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Inflammatory Response Following Hemorrhagic Stroke: The Role of Cytokines

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Abstract

Hemorrhagic stroke is a dangerous form of stroke resulting from the rupturing of weakened blood vessels, releasing blood that increases intracranial pressure and causes the death of surrounding tissue. Treatment options are improving but remain limited, as evidenced by this condition being characterized by high rates of mortality as well as long-term morbidity. Unregulated inflammatory responses that occur following injury may be partially to blame for these poor outcomes. In response to any injury, the immune system releases cytokines to recruit immune cell activation and promote inflammation. But after hemorrhagic stroke, whether aneurysmal subarachnoid hemorrhage (aSAH) or intracerebral hemorrhage (ICH), the rapid increase in inflammation and an imbalance of inflammatory cytokines can lead to the development of secondary ischemic injury. Several animal models have been developed to investigate these forms of stroke, and despite their shortcomings, these models have been crudely applied for the study of neuroinflammation post-stroke. There is an imperative need to establish a clear and robust animal model that accurately represents the condition taking place in human patients. This dissertation work began with a systematic review of the literature to more clearly understand the work that has been done in this area. Next, the endovascular puncture model of aSAH was utilized in rats. Tissue samples were collected and compared to human blood and cerebrospinal fluid samples to assess for cytokine changes. Lastly, collagenase mouse models for ICH were utilized to understand the changes that take place in the TXNIP-NLRP3 inflammasome following stroke and the therapeutic effects of verapamil on inflammatory, functional, and behavioral outcomes. This dissertation work adds to the body of literature on the relationship between inflammation, cytokine release, and outcomes, which will ultimately allow for the development of improved treatment protocols.

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UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTORAL DISSERTATION

Inflammatory Response Following Hemorrhagic Stroke: The Role of Cytokines

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DEDICATION

I would like to dedicate this work to my parents, Jeff and Gwen Devlin, and to my brother, Ryan Devlin. Without their love and support throughout the years, my ability to carry out this work would have not been possible. I would also like to thank all of my teachers, mentors, family, friends, coaches, and teammates. In their own unique ways, each of them provided guidance and the solid foundation needed to guide me to this point in my scientific career.

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ABSTRACT

Hemorrhagic stroke is a dangerous form of stroke resulting from the rupturing of weakened blood vessels, releasing blood that increases intracranial pressure and causes the death of surrounding tissue. Treatment options are improving but remain limited, as evidenced by this condition being characterized by high rates of mortality as well as long-term morbidity. Unregulated inflammatory responses that occur following injury may be partially to blame for these poor outcomes. In response to any injury, the immune system releases cytokines to recruit immune cell activation and promote inflammation. But after hemorrhagic stroke, whether aneurysmal subarachnoid hemorrhage (aSAH) or intracerebral hemorrhage (ICH), the rapid increase in inflammation and an imbalance of inflammatory cytokines can lead to the development of secondary ischemic injury. Several animal models have been developed to investigate these forms of stroke, and despite their shortcomings, these models have been crudely applied for the study of neuroinflammation post-stroke. There is an imperative need to establish a clear and robust animal model that accurately represents the condition taking place in human patients.

This dissertation work began with a systematic review of the literature to more clearly understand the work that has been done in this area. Next, the endovascular puncture model of aSAH was utilized in rats. Tissue samples were collected and compared to human blood and cerebrospinal fluid samples to assess for cytokine changes. Lastly, collagenase mouse models for ICH were utilized to understand the changes that take place in the TXNIP-NLRP3 inflammasome following stroke and the therapeutic effects of verapamil on inflammatory, functional, and behavioral outcomes.

This dissertation work adds to the body of literature on the relationship between inflammation, cytokine release, and outcomes, which will ultimately allow for the development of improved treatment protocols.

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LIST OF ABBREVIATIONS

aSAH	Aneurysmal subarachnoid hemorrhage
ASC	Apoptosis-associated speck-like protein
BBB	Blood-brain barrier
CSF	Cerebrospinal fluid
CT	Computerized tomography
DAMPs	Damage-associated molecular patterns
EVD	External ventriculostomy drain
GOS	Glasgow Outcome Scale
IL-	Interleukin
ICH	Intracerebral hemorrhage
I/R	Ischemic reperfusion
MrS	Modified Rankin Scale
MWM	Morris water maze
NLRP3	NOD-like receptor pyrin domain-containing-3 junction proteins
NF-κB	Nuclear factor kappa B
TNF-α	Tumor necrosis factor-alpha
TRX	Thioredoxin
TXNIP	Thioredoxin interacting protein
WFNSS	World Federation of Neurological Surgeons Scale
ZO-1	Zonula occludens-1

