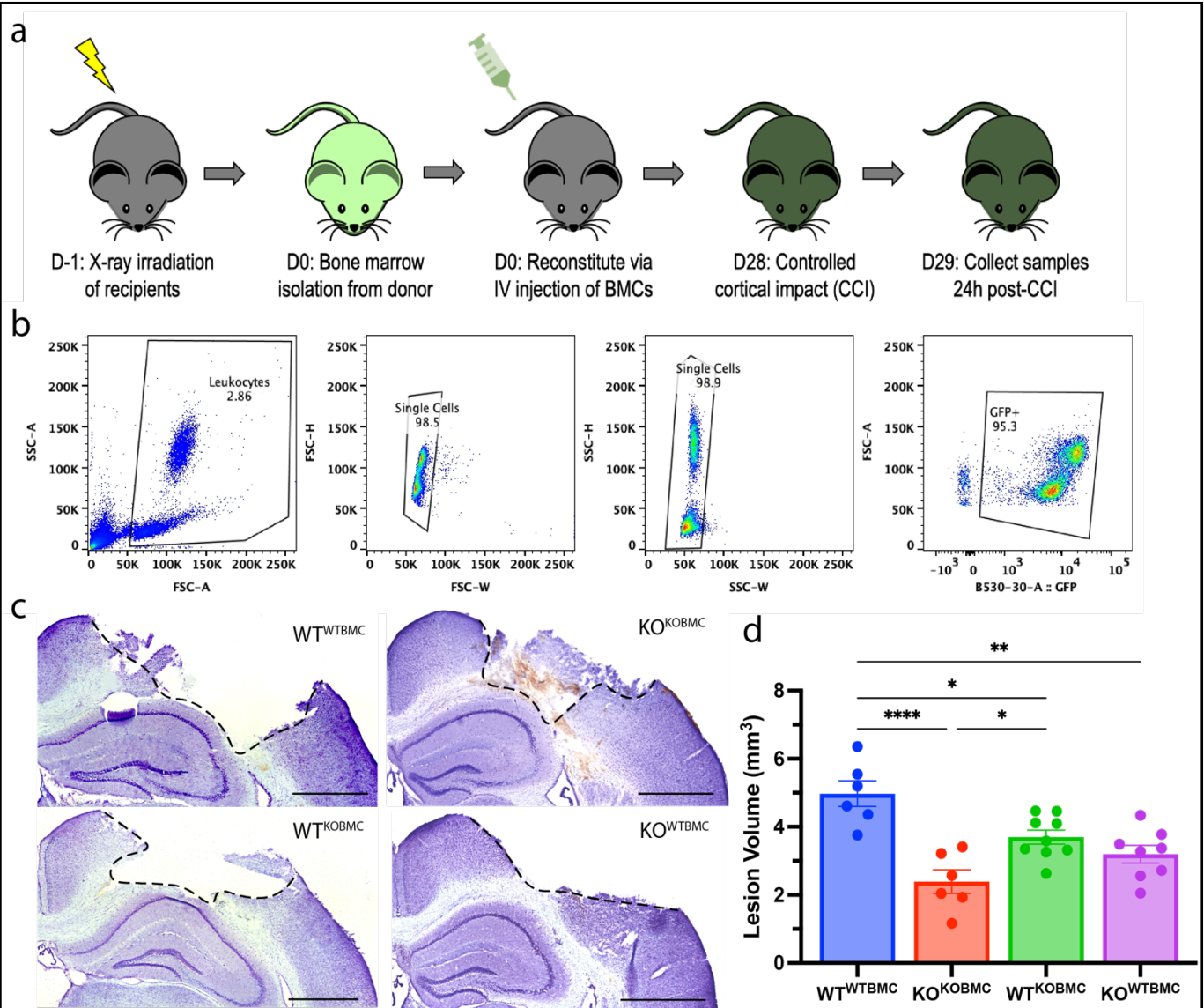
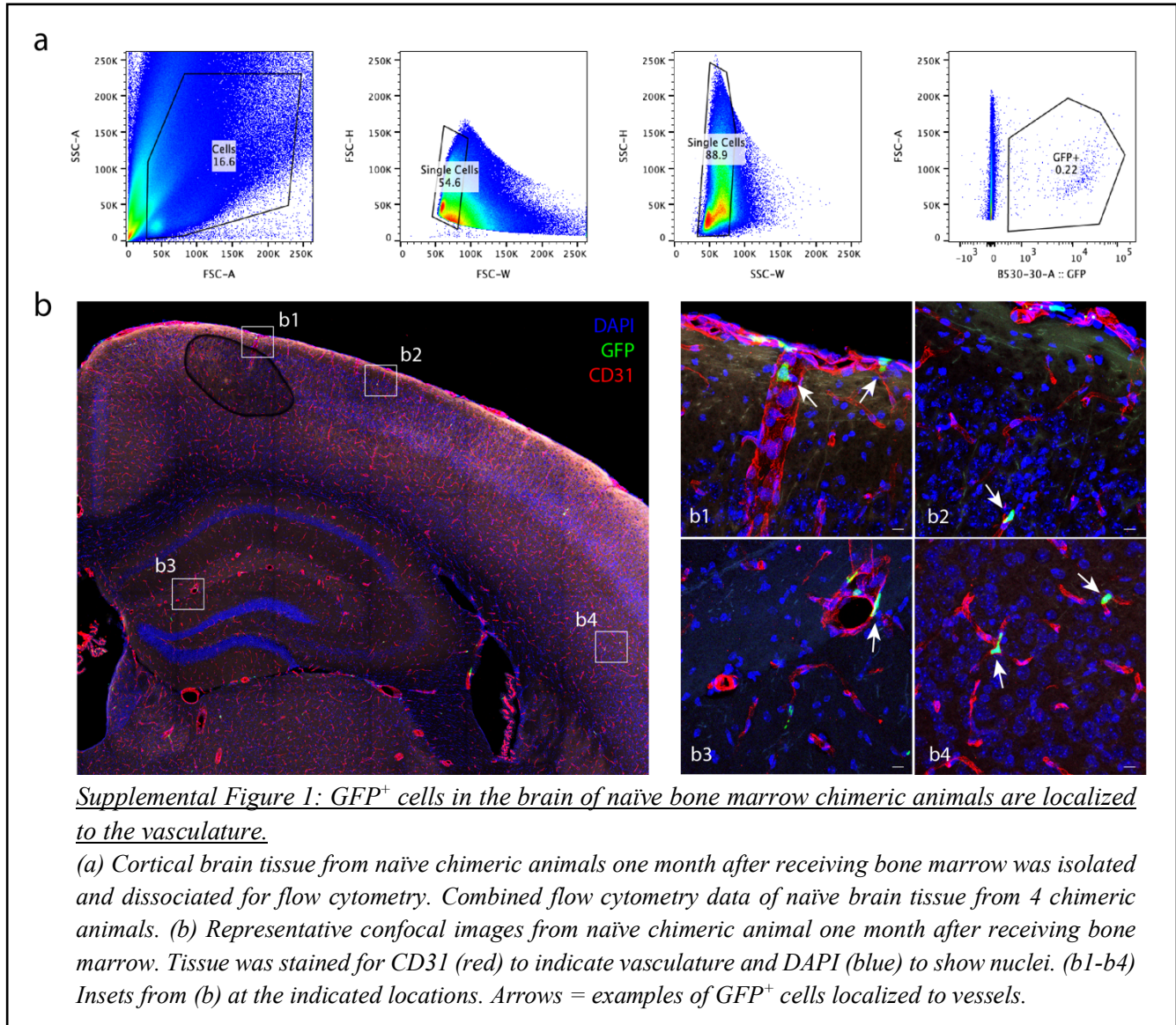


Figure 1: Loss of STING in the brain or peripheral immune cells confers neuroprotection.

(a) Model for developing bone marrow chimeric animals. Following irradiation, recipient animals received 4 million bone marrow cells from GFP⁺ donor animals via tail vein injection. Animals underwent CCI surgery 28 days later to allow time for reconstitution of the immune system. (b) Flow cytometry of white blood cells confirming successful reconstitution of immune system in chimeric animals. Plots are representative from one animal. Average of $n = 4$ was 94.78% ($\pm 0.88\%$ SD). (c) Representative images of sectioned Cresyl violet stained brains 24 hours after CCI from bone marrow chimeric animals. Scale bar = 1mm. (d) Quantification of lesion size 24 hours after injury. Each dot represents one animal. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Error bars = SEM.

in the KO^{KOBMC} animals, there was no significant difference between the WT^{KOBMC} and KO^{WTBMC} group or the KO^{WTBMC} and KO^{KOBMC} animals (Figure 1c-d). These data suggest that both brain and peripheral STING signaling contributes to tissue loss after TBI, and loss of STING in either compartment results in neuroprotection.



Full body, brain, or hematopoietic loss of STING reduces peripheral immune cell infiltration acutely after TBI

Given that STING signaling is known to promote production of chemokines such as monocytes chemoattractant protein 1 (MCP1) that attract immune cells (Deshmane et al. 2009), we next sought to determine whether loss of brain-specific STING would affect peripheral immune cell infiltration. Ipsilateral cortical tissue was microdissected and prepared for flow cytometry, then the percentage of GFP^+