CHAPTER 1. INTRODUCTION

Background

Hemorrhagic Stroke

Hemorrhagic stroke is a form of stroke that results from a blood vessel breaking and releasing blood into the brain. This can happen due to the spontaneous bursting of the arterial wall, head trauma, arteriovenous malformations, or a bleeding aneurysm. The likelihood of having a hemorrhagic event is influenced by many factors such as age, sex, diet, race, alcohol use, drug use, and hypertension (An et al., 2017; Grysiewicz et al., 2008). Death of brain tissue in the affected area occurs as a result of increased intracranial pressure and a cascading inflammatory response. Due to the complex vasculature of the brain, these strokes can occur anywhere in the brain and affect many inflammatory response systems. General prognosis is largely dependent upon the location and size of the bleed, although each case is unique. The two main subtypes of hemorrhagic strokes are aneurysmal subarachnoid hemorrhage (aSAH) and intracerebral hemorrhage (ICH).

aSAH is caused when a ruptured cerebral aneurysm bleeds into the subarachnoid space of the brain. The incidence of new cases of aSAH in the United States is about 30,000/y or 10/100,000 persons, which accounts for ~5% of all strokes (D'Souza, 2015). Despite being a less-common form of stroke, aSAH accounts for about 40% of total stroke deaths, with aSAH survivors often experiencing severe long-term disability and reduced quality of life (Lichtman et al., 2011). While improvements in the treatment methods used are reducing mortality rates, there remains a high burden of morbidity in this group.

Similarly, although ICH accounts for only 10-15% of all strokes, mortality rates after ICH can reach >50% (Rymer, 2011). Approximately 74% of those who do survive experience some degree of physical impairment; affective and cognitive changes may also occur (van Asch et al., 2010). Like aSAH, ICH occurs when vessels inside the brain rupture. However, in ICH, the blood released forms a hematoma in the surrounding tissue. As cerebral blood flow is altered, additional secondary events can occur, including edema, increased intracranial pressure, and herniation of brain tissue. Further brain damage results from activation of immune cells, such as macroglia, which drives cytotoxic and oxidative responses with neurotoxic effects. Ultimately, these processes culminate in cell death (Shao et al. 2019).

Common to both subtypes are the cascading effects of unmitigated inflammation that can create additional sequelae that can lead to poor outcomes. Thus, whether considered in the context of aSAH or ICH, an improved understanding of the inflammatory response following hemorrhagic stroke will inform treatment options to minimize mortality and morbidity for these clinical populations.

Current Treatment Methods for Hemorrhagic Stroke

Over the past years, improved treatment options to stop or slow cerebral bleeding have greatly decreased mortality of hemorrhagic stroke patients in the hyperacute period (Cesarini et al., 1999). Upon presentation for emergency care, a diagnosis of aSAH or ICH is made by computerized tomography (CT) scan, Magnetic resonance imaging (MRI), or cerebral angiography (Josephson et al., 2014). Treatment should be initiated rapidly; goals must be established that focus on interventions to stop or reduce bleeding and maintain acceptable intracranial pressures. Induced hypertension is commonly used to prevent cerebral ischemia (rather than the outdated triple H regimen). In the context of aSAH, the repair of vascular damage is also a goal, with endovascular coils (some biologically active), stents, and neurosurgical clips serving as options. Even in the best-case scenario, all of these treatments are initiated some time after the hemorrhage has occurred. This means that blood flow has already reduced to cerebral tissue and damage has already begun. Gains in recovery rates could be found in the development of an effective treatment that specifically manages the inflammatory response and could be rapidly initiated after stroke.

The Role of Cytokines and the Neuroinflammatory Response

In the context of hemorrhagic stroke, breach of the blood-brain barrier (BBB) disrupts normal inflammatory system homeostasis, setting up an environment ripe for the development of further damage (Butler et al., 2020). Vessel rupture alone causes an immediate, local inflammatory response. In this circumscribed area, the immune system works to recruit fast-acting immune cells (such as neutrophils and platelets) to respond to the bleeding. These immune cells release cytokines at the site of the rupture; their release recruits additional immune cells to the damaged area. This signaling in turn increases inflammation throughout the body, triggering a systemic response that can have many downstream effects on systems around the body.

Most critical to this homeostasis is the proper regulation of T cells controlled by external signals, such as those generated by cytokines (Smigiel et al., 2014). Cytokines can be produced by leukocytes upon appropriate stimulation and induction of inflammation (Zhang et al., 2007). Eight different cytokines, broadly recognized for their role in inflammation, have also been described in the literature as playing an important role in hemorrhagic stroke (Interleukin-1α [IL-1α], Interleukin-1β [IL-1β], Interleukin-4 [IL-4], Interleukin-6 [IL-6], Interleukin-8 [IL-8], Interleukin-10 [IL-10], Interleukin-12 [IL-12], Interleukin-13 [IL-13], and Tumor necrosis factor-alpha [TNF-α]). Some of these cytokines act as pro-inflammatory signals, while others act as anti-inflammatory signals (Zhang et al., 2007). For there to be proper homeostasis, there must be an appropriate balance between anti- and pro-inflammatory cytokines, with some aiding in immune cell response signaling and others acting as mediators to regulate the inflammatory response and return the system to homeostasis. A loss of balance has been well-described in the etiology of several diseases, including cancer, lung disease, and osteoarthritis (Furman et al., 2019).

Perhaps one of the most widely studied and influential cytokines in hemorrhagic stroke is Interleukin-6 (IL-6). This cytokine is important for the initial development of inflammatory response as well as the regulation of metabolic and regenerative processes as inflammation resolves (Tanaka et al., 2014). As a result of the role it plays, increased levels are associated with the development of ICH and aSAH, the severity of these strokes, and complications after a stroke has occurred (Maas and Furie, 2009). Correlation between increased IL-6 levels and poor patient outcomes post-aSAH and ICH have also been noted in various studies (Chaudhry et al., 2017; Leasure et al., 2021).

Other specific cytokines have been noted for their anti-inflammatory responses. For example, Interleukin-10 (IL-10) decreases the inflammatory response by inhibiting lipopolysaccharide-induced glucose uptake and glycolysis, which then encourages oxidative phosphorylation (Ip et al., 2017). As a result, an increase in IL-10 decreases overall inflammation in the system and may protect vascular endothelium in the context of stroke by counteracting some of the negative effects of excess inflammation (Garcia et al., 2017).

Current Hemorrhagic Stroke Animal Models

Work in animal models of hemorrhagic stroke has demonstrated the aforementioned inflammatory cytokine changes and suggested relationships between certain cytokines, inflammatory responses, and disability status after the stroke event (Ramiro et al., 2018). Yet despite the promise of these markers, the contributions of cytokines in aSAH and ICH inflammatory response have not jumped the translational gap to be tested for relevance in human patients. It is possible that the local and systemic changes in cytokines may differ between animal models and humans, suggesting that different mechanisms for inflammatory response are at play in these systems. But when considering animal models of hemorrhage stroke, it is much more likely that there are gaps in the ability to accurately produce the same conditions seen in humans versus animals.

In the real world, a hallmark of hemorrhagic stroke is its sudden onset and unpredictable course. In contrast, animal models must artificially induce such an event, introducing additional variables that are not present in a naturally occurring event. Various models introduce these to a greater or lesser degree; the most commonly used animal models for aSAH and ICH are described in turn below. While all these models are successful at showing some inflammatory effects, they are not completely accurate for mimicking the actual physiological reaction that takes in the human brain—and the reaction systemically—during the vascular event and early post-acute period. There is a need for better and more representative models of vascular disruption.

aSAH single-blood-injection model

One common aSAH model is the single-blood-injection model, whereby blood is injected directly into the subarachnoid space (Kamp et al., 2014). This model partially

accounts for the perfusion of blood into the subarachnoid space, which would result in an increase of pressure and damage to the surrounding tissue. However, from a mechanistic perspective, there is no vascular rupture. Thus, from an inflammatory perspective, such an injection would create a different environment relative to an aneurysm rupture. Due to this inaccuracy, any cytokine change results (or generalized inflammatory results) collected from this model may not be fully generalizable to the human subjects. Further, this model does not account for the fluctuating perfusion of blood that can take place during the aSAH event as bleeding, slowing, and rebleeding occurs in the hyperacute period. This main downfall ignores this process, which is likely a major player in the inflammatory response post-aSAH and a contributor to secondary complications.

aSAH endovascular puncture model

Another common model, the endovascular puncture model, is performed through the insertion of a suture into the internal carotid artery and through to the circle of Willis. At this point, the suture punctures the vessel wall (Kooijman et al., 2014). This destruction of the vessel results in instantaneous bleeding into the subarachnoid space, mimicking the aneurysm rupture that occurs in aSAH. While it may be mechanistically similar to the human condition, the artificial condition is much more abrupt, with no buildup of pressure inside the aneurysm and vascular wall. This sudden, rather than progressive, nature of these events may result in different specific inflammatory responses. When compared to the single-blood-injection model, this model better replicates the mechanical action that occurs during aSAH. While it is not a spontaneous, progressive rupture seen in humans, it does result in the destruction of a blood vessel and the perfusion of blood into the subarachnoid space. Some differences in the inflammatory response may occur due to the mechanical induction of this injury; however, such a procedure most closely recreates the local inflammatory environment observed in aSAH.

Several animal models have also been established for ICH. These models provide technical challenges in accessing and causing hematoma in deeper brain structures without causing immediate mortality.

ICH single-blood-injection model

In contrast to the aSAH single-blood-injection model, this model injects blood into the intracerebral space to create a hematoma, similar to what has been observed in ICH. Still, as in the aSAH single-blood-injection model, there is again a similar disadvantage that no vascular rupture is present.