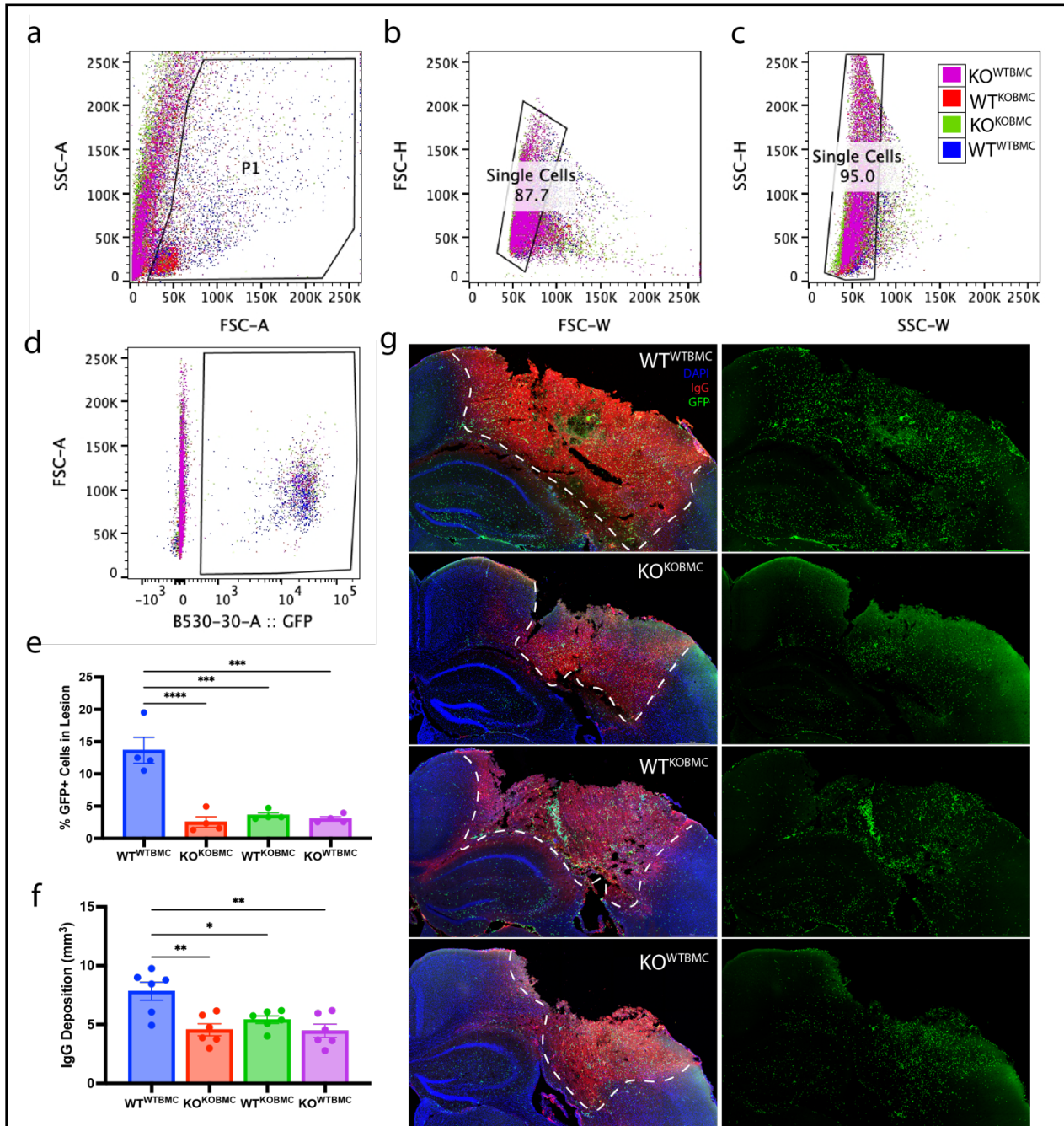


Figure 2: Full body, brain, or hematopoietic loss of STING reduces peripheral immune cell infiltration acutely after TBI.

(a-d) Cortical brain tissue from bone marrow chimeric animals was isolated 24 hours after injury and dissociated for flow cytometry. Plots are combined data from four animals for each group. Gating scheme included debris removal (a) and selection of single cells (b and c) prior to gating for GFP+ cells (d). GFP gating was set based on white blood cells from GFP+ control animals. (e) Quantification of GFP+ cells identified by flow cytometry, given as a percentage of single cells. (f) Quantification of IgG deposition 24 hours after injury. (g) Representative confocal images showing GFP+ cell infiltration and IgG deposition (red). Nuclei were stained with DAPI (blue). For (e) and (f), each dot represents an individual animal. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars = SEM.

cells from the entire region was assessed to provide a comparison of the level of infiltration (Figure 2a-d). As anticipated, WT^{WTBMC} animals had significantly higher levels of peripheral immune cell infiltration when compared to KO^{KOBMC} animals (Figure 2e,g). Interestingly, both groups of crossed genotypes had comparable levels of infiltration compared to KO^{KOBMC} (Figure 2e,g), suggesting complex cross-talk between the brain and periphery that influences peripheral immune cell recruitment. We further sought to evaluate whether changes in blood brain barrier (BBB) permeability may account for the similar infiltration by staining for IgG deposition. We observed comparable reductions in BBB permeability indicated by reduced IgG staining for KO^{KOBMC}, KO^{WTBMC}, and WT^{KOBMC} animals compared to WT^{WTBMC} animals (Figure 2f-g), suggesting that animals lacking STING had reduced BBB breakdown after TBI.

To determine if this pattern of infiltration may be explained by an alteration in the number of white blood cells between genotypes, complete blood counts (CBCs) were performed with naïve global STING KO and WT animals. The total WBC count was not altered between genotypes, and while there was a significant difference in the number of platelets and monocytes (Table 1), both values were within normal range (O'Connell et al. 2015).

	Wildtype (mean ± SD)	STING KO (mean ± SD)
Cell Counts		
Red blood cells (RBCs; 10 ⁶ cells/μL)	10.3 ± 0.180	10.1 ± 0.5
White blood cells (WBCs; 10 ³ cells/μL)	4.5 ± 0.748	6.0 ± 1.7
Lymphocytes (10 ³ cells/μL)	3.7 ± 0.408	4.5 ± 1.3
Seg Neutrophils (10 ³ cells/μL)	0.5 ± 0.335	0.8 ± 0.3
Monocytes (10 ³ cells/μL)	0.2 ± 0.119 *	0.4 ± 0.1
Eosinophils (10 ³ cells/μL)	0.1 ± 0.035	0.1 ± 0.1
Basophils (10 ³ cells/μL)	0.0 ± 0.0	0.0 ± 0.0
Reticulocyte # (10 ³ cells/μL)	327.1 ± 36.8	358.4 ± 25.9
Platelets (10 ³ cells/μL)	1678.7 ± 132.3 *	1415.2 ± 150.5
Other Blood Components		
Hemoglobin (g/dL)	15.3 ± 0.252	14.9 ± 0.5
Hematocrit (%)	49.5 ± 1.56	49.2 ± 1.1
Mean Corpuscular Volume (MCV; fL)	48.1 ± 0.723	48.5 ± 0.7
Mean Corpuscular Hemoglobin (MCH; pg)	14.8 ± 0.0577	14.8 ± 0.2
Mean Corpuscular Hemoglobin Concentration (MCHC; g/dL)	30.9 ± 0.513	30.4 ± 0.3
Mean Platelet Volume (MPV; fL)	6.2 ± 0.1	6.1 ± 0.1

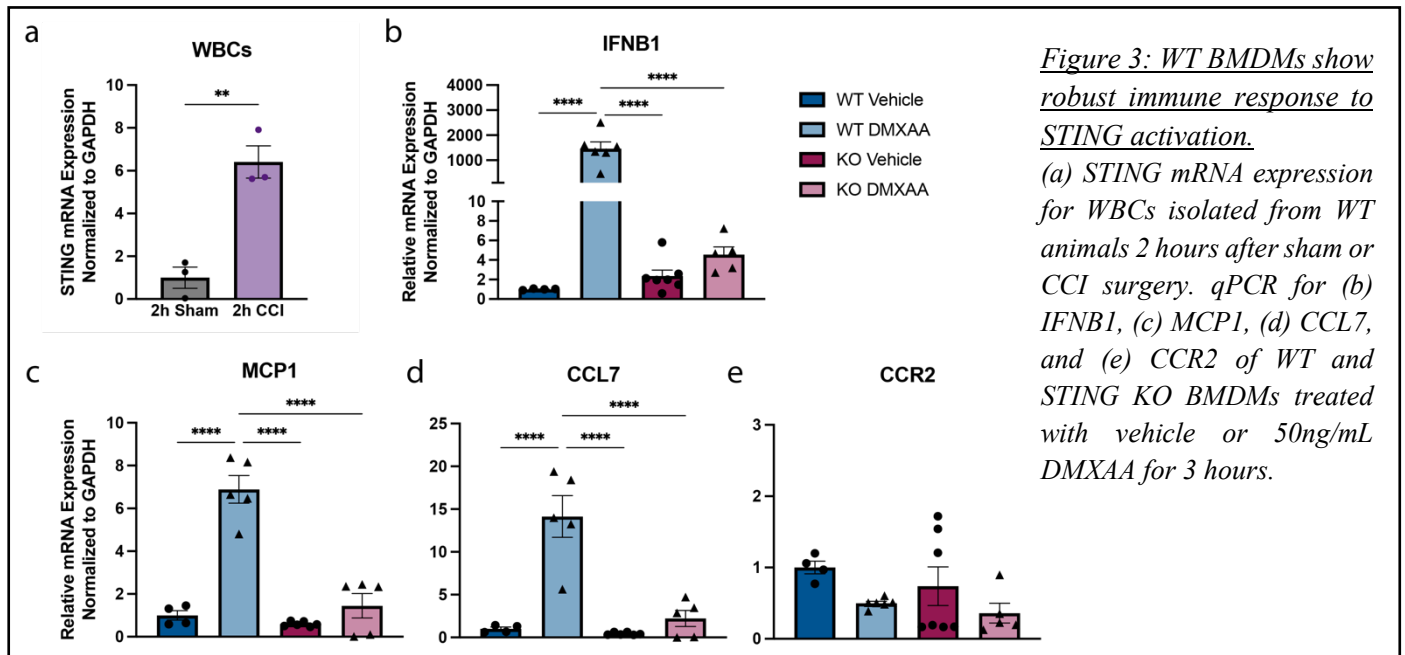
*Table 1: Complete blood count (CBC) data from naïve WT and STING KO animals. n = 3-5/genotype. * p < 0.05.*

To address whether peripheral immune cells were responding to IFNs or chemokines released after injury, WBCs were isolated from sham or injured WT animals 2 hours after surgery. WBCs from injured animals showed a significant upregulation in STING mRNA at this early timepoint (Figure 3a), suggesting that peripheral immune cells utilize STING signaling to respond to injury. In order to determine how activation of STING in peripheral immune cells may alter their reactivity to recruitment signals by the brain, we isolated bone marrow derived macrophages (BMDMs) from WT and STING KO animals. After treating BMDMs with the STING agonist, DMXAA, we saw a robust increase in IFNβ expression in WT cells as expected (Figure 3b). STING KO cells did not show any significant changes in response to DMXAA treatment, confirming specificity of the agonist to STING (Figure 3b-e). WT BMDMs had significant increases in the expression of the chemokines MCP1 and CCL7, but was not upregulated in KO BMDMs (Figure 3b-c). Expression of the receptor CCR2, which serves as the only receptor for MCP-1 and one of the primary receptors for CCL7 (Palomino and Marti 2015), was unaltered by STING activation *in vitro* (Figure 3e). Taken together, these data suggest that changes in BBB permeability and

STING signaling by both brain-resident cells and circulating WBCs influences peripheral immune influx into the brain after injury.

Loss of microglial-specific STING is neuroprotective

After determining that cells in both the brain and peripheral immune system play a role in STING-mediated neuroinflammation, we sought to clarify which cell type in the brain is responsible for this response. Our previous work suggested that microglia are the primary brain-resident cell type expressing STING (Fritsch et al. 2022); therefore, we developed a tamoxifen-inducible microglia-specific conditional STING KO ($STING^{fl/fl} CX3CR1^{CreER/+}$; cKO; Figure 4a). CX3CR1 is chemokine receptor that is present on many myeloid-derived cells, including monocytes, natural killer cells, dendritic cells, and microglia (Jung et al. 2000). In this model, tamoxifen administration conditionally knocks out STING in CX3CR1-expressing cells. Because of the rapid turnover of peripheral immune cells, but not microglia (Réu et al. 2017), this loss of STING can be localized to microglia by allowing repopulation of peripheral CX3CR1-expressing cells from unaffected myeloid progenitor cells in the bone marrow. One month after tamoxifen administration, white blood cells isolated from naïve animals showed no change in STING mRNA in peripheral immune cells between genotypes, confirming specificity of the model (Figure 4b). Microglia isolated from naïve cKO animals showed approximately a 75-fold decrease in STING mRNA relative to control animals ($STING^{fl/fl}$; Figure 4c-d). To evaluate whether loss of microglia-specific STING would show the same protection as was seen in the bone marrow chimeric KO^{WTBMC} , lesion size was assessed 24 hours after injury. We observed a significant reduction in lesion volume in cKO animals relative to $STING^{fl/fl}$ and $CX3CR1^{CreER/+}$ controls at this acute timepoint (Figure 4e-f). Furthermore, cKO animals showed a significant reduction in cell death when assessed via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Figure 5a-c). Within the lesion site, the density of cell death (indicated by TUNEL⁺ cells/mm³) was reduced in cKO animals compared to controls (Figure 5a). While



the density of apoptotic non-neuronal cells was comparable between cKO and both control genotypes, the density of apoptotic neurons was significantly reduced in the cKO animals (Figure 5b), suggesting neurons in particular are protected by the loss of microglial STING.