ICH collagenase-injection model

On the other hand, the collagenase injection model results in the bursting of blood vessels through the breaking down of collagen and the weakening of surrounding vessels. In most cases, the collagenase injection method is carried out through the use of a stereotactic apparatus to allow precise targeting. The exact brain area for the injection is determined, and coordinates are used to inform the injection location. The needle

containing the collagenase is then slowly lowered into the brain in order to ensure that no mechanical damage is done to unrelated areas. The collagenase is then injected and the needle withdrawn. By performing this precise injection technique, there are minimal off-target effects, thus allowing for the creation of very replicable and specific hematoma sites.

When comparing hematoma size, the single-blood-injection and the collagenase injection models are similar. However, there are important differences between the two in other factors. The damage caused by the collagenase injection results in greater disruption of the BBB, increased neuron loss, increased tissue damage, an absence of spontaneous neurological recovery, and the expansion of the hematoma over time when compared to single-blood-injection (Jia et al., 2021). For all these reasons, the collagenase model may be a more representative model for ICH in humans.

Aims

This dissertation aims to explore the inflammatory effects that occur in animal models of hemorrhage stroke and compare these effects with those found in patient samples. Our general premise for this work is that much of the information known about the inflammatory environment post-hemorrhagic stroke has been gleaned from animal models, which may be imperfect representations of the conditions found in hemorrhagic stroke. These differences can greatly influence the translational abilities of potential treatments that are tested in the models for clinical use in humans. In short, there is a gap that must be filled to determine which model is the best mimic of the inflammatory effects of hemorrhagic stroke in humans and is thus adequate for measuring and testing anti-inflammatory treatments. The goal of this body of work was to definitively describe the Inflammatory environment following hemorrhagic stroke, highlight the differences between animal models and the human condition, and propose possible targets for treatments that may be able to bridge the translational hurdles that face hemorrhagic stroke research. These aims were investigated in multiple different projects.

The first project, described in Chapter 2, is a systematic review published in *Translational Stroke Research.* This manuscript aimed to discover the breadth of the literature on cytokine change and its role in the inflammatory response following aSAH. Both animal models and human studies were considered in order to understand how research in these areas fundamentally differed. Information such as the tissue type used, the purpose of each study, cytokines investigated, and animal model utilized was collected. Through systematic searching of electronic databases, important insights into the current literature on the topic was collected and used to inform our projects moving forward.

The second project, described in Chapter 3, investigated the inflammatory environment found in rats after experimentally induced (endovascular puncture) aSAH and humans who suffered the same condition. In humans, cytokine levels were measured via cerebrospinal fluid (CSF) and plasma samples of aSAH patients collected at three

different timepoints: post-rupture day 1-3, 4-6, and 7-9. The plasma was collected intravenously, and the CSF samples were collected noninvasively from an external ventriculostomy drain (a standard aSAH practice used to manage intracranial pressure changes caused by aneurysm rupture). Rat cytokine levels directly after SAH were compared to give an indication of the systemic and local inflammatory environment surrounding the brain. The change over time and the absolute levels of cytokines measured were used to better understand the inflammatory environment following aSAH both locally (CSF) and systemically (plasma). Following aSAH, there was a particularly significant increase in inflammatory cytokines IL-6 and IL-8. Further, these changes were associated with demographic and clinical data to understand the overall role these specific cytokines could possibly play in patient outcomes. Moving forward, these results will establish commonly shared and significantly expressed cytokines, which will further validate this rat model for aSAH.

The final project has been submitted to *Neurochemistry International*. This project investigated ICH and its unique inflammatory environment. In particular, it investigated the efficacy of a drug known as Verapamil on influencing the inflammatory response, motor function, and cognitive ability following stroke. For this project, a mouse collagenase model was utilized, brain samples were analyzed, behavioral tests were conducted, and immune markers were measured. The project ultimately demonstrated that Verapamil may have several beneficial effects on recovery following ICH.

The following chapters will explore each of these projects undertaken for the completion of this dissertation. They aim to explore the available literature on the topic, to discover possible mechanisms that can influence the inflammatory response, and to compare the translational ability these animal models have in the actual human phenomenon. The overall goal of this body of work is to better understand the inflammatory environment post-hemorrhagic stroke to improve treatment and recovery.

CHAPTER 2. A SYSTEMATIC REVIEW OF INFLAMMATORY CYTOKINE CHANGES FOLLOWING ANEURYSMAL SUBARACHNOID HEMORRHAGE IN ANIMAL MODELS AND HUMANS[1]

Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) is a form of stroke that occurs when a cerebral aneurysm ruptures and bleeds into the subarachnoid space surrounding the brain. The incidence of new cases of aSAH in the United States is about 30,000/year or 10/100,000 persons, accounting for ~5% of all strokes ("Souza, 2015; Feigin et al., 2003). Despite being a less-common form of stroke, aSAH accounts for about 40% of the total number of stroke deaths nationally, and those who do survive often experience severe long-term disability and reduced quality of life (Lantigua et al., 2015; Aisiku et al., 2014). Mortality and morbidity rates are increased further for those patients who experience cerebral vasospasm, a common complication that decreases cerebral blood flow and can lead to delayed cerebral ischemia and secondary infarction. Inflammatory dysregulation is thought to be a major determinant of these complications and poor outcomes (van Lieshout et al., 2018). Thus, developing better treatment options requires understanding the factors involved in pro-inflammatory immune response regulation and disruption as they occur in the context of aneurysm rupture.

After the rupture of a brain aneurysm, neuroinflammation increases neural cell death by interfering with endogenous repair mechanisms, acting through immune cells, microglia, cytokines, chemokines, and other inflammatory molecules. Restoration of immune homeostasis relies on proper regulation of T cells controlled by external signals, such as those generated by cytokines. Cytokines are important signaling proteins vital for inflammation and response to cellular damage, and individual cytokines can have pro- or anti-inflammatory effects (**Table 2-1**). After aSAH, cytokine levels in brain tissue and CSF increase in response to the rupture, indicating a rapidly changing inflammatory response within the brain (Zeiler et al., 2017). Similar increases have been demonstrated in blood plasma and serum, indicating systemic inflammatory processes are also altered. Work in animal models has demonstrated persistent, longer-term inflammatory cytokine change and suggested relationships between the neuro-specific and systemic inflammatory effects, but many of these findings have yet to jump the translational gap to be tested in the clinical arena. In this systematic review, we will describe the state of the science in this area, considering animal models and aSAH patient samples. A secondary goal of this review is to illuminate the translational hurdles that often arise between animal experimental models and humans and how this affects this venue of aSAH research. Finally, we suggest directions for future research in this area to fill these gaps.

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[1]Reprinted by permission from Springer Nature: Springer Nature. Translation Stroke Research. A Systematic Review of Inflammatory Cytokine Changes Following Aneurysmal Subarachnoid Hemorrhage in Animal Models and Humans. Devlin P, Ishrat T, Stanfill AG. (2022). https://doi.org/10.1007/s12975-022-01001-y

Table 2-1. Main cytokines of interest.

Name	Symbol	Inflammatory Type	
		Pro-	Anti-
Interleukin-1	IL-1 α/ β/R	X	
Interleukin-4	IL-4		X
Interleukin-5	IL-5	X	
Interleukin-6	IL-6	X	
Interleukin-7	IL-7	X	
Interleukin-8	IL-8	X	
Interleukin-10	IL-10		X
Interleukin-12	IL-12	X	
Interleukin-13	IL-13		X
Interleukin-15	IL-15	X	X
Interleukin-17	IL-17	X	
Interleukin-18	IL-18	X	
Interleukin-22	IL-22		
Interleukin-23	IL-23		
Interleukin-33	IL-33		
Tumor Necrosis Factor Alpha	TNF-α	X	
Interferon-Gamma	IFN-γ		X
Transforming Growth Factor Beta	TGF-β	X	X
Macrophage migration inhibitory factor	MIF	X	
Monocyte Chemoattractant Protein-1	MCP-1	X	

Methods

A systematic review of the literature was conducted by utilizing the following three different databases: PubMed, SCOPUS, and the Cochrane Library. Our procedures followed the established Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2015). Three different search phrases were established and applied to each of the three databases: "cytokines and subarachnoid hemorrhage," "cytokine levels and subarachnoid hemorrhage," and "cytokine measurement and subarachnoid hemorrhage." Our inclusion criteria were: 1) written in English; 2) published between January 2015 and September 2021; and 3) primary data-based articles measuring changes for any cytokines in plasma, serum, CSF, and brain tissue post-aSAH in either animal models and/or humans. For human studies, all participants must have been 18 or older. For animal studies, the animals must be representative of this age group (i.e., adults). There were no limitations on the animal model species used. Review papers and meta-analyses were excluded.

Each search term combination was applied to each database, and an initial list of possible articles was identified. From this list, duplicates were removed. Article titles and abstracts were initially screened to determine adherence to inclusion/exclusion criteria. Two authors independently reviewed this list, and any articles failing this test were removed. Then, articles were separated into animal models or human subjects research, and each set of articles were reviewed in full by these same two authors. Each individual independently reviewed the list to ensure all inclusion/exclusion criteria were met; any article not meeting criteria was then removed.

Results

Search Results

A total of 856 papers were returned from our selected databases. Of these, duplicate papers across databases were removed. We then scanned titles and abstracts of the remaining papers. Next, we removed papers for failing inclusion and exclusion criteria in the title and/or abstract, leaving papers remaining for full text review. Of these, 95 used preclinical animal models, and 41 papers used specimens obtained from patients during clinical care (**Figure 2-1**). Four papers used animal models and human samples and were reported in both categories. These papers were then separated into animal model and human studies. Publication dates and number of papers published from year-to-year between 2015-2021 varied over time (**Figure 2-2**).