Loss of microglial STING reduces motor deficit and lesion size after TBI.

To determine whether the neuroprotection seen in cKO animals corresponded to changes in functional outcome, we assessed gross motor deficit with rotarod behavior testing in cKO and CX3CR1^{CreER/+} animals. Baseline (pre-injury) performance was comparable between groups (CX3CR1^{CreER/+} baseline time: 104.8 ± 6.6s; cKO baseline time: 109.0 ± 6.9s). Four days after injury, cKO animals had significantly reduced motor deficits than control animals (Figure 6a), suggesting loss of microglial STING offers

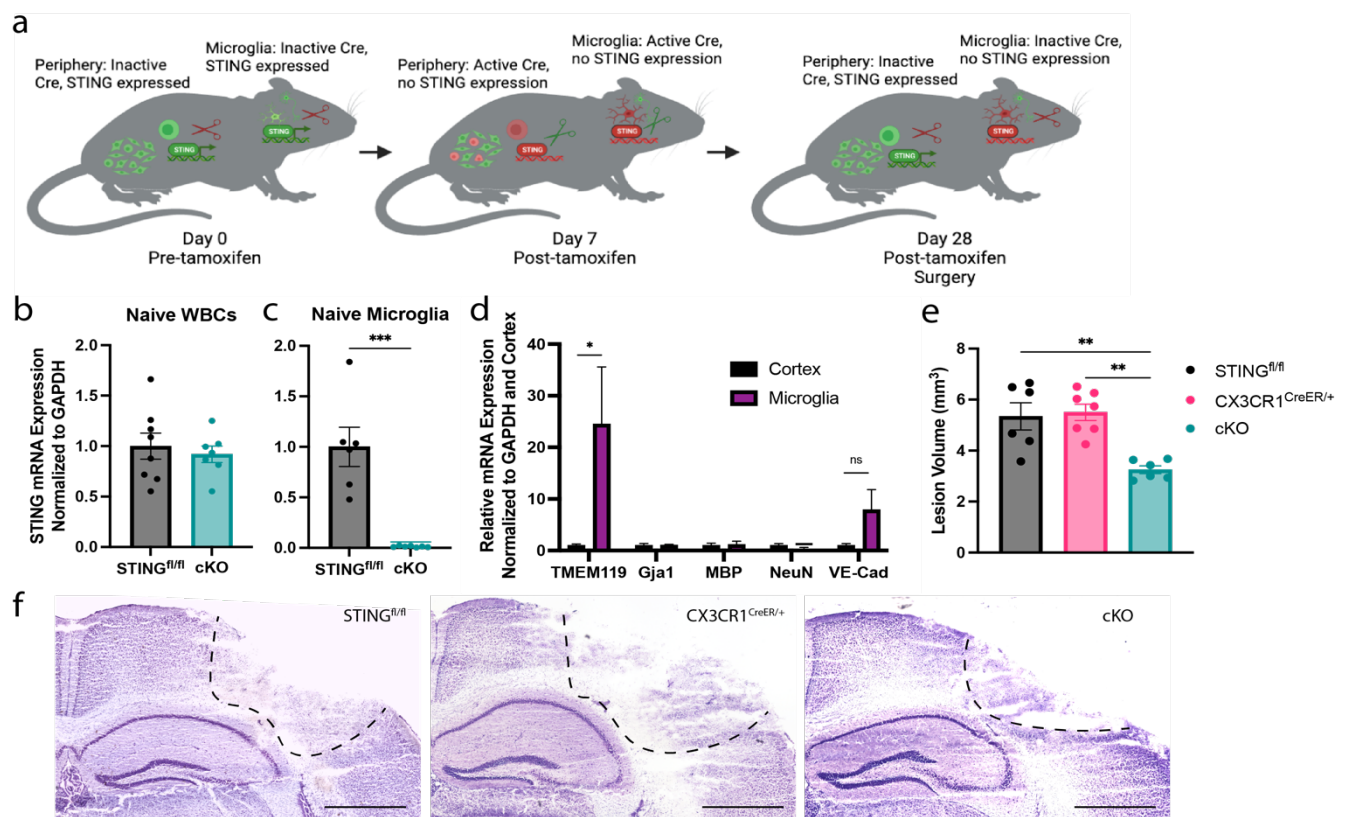
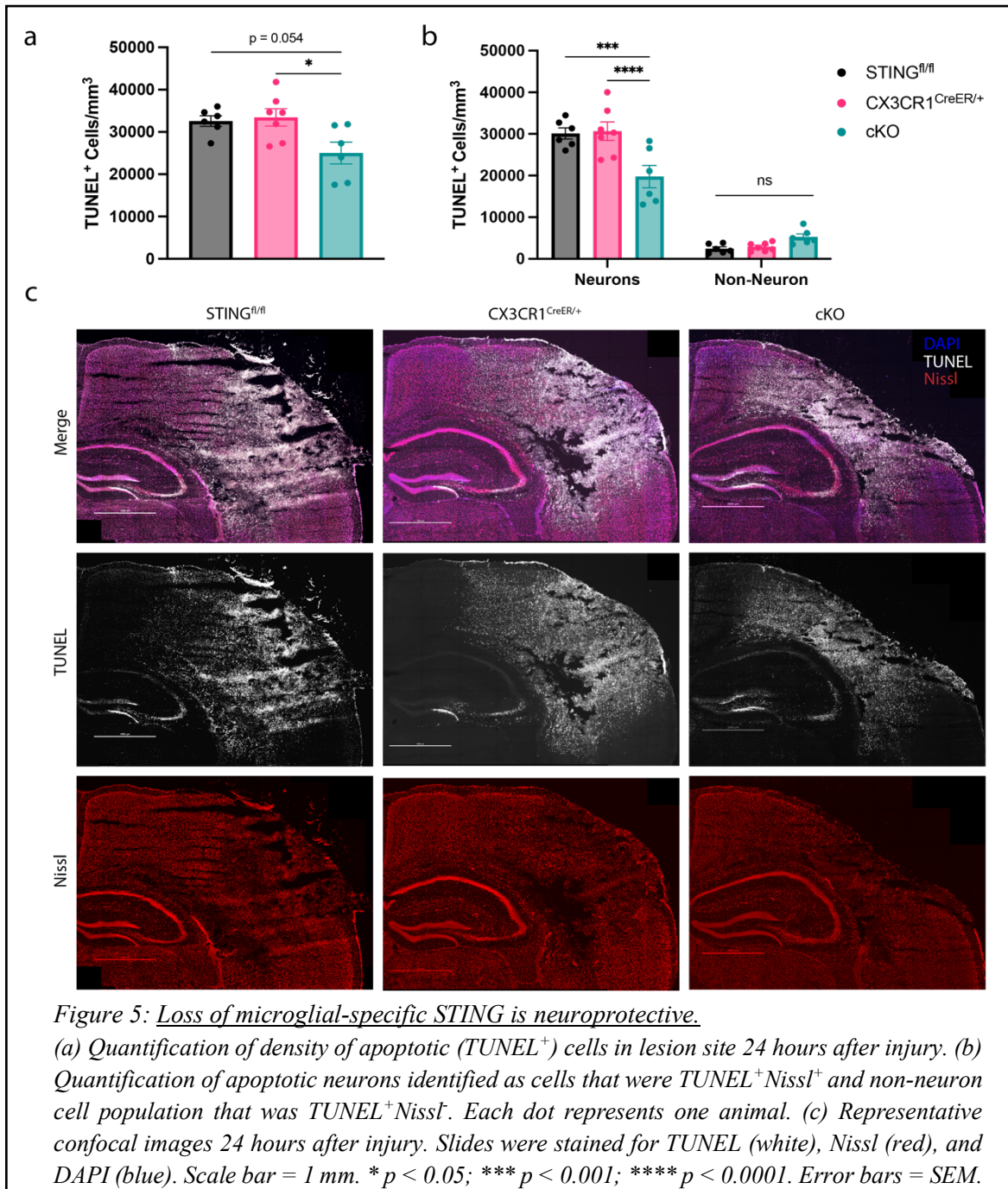


Figure 4: Loss of microglial-specific STING expression reduces lesion size 24 hours after injury.

(a) Model showing development of microglial-specific STING KO animals. All animals received tamoxifen for five consecutive days to remove STING from CX3CR1-expressing cells. Experiments were performed one month after the final dose of tamoxifen was administered. (b) Microglia were isolated from naïve cKO (STING^{fl/fl}, CX3CR1^{CreER/+}) and control STING^{fl/fl} animals. qPCR for STING was used to verify loss of microglial STING. *n* = 6-7/genotype. (c) qPCR for cell-type specific genes was used to confirm microglial purity of samples in (b). Genes represent microglia (TMEM119), astrocytes (Gja1), oligodendrocytes (MBP), neurons (NeuN), and endothelial cells (VE-cadherin). Samples were collected from both genotypes. *n* = 3-5. (d) Quantification of lesion size 24 hours after TBI in STING^{fl/fl}, CX3CR1^{CreER/+}, and cKO (STING^{fl/fl}, CX3CR1^{CreER/+}) animals. Each dot represents one animal. (e) Representative images of sectioned Cresyl violet stained brains 24 hours after injury. Scale bar = 1mm. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. Error bars = SEM.

functional improvement. No significant difference in performance was seen at more chronic timepoints, though this may be because both groups generally have substantial improvement on rotarod testing within one week of injury (Onyszchuk et al. 2007), making differences in performance difficult to detect. Regardless, cKO animals showed significantly reduced lesion size 14 days after TBI when compared to controls, suggesting that loss of microglial STING is beneficial beyond the 4-day timepoint (Figure 6b-c). Together, these data demonstrate that loss of microglial STING confers neuroprotection after brain injury.

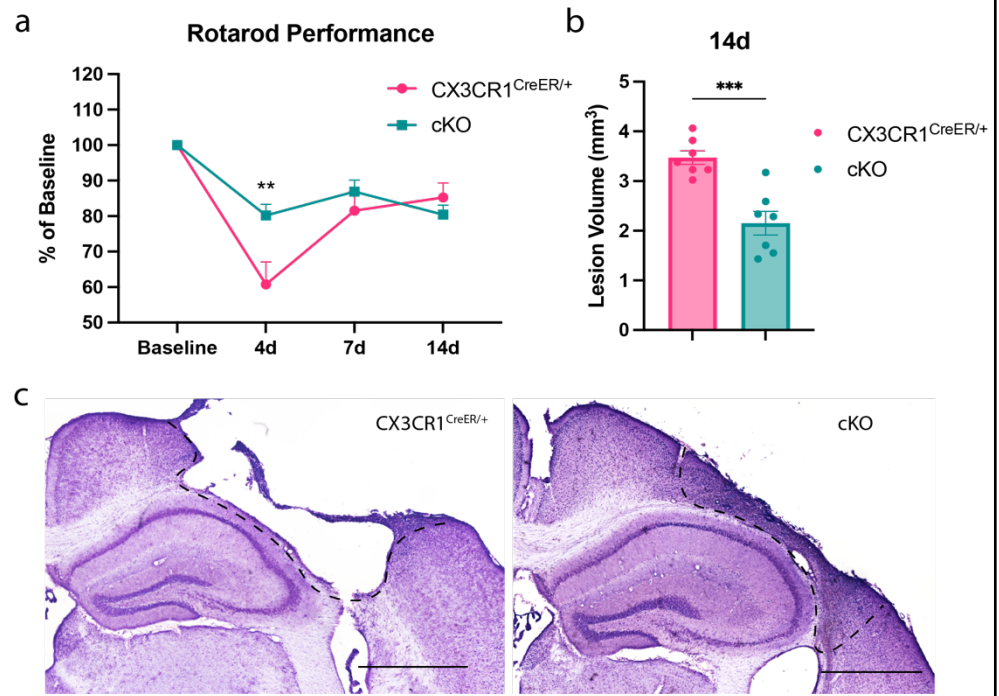


Discussion

Numerous recent studies have demonstrated that the Type I IFN response mediated by cGAS-STING signaling promotes detrimental neuroinflammation in the brain following injury (Fritsch et al. 2022; Abdullah et al. 2018; Barrett et al. 2020; Sen et al. 2020; Karve et al. 2016) and stroke (Kong et al. 2022; Gamdzyk et al. 2020; Peng et al. 2020). However, to date no studies have clarified which cell type(s) are responsible for promoting damage-induced STING-mediated neuroinflammation in the brain. To begin investigating which cell types are responsible for STING signaling in brain injury, we developed bone marrow chimeric animals lacking STING in the brain or peripheral immune cells. Mice lacking STING in either compartment showed reduced lesion size compared to WT^{WTBMC} animals, though there was a small increase in the lesion size in the WT^{KOBMC} group compared to KO^{KOBMC} animals (Figure 1c-d). This increase may indicate that STING signaling in the brain immediately after injury exacerbates tissue damage, but ultimately the combination of microglial and hematopoietic STING is more impactful than the inflammatory response produced by either population on its own. Interestingly, there was no difference in peripheral immune cell infiltration between the global chimeric STING KOs (KO^{KOBMC}) and mice lacking STING in the brain (KO^{WTBMC}) or periphery (WT^{KOBMC}) (Figure 2a-e). There are a number of factors that may influence immune cell infiltration, including permeability in the BBB and chemokine production. BBB disruption is known to enable increased infiltration (Chodobski, Zink, and Szmydynger-Chodobska 2011). IgG deposition was similarly reduced in the KO^{KOBMC}, KO^{WTBMC}, and WT^{KOBMC} groups (Figure 2f-g), suggesting that alterations in the BBB may at least partially explain the comparable reduction in immune cell infiltration. We have previously shown that global STING KO animals show a significant reduction in a number of pro-immune migratory cytokines, such as MCP-1 and CXCL10, in

Figure 6: Loss of microglial STING reduces motor deficits and lesion size after TBI at more chronic timepoints.

(a) Rotarod performance for CX3CR1^{CreER/+} and cKO (STING^{fl/fl}, CX3CR1^{CreER/+}) animals at the indicated timepoints after injury, given as a percentage of baseline. *n* = 10-12/genotype. (b) Quantification of lesion size 14 days after injury. Each dot represents an individual animal. (c) Representative Cresyl violet stained brain sections 14 days after TBI. ** *p* < 0.01; *** *p* < 0.001. Error bars = SEM.



the brain following TBI when compared to WT animals (Fritsch et al. 2022). This would suggest that loss of STING signaling in the brain would result in decreased infiltration of peripheral immune cells due to the blunted chemokine production.

A study evaluating CCR2 deficiency demonstrated considerable cross-talk between circulating monocytes and microglia following brain injury (Somebang et al. 2021). CCR2 is the receptor for CCL2/MCP-1, as well as one of the receptors for CCL7 (Palomino and Marti 2015), chemokines that recruit monocytes into the brain after neurotrauma in both humans (Semple et al. 2010) and rodents (Popiolek-Barczyk et al. 2020). CCR2 KO animals showed reduced peripheral immune cell infiltration, as well as reduced Type I IFN signaling in microglia after experimental TBI (Somebang et al. 2021), suggesting that infiltration of CCR2⁺ cells into the brain facilitates microglial IFN signaling. In support of a detrimental role for infiltration of CCR2⁺ cells after brain injury, another group utilizing bone marrow chimeric CCR2 KO mice showed that animals lacking hematopoietic CCR2 (WT^{KO^{BM}}) had reduced motor deficit compared to WT controls after intracerebral hemorrhage (ICH) (Hammond et al. 2014). The chimeric (WT^{KO^{BM}}) animals also had significantly reduced infiltration of inflammatory monocytes, but no difference in neutrophils (Hammond et al. 2014). The authors concluded that the infiltration of CCR2⁺Ly6C^{hi} inflammatory monocytes was largely responsible for the disability seen after ICH (Hammond et al. 2014). Unexpectedly, our work in BMDMs suggests that while STING activation causes an upregulation in chemokines that bind CCR2, CCR2 itself is unaltered *in vitro* (Figure 3e). This may indicate that activation of STING in peripheral immune cells does not make them more likely to migrate toward chemoattractant signals; however, further study is necessary to properly contextualize these findings. These studies, along with our work, suggests that while microglial IFN and chemokine production facilitates the recruitment of peripheral immune cells, signals from entering immune cells can also further stimulate microglia.

Much of what we know about STING in the brain comes from studies in viruses, yet this data is somewhat conflicting: Some studies of herpes simplex virus 1 (HSV-1) have shown that neurons are the primary cell type responsible for producing viral-suppressing Type I IFNs in HSV-1 infection (Low-Calle, Prada-Arismendy, and Castellanos 2014; Rosato and Leib 2015), while others have demonstrated that microglia are critical for restricting viral spread in the brain (Reinert et al. 2016; Katzilieris-Petras et al. 2022). *In vitro* work investigating rabies virus and vesicular stomatitis virus (VSV) suggests that Type I signaling in primary neurons helps reduce viral load (Chopy et al. 2011; Trottier, Palian, and Reiss 2005). However, RNA sequencing data suggests that microglia are the primary brain cell type expressing STING (Y. Zhang et al. 2016; Bennett et al. 2016). Regardless, considering the mounting evidence of Type I IFN activity in both microglia and neurons, it is likely that both cell types play some role in STING-mediated neuroinflammation. Neuron and astrocytes express the Type I IFN receptor, IFNAR (Y. Zhang et al. 2014; Chhatbar et al. 2018), therefore it is possible that microglia utilize STING-dependent mechanisms to produce Type I IFNs that then bind neuronal IFNAR to prompt inflammatory signaling. Future studies utilizing other cell-type specific STING KO animals would help clarify whether other brain cell types contribute to STING-mediated neuroinflammation in TBI. In order to determine whether microglial STING specifically influences TBI outcome, we developed conditional microglial-specific STING KO animals (STING^{fl/fl} CX3CR1^{CreER/+}, Figure 4a-d). Loss of microglial STING resulted in reduced lesion

size (Figure 4e-f, Figure 6b-c) and cell death (Figure 5), as well as decreased motor deficit (Figure 6a). While these data cannot rule out potential contributions from neurons, astrocytes, or other brain cell types, they do confirm that microglia have a key role in promoting detrimental STING signaling after TBI.

Several questions remain that should be addressed with future studies. First, further characterizing the phenotype of infiltrating immune cells in bone marrow chimeric or conditional knockout animals would be highly informative. The present work shows that animals lacking STING in the brain or periphery have comparable levels of immune infiltration to global chimeric KOs after injury. However, it remains unclear if phenotypic differences exist in the infiltrating cells across groups. Assessing whether the cells are particularly pro- or anti-inflammatory would help clarify whether it is more beneficial to inhibit STING in a particular cell population. Second, a key limitation to this study is the inclusion of only acute timepoints for the bone marrow chimera study. While histological data from the conditional KO animals suggests that loss of STING is beneficial long-term, future study is needed to determine how long STING signaling needs to be blunted to provide protection. Third, while our data suggests a coordinated effort between microglia and peripheral immune cells, how these cells are communicating was not investigated. Additional study into how loss of STING affects this effort would offer mechanistic insight into how this cross-talk occurs. Finally, the goal of this study was to build upon prior studies utilizing global KO animals in order to evaluate the roles of specific cell types. Consequently, only male animals were used. Before we can consider the use of antagonists in the cGAS-STING-IFN pathway, it is critical that these studies be repeated in females.

Taken together, we have shown that both microglia and peripheral immune cells contribute to acute STING-mediated neuroinflammation in neurotrauma. Importantly, these data suggest that limiting peripheral immune cell or microglial STING activation may be beneficial, which could be therapeutically relevant in the future. Of note, a number of small molecule inhibitors for STING are commercially available (Haag et al. 2018), making targeting this pathway in pre-clinical studies possible even if these compounds do not significantly cross the BBB. Our findings confirm that neuroimmune signaling is highly complex, and highlight the need to verify which cell type or types contribute to specific responses.

Material and Methods

Animals

All mice were housed in pathogen-free facility on a 12-hour light/dark cycle at Virginia Tech and provided standard rodent diet and water *ad libitum*. Male C57BL/6J (wildtype, stock no. 000664), C57BL/6J-TMEM173^{g1}/J (*STING*^{-/-}; stock no. 017537) (Sauer et al. 2011), B6;SJL-*Sting*^{tm1.1Camb/J} (*STING*^{fl/fl}; stock no. 031670) (Jin et al. 2011), B6.129P2(Cg)-*Cx3cr1*^{tm2.1(cre/ERT2)Litt/WganJ} (*Cx3CR1*^{CreER/CreER} knockin/knockout with EYFP; stock no. 021160) (Sahasrabudde and Ghosh 2022), and C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ (GFP⁺ WT; stock no. 006567) (Okabe et al. 1997) mice were purchased from Jackson Laboratories (Ellsworth, ME, USA). GFP⁺ *STING*^{-/-} mice were generated by crossing GFP⁺ WT and *STING*^{-/-} mice. Mice were genotyped according to protocols provided by Jackson Laboratories. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under approval of the Virginia Tech Institutional Animal Care and Use Committee.

Generation of microglial-specific STING KO animals

Microglial-specific STING^{-/-} (cKO; STING^{fl/fl}, Cx3CR1^{CreER/+}) animals were generated by crossing Cx3CR1^{CreER/CreER} and STING^{fl/fl} animals. Cx3CR1^{CreER/+} animals have one copy of Cx3CR1 replaced with Cre^{ERT2} and EYFP protein; therefore, these animals were used as controls to confirm that any detected changes were due to loss of microglial STING and not partial loss of Cx3CR1. To activate Cre recombinase, 6-week old male animals received 100mg/kg tamoxifen (Sigma-Aldrich, St. Louis, MO) in corn oil via intraperitoneal injection for 5 consecutive days. Animals underwent CCI surgery one month after the last dose of tamoxifen. Microglia and white blood cells were isolated from 20% of animals at this time point to verify intact peripheral STING and knocked-out microglial STING.