Preclinical Animal Model Investigations

A total of 95 preclinical animal model studies are included in this review. These studies analyzed cytokines for the following: illustrate the treatment effect of various

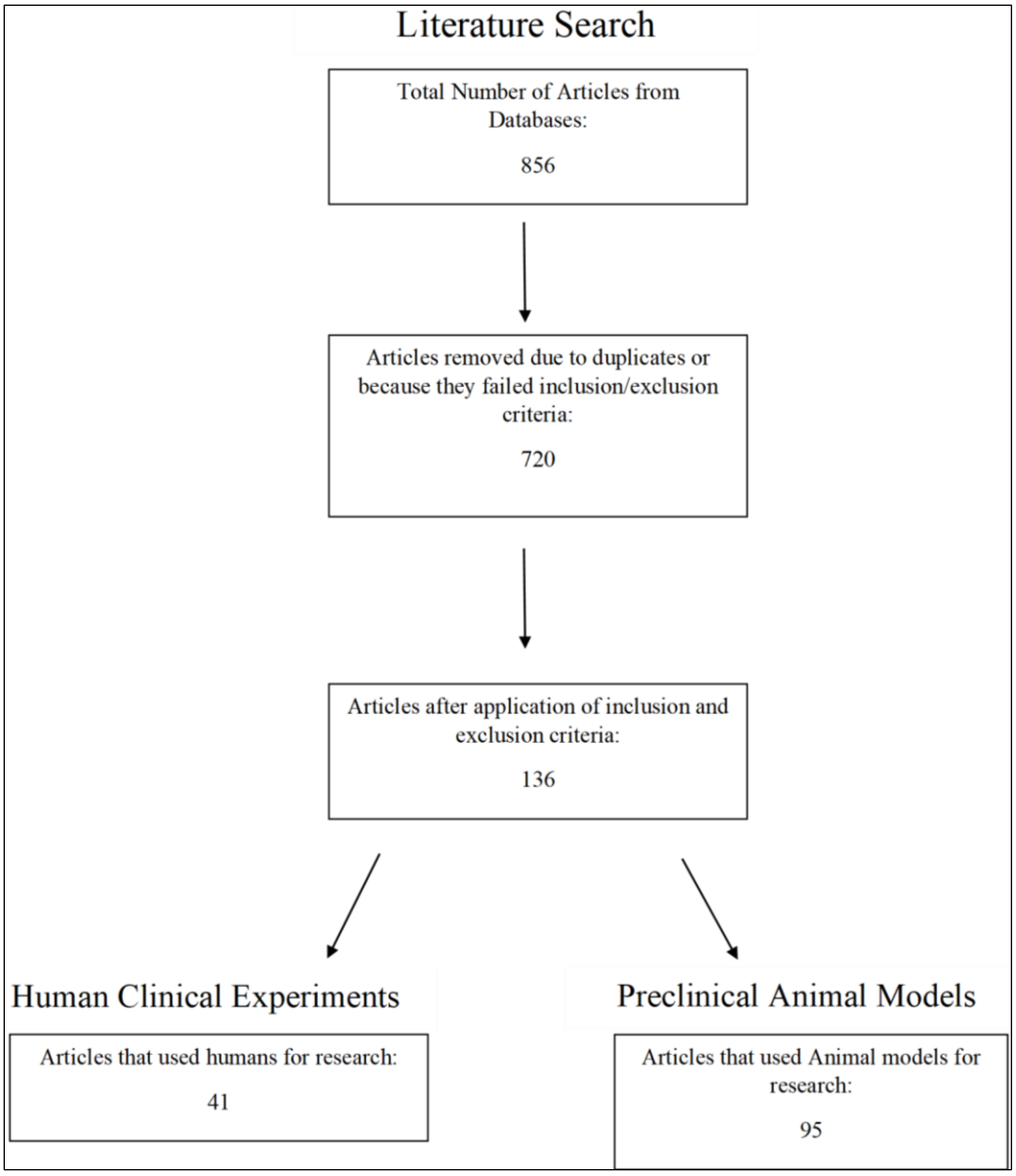

Figure 2-1. Study selection flowchart for systematic literature search procedure and outcomes.