Generation of bone marrow chimeric animals

WT and STING KO mice were X-ray irradiated with two doses of 550 rad spaced 6 hours apart to ablate bone marrow cells. Animals were placed on 1 mg/mL gentamicin sulfate water for three days prior to and two weeks following irradiation. Bone marrow cells from GFP⁺ donor animals were collected by flushing bone marrow and lysing red blood cells before resuspending in sterile PBS. Within 24 hours of the second dose of irradiation, recipient animals were reconstituted with 4 million/each bone marrow cells via tail vein. CCI surgery was performed one month after reconstitution.

Controlled cortical impact (CCI) injury

Male mice age 8-12 weeks were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), then positioned in a stereotactic frame. Body temperature was maintained at 37°C with an autoregulated heating pad. A 4 mm craniotomy was made with a portable drill over the right parietal-temporal cortex (-2.5 mm A/P and 2.0 mm lateral from bregma). Moderate CCI was induced with an eCCI-6.3 device (Custom Design and Fabrication, Richmond, VA, USA) using a 3 mm impact tip at an angle of 70°, 5.0 m/s velocity, 2.0 mm impact depth, and 100 ms dwell period. The incision was closed with Vetbond tissue adhesive (3M, St. Paul, MN, USA), and animals received subcutaneous Buprenorphine SR after surgery (1 mg/kg, ZooPharm, Windsor, CO, USA). Animals were monitored in a heated cage until recovered. Sham animals received a craniotomy only.

Rotarod

Gross motor function was evaluated by Rotarod (Columbus Instruments, Columbus, OH, USA) testing from 4 to 14 days post-TBI. Ten-week old animals were trained for 4 consecutive days, then a baseline measurement was taken on the fifth day using an initial velocity was 5 rpm and an acceleration of 0.1 rpm/s. Each animal underwent four trials per day with a 2-minute rest between each trial. The average time of the four trials was used for analysis. Animals underwent sham or CCI surgery, then rotarod performance was evaluated at 4-, 7-, and 14-days post-surgery. Each animal's performance was compared to its baseline measurement, and average performance for all animals was reported. After the final day of testing, animals were euthanized for histology as described.

Histology and TUNEL staining

Mice were anesthetized by isoflurane (IsoFlo[®], Zoetis, Parsippany-Troy Hills, NJ, USA) and euthanized by cervical dislocation at the indicated time points. Brains were fresh frozen on dry ice and embedded in O.C.T. (Tissue-Plus[™] O.C.T. Compound, Fisher HealthCare, Houston, TX, USA), coronally sectioned (30 μ m thickness) through the lesion site (-1.1 to -2.6 mm posterior to bregma) using a cryostat (CryoStar NX50, Thermo Scientific, Waltham, MA, USA). Serial sections 300 μ m apart were stained with Cresyl violet (Electron Microscopy Sciences, Hatfield, PA, USA).

To identify cells undergoing apoptosis, slides were fixed in 10% formalin (Fisher Chemicals, Pittsburgh, PA) for 5 min, washed with 1X PBS, permeabilized in 2:1 ethanol:acetic acid at -20°C for 10 minutes and 0.4% Triton for 10 minutes, then washed with 1X PBS and TUNEL stained according to the manufacturer's suggestions (DeadEnd[™] Fluorometric TUNEL System, Promega, Madison, WI). To label neurons, slides were blocked in 0.2% Triton, 2% cold water fish gel in PBS for 30 minutes then stained with Nissl (1:100 in block, NeuroTrace[™] 530/615 Red Fluorescence Nissl, Invitrogen, Carlsbad, CA, USA) and mounted with DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Representative images were taken at 20x on a Nikon C2 confocal microscope. Maximum intensity projections were created in Nikon NIS-Elements.

Estimating lesion size and TUNEL⁺/Nissl⁺ cells

StereoInvestigator software (MicroBrightField, Williston, VT, USA) and an Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA, USA) was used by a blinded investigator to estimate lesion size and number of TUNEL⁺/Nissl⁺ cells. Five coronal serial sections spaced 300 μ m apart around the lesion center were used for each animal. For lesion volume (mm³), Cresyl violet stained slides (described above) were visualized at 4x under brightfield illumination using StereoInvestigator's Cavalieri estimator. A grid with 100 μ m spacing was overlaid on the ipsilateral lesion site and markers were placed over contused tissue, identified by reduced staining intensity, abnormal morphology, and pyknotic neurons as previously described (Fritsch et al. 2022). The Cavalieri estimator software used the number of markers, section thickness, section interval, and number of sections to estimate volume of the lesion.

To approximate the number of apoptotic (TUNEL⁺) cells and apoptotic neurons (TUNEL⁺Nissl⁺), StereoInvestigator Optical Fractionator was used with a grid size of 500 x 500 μ m and counting frame of 100 x 100 μ m. A contour was drawn around the lesion site (indicated by high density of TUNEL⁺ cells) at 4x, then approximately 100 randomized sites were evaluated at 60x for each animal. Section thickness was estimated every 5 sites to improve cell count estimate accuracy. The number of cells per contour, average estimated section thickness, section interval, and number of sections were used to estimate the number of cells within the lesion.

Flow cytometry

White blood cells were collected as described and resuspended in PBS with 1% BSA. For evaluating peripheral immune cell infiltration in bone marrow chimera mice, animals were deeply anesthetized with a ketamine (500 mg/kg)/xylazine(10 mg/kg) cocktail and hand perfused with cold PBS

to remove blood. Cortical tissue was rapidly dissected in ice cold PBS and dissociated in papain following the Worthington Papain Dissociation System protocol (Worthington Biochemical Corporation, Lakewood, NJ, USA). Cells were resuspended in PBS with 1% BSA. GFP⁺ single cells were identified with a BD FACSAria Fusion Cell Sorter (BD, Franklin Lakes, NJ, USA) using the 530nm laser. Analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR, USA). Data are given as the percentage of single cells that were GFP⁺.

White blood cell isolation and complete blood counts

Mice were deeply anesthetized with a ketamine (500 mg/kg)/xylazine(10 mg/kg) cocktail and approximately 1mL of whole blood was collected and immediately mixed with 100ul of 500mM EDTA. For complete blood counts, samples were processed by Virginia Tech Animal Laboratory Services (ViTALS; Blacksburg, VA, USA). For white blood cell isolation, red blood cells were lysed with ACK Lysis Buffer (Quality Biological, Gaithersburg, MD, USA) twice, then washed with 1X PBS and resuspended for the appropriate downstream application.

Microglia isolation

Microglia were isolated by plating the cells collected following the Worthington Papain Dissociation System protocol (Worthington Biochemical Corporation, Lakewood, NJ, USA). Briefly, mice were deeply anesthetized with a ketamine (500 mg/kg)/xylazine(10 mg/kg) cocktail and hand perfused with cold PBS to remove blood. Cortical tissue was rapidly dissected in ice cold PBS and dissociated in papain, then resuspended in media. Cells were incubated for one hour at 37°C and non-adherent cells were washed off, leaving microglia adherent to the plate.

Bone marrow derived macrophages

Following euthanasia via isoflurane, femur and tibia bones were isolated from WT and STING KO animals. Bones were cleaned and the ends were cut off, then flushed with complete RPMI containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. Cells were spun down and resuspended in ACK Lysis Buffer (Thermo Scientific, Waltham, MA, USA) to lyse red blood cells and washed with sterile PBS. Three million bone marrow cells were plated per well in six-well tissue culture treated plates with RPMI containing 10 ng/mL M-CSF (PeproTech, Cranbury, NJ, USA). Three days after plating, fresh media was added. Morphology was verified after 6 days in culture, then adherent cells were treated with 50 µg/mL DMXAA (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO or vehicle (DMSO alone) for 3 hours. Cells were washed with cold HBSS, then directly resuspended in TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation.

Real Time qPCR

A 4x4mm section of the injured cortex tissue was micro-dissected from the parietal cortex each animal and immediately submerged in TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Ipsilateral sham cortices or contralateral cortices from injured animals were used as controls. Following mechanical homogenization, mRNA from cortical tissue was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad,

CA, USA) per the manufacturer's protocol. cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), then qPCR was run on the CFX96 System (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA), 10 ng of cDNA and 0.4 mM of each primer set. Each sample was run in technical triplicates, and expression levels were normalized to GAPDH. Fold change was calculated by comparative C_T method (Schmittgen and Livak 2008) using CFX Maestro software (Bio-Rad, Hercules, CA, USA). Primer efficiency was determined using a 4-point log concentration curve (Bio-Rad, Hercules, CA, USA).

Gene	Forward Seq. (5' - 3')	Reverse Seq. (5' - 3')
CCL7	GTC CCT GGG AAG CTG TTA TCT TCA	TGC TAT AGC CTC CTC GAC CC
CCR2	GGG CTG TGA GGC TCA TCT TT	TGC ATG GCC TGG TCT AAG TG
GAPDH	ATT GTG TCC GTC GTG GAT CTG A	AGA TGC CTG CTT CAC CAC CTT CTT
Gja1	CGG AAG CAC CAT CTC CAA CT	CCA CGA TAG CTA AGG GCT GG
IFNB1	AAC TCC ACC AGC AGA CAG TG	GGT ACC TTT GCA CCC TCC AG
MCP1	TCA CCT GCT GCT ACT CAT TCA CCA	TAC AGC TTC TTT GGG ACA CCT GCT
MBP	TCA CGA CCC CGG AAC ATA GT	TGG GGT GTT CAA GAG TGG TG
NeuN	CAC TCT CTT GTC CGT TTG CTT C	CAC TCT CTT GTC CGT TTG CTT C
STING	GCC TTC AGA GCT TGA CTC CA	GTA CAG TCT TCG GCT CCC TG
TMEM119	AGA CCC TTC TGC TTC CCC TT	CCC AGT ATG TGG GGT CAC TG
VE-Cadherin	AGG ACA GCA ACT TCA CCC TCA	AAC TGC CCA TAC TTG ACC GTG

Statistical Analysis

Data were analyzed with GraphPad Prism 9 (GraphPad, San Diego, CA, USA). A Student's two-tailed t-test was used for comparison of two groups, and a one-way or two-way ANOVA with Tukey's multiple comparison test was used to compare more than two groups as appropriate. Differences were considered statistically significant at $p < 0.05$. Data are reported as mean $\pm$ SEM with n values given in the figure legend.

Author Contributions

L.E.F. wrote the first draft, performed experiments. and analyzed data. N.D., C.K., S.P., H.O., and J.J performed experiments and analyzed data. A.M.P. designed the project, performed experiments, analyzed data, and wrote the manuscript. All authors read, edited, and approved the manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

Acknowledgements

This work was supported by the National Institutes of Health (R35GM142368) and Commonwealth Heath Research Board (#208-05-20) (AMP).

References

Abdullah, Amar, Moses Zhang, Tony Frugier, Sammy Bedoui, Juliet M. Taylor, and Peter J. Crack. 2018. "STING-Mediated Type-I Interferons Contribute to the Neuroinflammatory Process and

- Detrimental Effects Following Traumatic Brain Injury.” *Journal of Neuroinflammation* 15 (1). <https://doi.org/10.1186/S12974-018-1354-7>.
- Ablasser, Andrea, Marion Goldeck, Taner Cavlar, Tobias Deimling, Gregor Witte, Ingo Röhl, Karl Peter Hopfner, Janos Ludwig, and Veit Hornung. 2013. “CGAS Produces a 2’-5’-Linked Cyclic Dinucleotide Second Messenger That Activates STING.” *Nature* 498 (7454): 380–84. <https://doi.org/10.1038/NATURE12306>.
- Ahn, Jeonghyun, Tianli Xia, Hiroyasu Konno, Keiko Konno, Phillip Ruiz, and Glen N. Barber. 2014. “Inflammation-Driven Carcinogenesis Is Mediated through STING.” *Nature Communications* 2014 5:1 5 (1): 1–9. <https://doi.org/10.1038/ncomms6166>.
- Barrett, James P., Rebecca J. Henry, Kari Ann Shirey, Sarah J. Doran, Oleg D. Makarevich, Rodney R. Ritzel, Victoria A. Meadows, et al. 2020. “Interferon- β Plays a Detrimental Role in Experimental Traumatic Brain Injury by Enhancing Neuroinflammation That Drives Chronic Neurodegeneration.” *The Journal of Neuroscience*, February, 2516–19. <https://doi.org/10.1523/jneurosci.2516-19.2020>.
- Bennett, Mariko L., F. Chris Bennett, Shane A. Liddelow, Bahareh Ajami, Jennifer L. Zamanian, Nathaniel B. Fernhoff, Sara B. Mulinyawe, et al. 2016. “New Tools for Studying Microglia in the Mouse and Human CNS.” *Proceedings of the National Academy of Sciences* 113 (12): E1738–46. <https://doi.org/10.1073/pnas.1525528113>.
- Chhatbar, Chintan, Claudia N. Detje, Elena Grabski, Katharina Borst, Julia Spanier, Luca Ghita, David A. Elliott, et al. 2018. “Type I Interferon Receptor Signaling of Neurons and Astrocytes Regulates Microglia Activation during Viral Encephalitis.” *Cell Reports* 25 (1): 118-129.e4. <https://doi.org/10.1016/J.CELREP.2018.09.003>.
- Chodobski, Adam, Brian J. Zink, and Joanna Szmydynger-Chodobska. 2011. “Blood-Brain Barrier Pathophysiology in Traumatic Brain Injury.” *Translational Stroke Research* 2 (4): 492. <https://doi.org/10.1007/S12975-011-0125-X>.
- Chopy, Damien, Claudia N. Detje, Mireille Lafage, Ulrich Kalinke, and Monique Lafon. 2011. “The Type I Interferon Response Bridges Rabies Virus Infection and Reduces Pathogenicity.” *Journal of Neurovirology* 17 (4): 353–67. <https://doi.org/10.1007/S13365-011-0041-6>.
- Civril, Filiz, Tobias Deimling, Carina C. De Oliveira Mann, Andrea Ablasser, Manuela Moldt, Gregor Witte, Veit Hornung, and Karl Peter Hopfner. 2013. “Structural Mechanism of Cytosolic DNA Sensing by CGAS.” *Nature* 498 (7454): 332. <https://doi.org/10.1038/NATURE12305>.
- Deshmane, Satish L., Sergey Kremlev, Shohreh Amini, and Bassel E. Sawaya. 2009. “Monocyte Chemoattractant Protein-1 (MCP-1): An Overview.” *Journal of Interferon & Cytokine Research* 29 (6): 313. <https://doi.org/10.1089/JIR.2008.0027>.
- Dewan, Michael C., Abbas Rattani, Saksham Gupta, Ronnie E. Baticulon, Ya Ching Hung, Maria Punchak, Amit Agrawal, et al. 2018. “Estimating the Global Incidence of Traumatic Brain Injury.” *Journal of Neurosurgery* 130 (4): 1080–97. <https://doi.org/10.3171/2017.10.JNS17352>.
- Donnelly, Christopher R., Changyu Jiang, Amanda S. Andriessen, Kaiyuan Wang, Zilong Wang, Huiping Ding, Junli Zhao, et al. 2021. “STING Controls Nociception via Type I Interferon Signalling in Sensory Neurons.” *Nature* 591 (7849): 275–80. <https://doi.org/10.1038/S41586-020->

03151-1.

- Fritsch, Lauren E., Jing Ju, Erwin Kristobal Gudenschwager Basso, Eman Soliman, Swagatika Paul, Jiang Chen, Alexandra M. Kaloss, et al. 2022. "Type I Interferon Response Is Mediated by NLRX1-CGAS-STING Signaling in Brain Injury." *Frontiers in Molecular Neuroscience* 0 (February): 53. <https://doi.org/10.3389/FNMOL.2022.852243>.
- Frugier, Tony, Maria Cristina Morganti-Kossmann, David O'Reilly, and Catriona A. McLean. 2010. "In Situ Detection of Inflammatory Mediators in Post Mortem Human Brain Tissue after Traumatic Injury." *Journal of Neurotrauma* 27 (3): 497–507. <https://doi.org/10.1089/neu.2009.1120>.
- Gamdzyk, Marcin, Desislava Met Doycheva, Camila Araujo, Umut Ocak, Yujie Luo, Jiping Tang, and John H. Zhang. 2020. "CGAS/STING Pathway Activation Contributes to Delayed Neurodegeneration in Neonatal Hypoxia-Ischemia Rat Model: Possible Involvement of LINE-1." *Molecular Neurobiology* 57 (6): 2600–2619. <https://doi.org/10.1007/S12035-020-01904-7>.
- Grassivaro, Francesca, Ramesh Menon, Massimo Acquaviva, Linda Ottoboni, Francesca Ruffini, X. Andrea Bergamaschi, Luca Muzio, Cinthia Farina, and X. Gianvito Martino. 2020. "Convergence between Microglia and Peripheral Macrophages Phenotype during Development and Neuroinflammation." *Journal of Neuroscience* 40 (4): 784–95. <https://doi.org/10.1523/JNEUROSCI.1523-19.2019>.
- Gyoneva, Stefka, and Richard M. Ransohoff. 2015. "Inflammatory Reaction after Traumatic Brain Injury: Therapeutic Potential of Targeting Cell-Cell Communication by Chemokines." *Trends in Pharmacological Sciences* 36 (7): 471. <https://doi.org/10.1016/J.TIPS.2015.04.003>.
- Haag, Simone M., Muhammet F. Gulen, Luc Reymond, Antoine Gibelin, Laurence Abrami, Alexiane Decout, Michael Heymann, et al. 2018. "Targeting STING with Covalent Small-Molecule Inhibitors." *Nature* 559 (7713): 269–73. <https://doi.org/10.1038/s41586-018-0287-8>.
- Hammond, Matthew D., Roslyn A. Taylor, Michael T. Mullen, Youxi Ai, Hector L. Aguila, Matthias Mack, Scott E. Kasner, Louise D. McCullough, and Lauren H. Sansing. 2014. "CCR2+Ly6Chi Inflammatory Monocyte Recruitment Exacerbates Acute Disability Following Intracerebral Hemorrhage." *The Journal of Neuroscience* 34 (11): 3901. <https://doi.org/10.1523/JNEUROSCI.4070-13.2014>.
- Harding, Shane M., Joseph L. Benci, Jerome Irianto, Dennis E. Discher, Andy J. Minn, and Roger A. Greenberg. 2017. "Mitotic Progression Following DNA Damage Enables Pattern Recognition within Micronuclei." *Nature* 548 (7668): 466–70. <https://doi.org/10.1038/nature23470>.
- Ishikawa, Hiroki, and Glen N. Barber. 2008. "STING Is an Endoplasmic Reticulum Adaptor That Facilitates Innate Immune Signalling." *Nature* 455 (7213): 674–78. <https://doi.org/10.1038/nature07317>.
- Ishikawa, Hiroki, Zhe Ma, and Glen N. Barber. 2009. "STING Regulates Intracellular DNA-Mediated, Type I Interferon-Dependent Innate Immunity." *Nature* 2009 461:7265 461 (7265): 788–92. <https://doi.org/10.1038/nature08476>.
- Ivashkiv, Lionel B., and Laura T. Donlin. 2014. "Regulation of Type I Interferon Responses." *Nature Reviews Immunology* 14 (1): 36–49. <https://doi.org/10.1038/nri3581>.
- Jassam, Yasir N., Saef Izzy, Michael Whalen, Dorian B. McGavern, and Joseph El Khoury. 2017.

- “Neuroimmunology of Traumatic Brain Injury: Time for a Paradigm Shift.” *Neuron*. Cell Press. <https://doi.org/10.1016/j.neuron.2017.07.010>.
- Jin, Lei, Krista K. Hill, Holly Filak, Jennifer Mogan, Heather Knowles, Bicheng Zhang, Anne-Laure Perraud, John C. Cambier, and Laurel L. Lenz. 2011. “MPYS Is Required for IFN Response Factor 3 Activation and Type I IFN Production in the Response of Cultured Phagocytes to Bacterial Second Messengers Cyclic-Di-AMP and Cyclic-Di-GMP.” *Journal of Immunology (Baltimore, Md. : 1950)* 187 (5): 2595–2601. <https://doi.org/10.4049/JIMMUNOL.1100088>.
- Jung, Steffen, Julio Aliberti, Petra Graemmel, Mary Jean Sunshine, Georg W. Kreutzberg, Alan Sher, and Dan R. Littman. 2000. “Analysis of Fractalkine Receptor CX 3 CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion .” *Molecular and Cellular Biology* 20 (11): 4106–14. <https://doi.org/10.1128/MCB.20.11.4106-4114.2000/ASSET/5CCE0B3C-74FB-405E-A09B-AB7215CC96D5/ASSETS/GRAPHIC/MB110036705B.JPEG>.
- Karve, Ila P., Moses Zhang, Mark Habgood, Tony Frugier, Kate M. Brody, Maithili Sashindranath, C. Joakim Ek, et al. 2016. “Ablation of Type-I IFN Signaling in Hematopoietic Cells Confers Protection Following Traumatic Brain Injury.” *ENeuro* 3 (1): 89–93. <https://doi.org/10.1523/ENEURO.0128-15.2016>.
- Katzilieris-Petras, Georgios, Xin Lai, Ahmad S. Rashidi, Georges M. G. M. Verjans, Line S. Reinert, and Søren R. Paludan. 2022. “Microglia Activate Early Antiviral Responses upon Herpes Simplex Virus 1 Entry into the Brain to Counteract Development of Encephalitis-Like Disease in Mice.” *Journal of Virology* 96 (6). <https://doi.org/10.1128/JVI.01311-21/ASSET/73DB7756-6384-4815-B69F-48962E2D5019/ASSETS/IMAGES/LARGE/JVI.01311-21-F008.JPG>.
- Kong, Lingqi, Wenyu Li, E. Chang, Wuxuan Wang, Nan Shen, Xiang Xu, Xinyue Wang, et al. 2022. “MtDNA-STING Axis Mediates Microglial Polarization via IRF3/NF-KB Signaling After Ischemic Stroke.” *Frontiers in Immunology* 13 (April): 1538. <https://doi.org/10.3389/FIMMU.2022.860977/BIBTEX>.
- Liu, Yin, Adriana A. Jesus, Bernadette Marrero, Dan Yang, Suzanne E. Ramsey, Gina A. Montealegre Sanchez, Klaus Tenbrock, et al. 2014. “Activated STING in a Vascular and Pulmonary Syndrome.” *New England Journal of Medicine* 371 (6): 507–18. <https://doi.org/10.1056/nejmoa1312625>.
- Low-Calle, Ana Maria, Jeanette Prada-Arismendy, and Jaime E. Castellanos. 2014. “Study of Interferon- β Antiviral Activity against Herpes Simplex Virus Type 1 in Neuron-Enriched Trigeminal Ganglia Cultures.” *Virus Research* 180 (February): 49–58. <https://doi.org/10.1016/J.VIRUSRES.2013.12.022>.
- Maekawa, Hiroshi, Tsuyoshi Inoue, Haruki Ouchi, Tzu Ming Jao, Reiko Inoue, Hiroshi Nishi, Rie Fujii, et al. 2019. “Mitochondrial Damage Causes Inflammation via CGAS-STING Signaling in Acute Kidney Injury.” *Cell Reports*. <https://doi.org/10.1016/j.celrep.2019.09.050>.
- Mathur, Vidhu, Ritwik Burai, Ryan T. Vest, Liana N. Bonanno, Benoit Lehallier, Macy E. Zardeneta, Karishma N. Mistry, et al. 2017. “Activation of the STING-Dependent Type I Interferon Response Reduces Microglial Reactivity and Neuroinflammation.” *Neuron*. <https://doi.org/10.1016/j.neuron.2017.11.032>.
- O’Connell, Karyn E., Amy M. Mikkola, Aaron M. Stepanek, Andyna Vernet, Christopher D. Hall, Chia