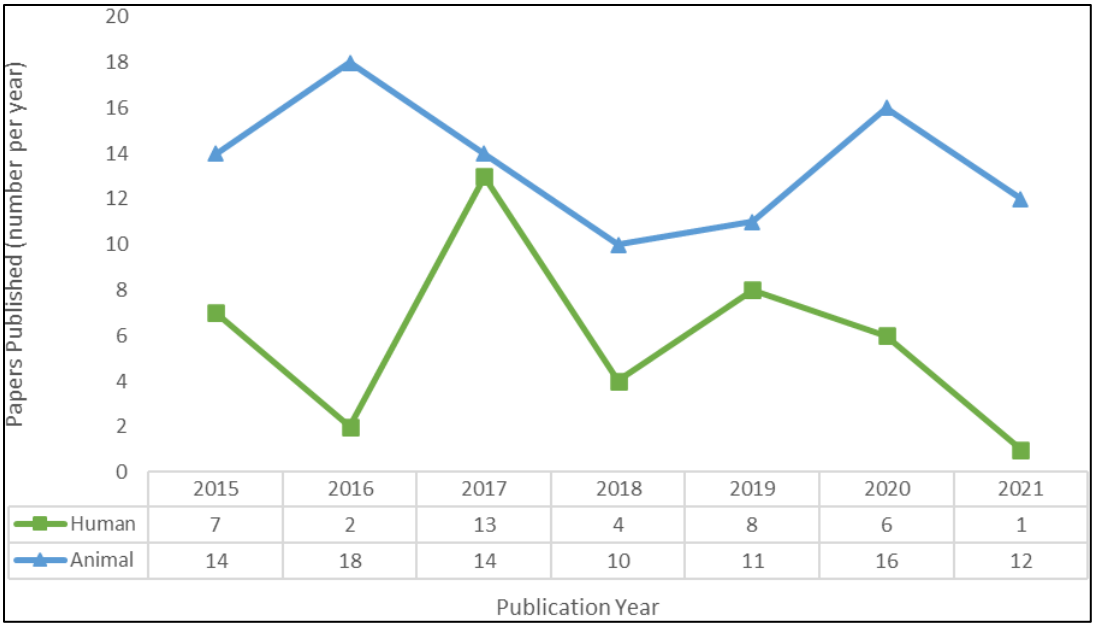

Figure 2-2. The change over time of articles being published in both human and animal model studies.

substances (n= 57); demonstrate cytokines' relationship with other inflammatory factors (n=36); serve as a biomarker for aSAH severity (n= 6); and show the change in cytokine levels of the brain environment over time (n=8). The vast majority of included studies (n=80) measured cytokine levels in brain tissue harvested post-mortem. The remainder used blood serum (n=9) and CSF (n=11) as the tissue of choice, while only one utilized plasma. Three different animal models were used in these projects, with most studies using rats (n=67), a few using mice (n=22), and a handful using rabbits (n=6). The aneurysm rupture model was performed most commonly by vascular puncture (n=55), followed by blood or hemolysate injection or infusion (n=35), followed by blood clot placement (n=21), extracranial-intracranial shunting (n=2), injection of enzymes (n=2) and arterial occlusion (n=1). These studies were broadly representative of investigations in pro- and anti-inflammatory cytokines, including the following: IL-1α, IL-1β, IL-4, IL-6, IL-7, IL-8, IL-10, IL-17, IL-18, IL-22, IL-33, TNF-α, IFN-γ, and TGF-β (**Appendix A**).

Individual cytokines have specific roles, either pro- or anti-inflammatory, and a consideration of this differential effect was apparent throughout the preclinical work. For example, three of the most prominent pro-inflammatory cytokines studied included IL-1β (n=71), IL-6 (n=58), and TNF-α (n=75). Following aSAH, the levels of each of these pro-inflammatory cytokines were found to significantly increase across studies, with each of them having varying behaviors depending on studies. They were thus most often used as markers to show the anti-inflammatory nature of various substances. One report demonstrated increases in IL-33 following injury, though other included studies did not measure this change (Gong et al., 2018).

Another important cytokine being studied in this area is IL-10 (n=12). This cytokine is well-established to act as an anti-inflammatory cytokine to dampen the inflammatory response following injury. A few studies demonstrated that treatment of aSAH (whether by the pharmaceuticals or use of stem cell therapeutics) was associated with increases in the levels of IL-10 in conjunction with decreasing overall inflammation indicated by changes of inflammatory cytokines IL-6, IL-1 β, and TNF-α (n=8).

Clinical Patient Investigations

A total of 41 clinical studies are included in this review. All human studies were conducted on patients who had experienced an aSAH and consented to collection of tissue samples themselves or via proxy. These studies focused on how cytokine change serves as a biomarker for aSAH severity (n= 30); show how cytokine levels change over time (n=16), investigate cytokines relationships with other inflammatory factors (n=14), and illustrate the treatment effects of various substances (n=2). The majority of these studies investigated cytokine levels in CSF (n=20) or in serum (n=22), while a few considered alterations in blood plasma (n=8) or interstitial fluid (n=3). The included studies investigated various specific cytokines, including the following: IL-1α, IL-1β, IL-1R, IL-4, IL-5, IL-6, IL-7, IL-8 IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-23, IL-33, TNF-α, IFN-γ, TGF-β, MIF, and MCP-1 (**Appendix A**). Clinical work revealed a high