- C. Sun, Eda Yildirim, John F. Staropoli, Jeannie T. Lee, and Diane E. Brown. 2015. “Practical Murine Hematopathology: A Comparative Review and Implications for Research.” *Comparative Medicine* 65 (2): 96. /pmc/articles/PMC4408895/.
- Okabe, Masaru, Masahito Ikawa, Katsuya Kominami, Tomoko Nakanishi, and Yoshitake Nishimune. 1997. “‘Green Mice’ as a Source of Ubiquitous Green Cells.” *FEBS Letters* 407 (3): 313–19. [https://doi.org/10.1016/S0014-5793\(97\)00313-X](https://doi.org/10.1016/S0014-5793(97)00313-X).
- Onyszchuk, Gregory, Baraa Al-Hafez, Yong Yue He, Mehmet Bilgen, Nancy E.J. Berman, and William M. Brooks. 2007. “A Mouse Model of Sensorimotor Controlled Cortical Impact: Characterization Using Longitudinal Magnetic Resonance Imaging, Behavioral Assessments and Histology.” *Journal of Neuroscience Methods* 160 (2): 187–96. <https://doi.org/10.1016/J.JNEUMETH.2006.09.007>.
- Palomino, Diana Carolina Torres, and Luciana C.avalheiro Marti. 2015. “Chemokines and Immunity.” *Einstein (Sao Paulo, Brazil)* 13 (3): 469–73. <https://doi.org/10.1590/S1679-45082015RB3438>.
- Peng, Yucong, Jianfeng Zhuang, Guangyu Ying, Hanhai Zeng, Hang Zhou, Yang Cao, Huaijun Chen, et al. 2020. “Stimulator of IFN Genes Mediates Neuroinflammatory Injury by Suppressing AMPK Signal in Experimental Subarachnoid Hemorrhage.” *Journal of Neuroinflammation* 17 (1): 1–20. <https://doi.org/10.1186/S12974-020-01830-4/FIGURES/10>.
- Popiolek-Barczyk, Katarzyna, Agata Ciechanowska, Katarzyna Ciapała, Katarzyna Pawlik, Marco Oggioni, Domenico Mercurio, Maria Grazia De Simoni, and Joanna Mika. 2020. “The CCL2/CCL7/CCL12/CCR2 Pathway Is Substantially and Persistently Upregulated in Mice after Traumatic Brain Injury, and CCL2 Modulates the Complement System in Microglia.” *Molecular and Cellular Probes* 54 (December): 101671. <https://doi.org/10.1016/J.MCP.2020.101671>.
- Reinert, Line S., Katarína Lopusná, Henriette Winther, Chenglong Sun, Martin K. Thomsen, Ramya Nandakumar, Trine H. Mogensen, et al. 2016. “Sensing of HSV-1 by the CGAS-STING Pathway in Microglia Orchestrates Antiviral Defence in the CNS.” *Nature Communications* 7 (1): 1–12. <https://doi.org/10.1038/ncomms13348>.
- Réu, Pedro, Azadeh Khosravi, Samuel Bernard, Jeff E. Mold, Mehran Salehpour, Kanar Alkass, Shira Perl, et al. 2017. “The Lifespan and Turnover of Microglia in the Human Brain.” *Cell Reports* 20 (4): 779. <https://doi.org/10.1016/J.CELREP.2017.07.004>.
- Rongvaux, Anthony, Ruaidhrí Jackson, Christian C.D. C.D. Harman, Tuo Li, A. Phillip Phillip West, Marcel R. de Zoete, Youtong Wu, et al. 2014. “Apoptotic Caspases Prevent the Induction of Type I Interferons by Mitochondrial DNA.” *Cell* 159 (7): 1563–77. <https://doi.org/10.1016/j.cell.2014.11.037>.
- Roozenbeek, Bob, Andrew I. R. Maas, and David K. Menon. 2013. “Changing Patterns in the Epidemiology of Traumatic Brain Injury.” *Nature Reviews Neurology* 9 (4): 231–36. <https://doi.org/10.1038/nrneurol.2013.22>.
- Rosato, Pamela C., and David A. Leib. 2015. “Neuronal Interferon Signaling Is Required for Protection against Herpes Simplex Virus Replication and Pathogenesis.” *PLOS Pathogens* 11 (7): e1005028. <https://doi.org/10.1371/JOURNAL.PPAT.1005028>.
- Sahasrabudhe, Vinaya, and Hiya Singhee Ghosh. 2022. “Cx3Cr1-Cre Induction Leads to Microglial

- Activation and IFN-1 Signaling Caused by DNA Damage in Early Postnatal Brain.” *Cell Reports* 38 (3): 110252. <https://doi.org/10.1016/J.CELREP.2021.110252>.
- Sauer, John Demian, Katia Sotelo-Troha, Jakob Von Moltke, Kathryn M. Monroe, Chris S. Rae, Sky W. Brubaker, Mamoru Hyodo, et al. 2011. “The N-Ethyl-N-Nitrosourea-Induced Goldenticket Mouse Mutant Reveals an Essential Function of Sting in the in Vivo Interferon Response to *Listeria Monocytogenes* and Cyclic Dinucleotides.” *Infection and Immunity* 79 (2): 688–94. <https://doi.org/10.1128/IAI.00999-10>.
- Schmittgen, Thomas D, and Kenneth J Livak. 2008. “Analyzing Real-Time PCR Data by the Comparative CT Method.” *Nature Protocols* 3 (6): 1101–8. <https://doi.org/10.1038/nprot.2008.73>.
- Semple, Bridgette D., Nicole Bye, Mario Rancan, Jenna M. Ziebell, and M. Cristina Morganti-Kossmann. 2010. “Role of CCL2 (MCP-1) in Traumatic Brain Injury (TBI): Evidence from Severe TBI Patients and CCL2–/– Mice.” *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism* 30 (4): 769. <https://doi.org/10.1038/JCBFM.2009.262>.
- Sen, Tanusree, Pampa Saha, Rajaneesh Gupta, Lesley M. Foley, Tong Jiang, Olena S. Abakumova, T. Kevin Hitchens, and Nilkantha Sen. 2020. “Aberrant ER Stress Induced Neuronal-IFN β Elicits White Matter Injury Due to Microglial Activation and T-Cell Infiltration after TBI.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 40 (2): 424–46. <https://doi.org/10.1523/JNEUROSCI.0718-19.2019>.
- Simon, Dennis W, Mandy J McGeachy, Hülya Bayır, Robert S B Clark, David J Loane, and Patrick M Kochanek. 2017. “The Far-Reaching Scope of Neuroinflammation after Traumatic Brain Injury.” *Nature Reviews. Neurology* 13 (3): 171–91. <https://doi.org/10.1038/nrneurol.2017.13>.
- Somebang, Kerri, Joshua Rudolph, Isabella Imhof, Luyi Li, Erene C. Niemi, Judy Shigenaga, Huy Tran, et al. 2021. “CCR2 Deficiency Alters Activation of Microglia Subsets in Traumatic Brain Injury.” *Cell Reports* 36 (12): 109727. <https://doi.org/10.1016/J.CELREP.2021.109727>.
- Sun, Lijun, Jiaxi Wu, Fenghe Du, Xiang Chen, and Zhijian J Chen. 2013. “Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway.” *Science (New York, N.Y.)* 339 (6121): 786–91. <https://doi.org/10.1126/science.1232458>.
- Thim-uam, Arthid, Thaneas Prabakaran, Mookmanee Tansakul, Jiradej Makjaroen, Piriya Wongkongkathap, Naphat Chantaravisoot, Thammakorn Saethang, et al. 2020. “STING Mediates Lupus via the Activation of Conventional Dendritic Cell Maturation and Plasmacytoid Dendritic Cell Differentiation.” *IScience* 23 (9): 101530. <https://doi.org/10.1016/j.isci.2020.101530>.
- Trottier, Mark D., Beth M. Palian, and Carol Shoshkes Reiss. 2005. “VSV Replication in Neurons Is Inhibited by Type I IFN at Multiple Stages of Infection.” *Virology* 333 (2): 215–25. <https://doi.org/10.1016/J.VIROL.2005.01.009>.
- West, A. Phillip, William Khoury-Hanold, Matthew Staron, Michal C. Tal, Cristiana M. Pineda, Sabine M. Lang, Megan Bestwick, et al. 2015. “Mitochondrial DNA Stress Primes the Antiviral Innate Immune Response.” *Nature* 520 (7548): 553–57. <https://doi.org/10.1038/NATURE14156>.
- White, Michael J. J., Kate McArthur, Donald Metcalf, Rachael M. M. Lane, John C. C. Cambier, Marco J. J. Herold, Mark F. van Delft, et al. 2014. “Apoptotic Caspases Suppress MtDNA-Induced

STING-Mediated Type I IFN Production.” *Cell* 159 (7): 1549–62.

<https://doi.org/10.1016/j.cell.2014.11.036>.

Wu, Jiayi, Lijun Sun, Xiang Chen, Fenghe Du, Heping Shi, Chuo Chen, and Zhijian J. Chen. 2013.

“Cyclic-GMP-AMP Is An Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA.” *Science (New York, N.Y.)* 339 (6121): 826–30.

<https://doi.org/10.1126/SCIENCE.1229963>.

Zhang, Xu, Heping Shi, Jiayi Wu, Xuewu Zhang, Lijun Sun, Chuo Chen, and Zhijian J. Chen. 2013.

“Cyclic GMP-AMP Containing Mixed Phosphodiester Linkages Is An Endogenous High Affinity Ligand for STING.” *Molecular Cell* 51 (2): 226–35.

<https://doi.org/10.1016/J.MOLCEL.2013.05.022>.

Zhang, Ye, Kenian Chen, Steven A. Sloan, Mariko L. Bennett, Anja R. Scholze, Sean O’Keeffe, Hemali

P. Phatnani, et al. 2014. “An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 34 (36): 11929–47.

<https://doi.org/10.1523/JNEUROSCI.1860-14.2014>.

Zhang, Ye, Steven A. Sloan, Laura E. Clarke, Christine Caneda, Colton A. Plaza, Paul D. Blumenthal,

Hannes Vogel, et al. 2016. “Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse.” *Neuron* 89 (1): 37–

53. <https://doi.org/10.1016/J.NEURON.2015.11.013/ATTACHMENT/52207ACF-50BF-40FC-B0B3-B547B21C06FE/MMC5.XLSX>.

Chapter 5

Summary and Future Directions

Summary of Findings

Despite how common traumatic brain injuries are, there remains much to learn about how the brain-resident and peripheral immune system contributes to secondary injury. Several factors play a substantial role in the neuroinflammatory response to TBI, including cytokine signaling in the brain and the infiltration of peripheral immune cells. In this work, we have identified the cGAS-STING signaling pathway as a key mediator of this response following a mouse model of TBI. The following are the key findings presented in this dissertation:

(1) We show that the global loss of either cGAS or STING results in substantial neuroprotection. Mice lacking either gene have reduced cytokine production, lesion size, and cell death 24 hours after injury. Furthermore, loss of cGAS reduces motor deficits, indicating that the changes seen at 24 hours contribute to functional improvement.

(2) We provide evidence that mitochondrial DNA is present in the cytosol of injured cortical cells, suggesting that mtDNA may be the trigger for cGAS-STING signaling in brain injury.

(3) We identified an additional protein, NLRX1, that appears to regulate STING signaling in TBI. This gives an additional target for future therapeutics focused in this pathway.

(4) We show that both microglia and peripheral immune cells play a role in detrimental STING signaling after injury. Loss of STING in either cell population confers neuroprotection and reduces immune cell infiltration.

(5) We determine that loss of STING specifically in microglia is beneficial. Animals lacking microglial-STING have smaller lesion volumes and less neuronal cell death 24 hours after injury. In support of our findings in global KO animals, these animals also show reduced motor deficits.

Taken together, we conclude that peripheral immune cells and microglia contribute to detrimental STING signaling following brain injury. Broadly speaking, the loss of STING results in reduced lesion size, cell death, inflammatory cytokine production, and motor deficits. This work suggests that inhibiting cGAS-STING signaling following TBI may be beneficial; however, future study is needed to develop a more complete understanding of how we could target this complex neuroinflammatory pathway.

Future Directions

Substantial work is still needed to understand the mechanisms of STING activation and signaling in the context of brain trauma. First, while our work suggests that mtDNA may be

responsible for activation of cGAS, this evidence is indirect and only confirms the presence of mtDNA. This could be addressed more directly through the use of chromatin immunoprecipitation (ChIP); however, the lack of suitable cGAS antibodies makes this extremely challenging. To combat this, we attempted to utilize HA-flagged cGAS, but numerous technical challenges prevented this method from working. One alternative is tagging endogenous cGAS with a fluorescent protein using a transgenic mouse model. cGAS^{fl/fl} mice are commercially available; therefore, a mouse line expressing GFP-cGAS could be developed. This may allow for ChIP-sequencing studies that can directly test whether cGAS binds mtDNA after TBI. However, this method is costly, in terms of both time and finances. The development of better cGAS antibodies for both immunofluorescence and ChIP would be extremely helpful in providing stronger *in vivo* evidence for cGAS-mtDNA interactions.

The work presented in Chapter 4 concludes that both microglia and peripheral immune cells contribute to STING signaling in TBI. While our data in microglial-specific STING KO animals confirms that microglia are critical for this response, we were unable to completely rule out contributions from neurons or other CNS cell types. The use of additional conditional KO models would help clarify if neurons also play a role in STING-IFN signaling. Furthermore, we do not yet know exactly how microglia and peripheral immune cells coordinate the IFN response. Microglia could produce IFNs that bind peripheral immune IFNARs, or vice versa. While STING activation results in production of IFNs and ISGs, IFN signaling occurs independently of STING. However, that loss of STING specifically is beneficial suggests that both populations are producing Type I IFNs through STING which could bind IFNARs on any number of different cell types to exacerbate inflammation. Given that the loss of IFNAR on hematopoietic cells, but not brain-resident cells, is beneficial in TBI (Karve et al. 2016), it seems most likely microglia are primarily responsible for producing Type I IFNs through STING that bind hematopoietic IFNAR. This response is then amplified by additional STING signaling in peripheral immune cells, resulting in high levels of inflammation. Future study could utilize a combination of chimeric STING KO animals with IFNAR-blocking antibodies to help confirm or refute this hypothesis.

From the perspective of therapeutic development, several questions remain to be addressed. Perhaps most importantly, the presented work was only done in male animals. However, there are known differences in the immune systems of males and females (Späni, Braun, and Van Eldik 2018; Gupte et al. 2019). Before we consider targeting the cGAS-STING-IFN pathway as a therapeutic, we need to confirm these findings in females. Furthermore, the most chronic timepoint assessed in this work was two weeks post-TBI. Assessment of the effects several months after injury would be helpful in determining if these benefits seen acutely correspond to improvement more chronically. Finally, better understanding the mechanism of activation and cross-talk between microglia and peripheral immune cells is critical for determining how to target STING signaling. At present, it remains unclear how effectively small molecule STING antagonists cross the blood brain barrier. While the BBB is compromised in TBI, the level of permeability is

dependent upon injury severity. If STING antagonists are to be considered for treatment of mild TBI, we must clarify (a) if targeting peripheral immune cells alone is sufficiently beneficial, and (b) how well these, or other, molecules cross the BBB. Despite these unknowns, this work suggests that inhibition of STING-IFN, either directly or through a mediator such as NLRX1, may be a promising therapeutic target for brain injury.

Concluding Remarks

As discussed in Chapter 2, this work is not the first to investigate the role of cGAS-STING-IFN signaling in the context of neurological insult, which only confirms the importance of our findings. In the TBI field, several groups have investigated the effects of loss of STING (Abdullah et al. 2018), IFN β (Barrett et al. 2020; Sen et al. 2020), and INFAR (Karve et al. 2016) through genetic methods, whereas the stroke field has largely utilized small molecule inhibitors of cGAS (Ding et al. 2022; Li et al. 2020; Gamdzyk et al. 2020) and STING (Kong et al. 2022; Peng et al. 2020). To date, only one study has specifically addressed the effects of loss of IFN signaling in brain-resident versus peripheral immune cells (Karve et al. 2016), and no studies have studied the global loss of cGAS in TBI. Thus, this work fills two important gaps in the current knowledge of STING-IFN signaling the neurotrauma: First, we have shown that the global loss of either cGAS or STING is equally beneficial, indicating that canonical, cGAS-dependent signaling is occurring in the brain. Second, we provide compelling evidence that the STING-IFN response to TBI is more complex than previously thought and is dependent upon both microglia and peripheral immune cells. While it has been evident for several years that STING signaling is largely detrimental in TBI, the findings presented in this dissertation provide important information about where and how this response occurs, and may offer insight that could ultimately facilitate novel therapeutics.

References

- Abdullah, Amar, Moses Zhang, Tony Frugier, Sammy Bedoui, Juliet M. Taylor, and Peter J. Crack. 2018. "STING-Mediated Type-I Interferons Contribute to the Neuroinflammatory Process and Detrimental Effects Following Traumatic Brain Injury." *Journal of Neuroinflammation* 15 (1). <https://doi.org/10.1186/S12974-018-1354-7>.
- Barrett, James P., Rebecca J. Henry, Kari Ann Shirey, Sarah J. Doran, Oleg D. Makarevich, Rodney R. Ritzel, Victoria A. Meadows, et al. 2020. "Interferon- β Plays a Detrimental Role in Experimental Traumatic Brain Injury by Enhancing Neuroinflammation That Drives Chronic Neurodegeneration." *The Journal of Neuroscience*, February, 2516–19. <https://doi.org/10.1523/jneurosci.2516-19.2020>.
- Ding, Rui, Haiyan Li, Yaqi Liu, Weiyang Ou, Xifang Zhang, Huihui Chai, Xiaofei Huang, Weijie Yang, and Qiujing Wang. 2022. "Activating CGAS-STING Axis Contributes to Neuroinflammation in CVST Mouse Model and Induces Inflammasome Activation and Microglia Pyroptosis." *Journal of Neuroinflammation* 19 (1): 137. <https://doi.org/10.1186/S12974-022-02511-0/FIGURES/9>.
- Gamdzyk, Marcin, Desislava Met Doycheva, Camila Araujo, Umut Ocak, Yujie Luo, Jiping Tang, and John H. Zhang. 2020. "CGAS/STING Pathway Activation Contributes to

- Delayed Neurodegeneration in Neonatal Hypoxia-Ischemia Rat Model: Possible Involvement of LINE-1.” *Molecular Neurobiology* 57 (6): 2600–2619. <https://doi.org/10.1007/S12035-020-01904-7>.
- Gupte, Raeesa, William Brooks, Rachel Vukas, Janet Pierce, and Janna Harris. 2019. “Sex Differences in Traumatic Brain Injury: What We Know and What We Should Know.” *Journal of Neurotrauma* 36 (22): 3063. <https://doi.org/10.1089/NEU.2018.6171>.
- Karve, Ila P., Moses Zhang, Mark Habgood, Tony Frugier, Kate M. Brody, Maithili Sashindranath, C. Joakim Ek, et al. 2016. “Ablation of Type-1 IFN Signaling in Hematopoietic Cells Confers Protection Following Traumatic Brain Injury.” *ENeuro* 3 (1): 89–93. <https://doi.org/10.1523/ENEURO.0128-15.2016>.
- Kong, Lingqi, Wenyu Li, E. Chang, Wuxuan Wang, Nan Shen, Xiang Xu, Xinyue Wang, et al. 2022. “MtDNA-STING Axis Mediates Microglial Polarization via IRF3/NF-KB Signaling After Ischemic Stroke.” *Frontiers in Immunology* 13 (April): 1538. <https://doi.org/10.3389/FIMMU.2022.860977/BIBTEX>.
- Li, Qian, Yuze Cao, Chun Dang, Bin Han, Ranran Han, Heping Ma, Junwei Hao, and Lihua Wang. 2020. “Inhibition of Double-strand DNA-sensing CGAS Ameliorates Brain Injury after Ischemic Stroke.” *EMBO Molecular Medicine*. <https://doi.org/10.15252/emmm.201911002>.
- Peng, Yucong, Jianfeng Zhuang, Guangyu Ying, Hanhai Zeng, Hang Zhou, Yang Cao, Huaijun Chen, et al. 2020. “Stimulator of IFN Genes Mediates Neuroinflammatory Injury by Suppressing AMPK Signal in Experimental Subarachnoid Hemorrhage.” *Journal of Neuroinflammation* 17 (1): 1–20. <https://doi.org/10.1186/S12974-020-01830-4/FIGURES/10>.
- Sen, Tanusree, Pampa Saha, Rajaneesh Gupta, Lesley M. Foley, Tong Jiang, Olena S. Abakumova, T. Kevin Hitchens, and Nilkantha Sen. 2020. “Aberrant ER Stress Induced Neuronal-IFN β Elicits White Matter Injury Due to Microglial Activation and T-Cell Infiltration after TBI.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 40 (2): 424–46. <https://doi.org/10.1523/JNEUROSCI.0718-19.2019>.
- Späni, Claudia B., David J. Braun, and Linda J. Van Eldik. 2018. “Sex-Related Responses after Traumatic Brain Injury: Considerations for Preclinical Modeling.” *Frontiers in Neuroendocrinology* 50 (July): 52. <https://doi.org/10.1016/J.YFRNE.2018.03.006>.