degree of concordance with the changes demonstrated in preclinical models. For example, observed increases (from 1 to 14 days after injury) occurred in pro-inflammatory cytokines such as IL-1β (n=4), IL-6 (n=31), and TNF-α (n=14). Capitalizing on the previous animal work mentioned [7], one study investigated changes in IL-33 (Huang et al., 2015). As in the preclinical model, upregulation of IL-33 was found post-aSAH. This upregulation of cytokine levels in IL-33, as well as in IL-17 (n=5) and IL- 23 (n=1), were associated with clinical grading scales predicting poorer prognosis (Chaudhry et al., 2017). The cytokine IL-33, in particular, has a strong relationship to stroke severity scores following aSAH, whether considered from size of bleed on CT scan (modified Fisher grading scale) or from clinical grading scale(s) (e.g., World Federation of Neurological Surgeons Scale [WFNSS]). A few studies followed outcomes longitudinally by measuring Glasgow Outcome Scale (GOS). Some measured outcomes at 72 hours after rupture, while others followed the patients until 6 months post-stroke. For these, increases in IL-33 concentration resulted in worse outcomes for patients following aSAH (Gong et al., 2018).

Changes in the anti-inflammatory cytokine IL-10 were investigated in multiple studies and found to be associated with worse outcomes. One study showed elevated systemic IL-10 levels were found on day 7 post-aSAH in patients who suffered from complications including cerebral vasospasm, nosocomial infections, or shunt-dependent chronic hydrocephalus. Specifically, the data indicate that an increase in serum IL-10 levels on day 7 was able to predict chronic hydrocephalus and infections during the acute treatment period of SAH (70% sensitivity and ~60% specificity for each complication). This increase resulted in overall poorer outcomes, whether measured by modified Rankin Scale (mRS) or GOS (Chaudhry et al., 2020). Another study showed that elevated systemic IL-6 levels were a reliable biomarker for the development of common post-aSAH complications that included seizures, vasospasm, and chronic hydrocephalus (Chaudhry et al., 2017). Several other studies illustrated the correlation between changes in specific cytokines and patient outcomes or comorbidities over several timepoints following SAH **(Table 2-2)**.

Changes in the pro-inflammatory cytokine IL-6 also influenced patient outcomes (n=10). In one study, an increase in IL-6 and a decrease in IL-10 resulted in a poorer functional outcome, as measured by mRS, at 3 months following aSAH (Ahn et al., 2019). These results suggest a possible interrelated relationship between these cytokines that may work to maintain the homeostatic balance of the inflammatory effects that may influence common complications of aSAH, aSAH recovery, and long-term outcomes.

Discussion

This systematic review identified the state of what is known about cytokine change in aSAH in preclinical and clinical work. Understanding the similarities and differences in the results of these studies, as well as the knowledge gaps that are found, will inform future research in this area and may identify targets for therapeutic intervention.

Table 2-2. Relationship between cytokine change and patient outcomes.

Paper	Cytokine of Interest	Sample Collection Timeframe (After aSAH)	Timeframe Patient Outcomes Were Measured	Patient Outcomes	Associated Comorbidities
Chaudhry et al., 2017	IL-23, IL-17	Day 1 and 7	Discharge	GOS and mRS (No correlation)	-
Wenneberg et al., 2020	TNF-α, IL-6, IL-1R	Day 1	Discharge	GOS	-
Höllig et al., 2015	IL-6	Day 1	Discharge and 6-months after	mRS	-
Schiefecker et al., 2019	IL-6	2,8,12 hrs <24 hrs	Admittance and 3-months	FGS	Linked to fever Delayed Cerebral Infarction Metabolic distress
Al-Tamimi et al., 2019	IL-4, IL-6	Days 1-3, 5, 7 and 9	Day 10 and 6-months	mRS	Delayed ischemic Neurological Deficit
Savarraj et al., 2018	IL-6, IL-8, IL-10	24 hr	Discharge	HHS mRS	
Helbok et al., 2015	IL-6	Days 1-5	3-months	mRS	Delayed Cerebral Ischemia
Savarraj et al., 2018	IL-6	≤ 48 hours of admission	Discharge	HHS	Global Cerebral Edema
Chamling et al., 2017	IL-6	Days 1, 4, 7, 10, and 14	-	-	Delayed Cerebral Ischemia
Ďuriš et al., 2017	IL-6	Days 1-4	Discharge	GOS HHS	-
Wu et al., 2016	IL-6, TNF-α	Day 2	Discharge	HHS	Cerebral Vasospasm
Chaudhry et al., 2020	IL-10	Day 1 and Day 7	Discharge	mRS GOS	Infection Cerebral Vasospasm Chronic Hydrocephalus
Chaudhry et al., 2017	IL-6	Days 1, 3, 5, 7, 9, 11, and 13	Discharge	HHS	Increasing Age Seizures Cerebral Vasospasm Chronic Hydrocephalus Pneumonia
Righy et al., 2018	IL-6 and IL-8	Day 1, 2, 3	Day 7	Mortality	-
Righy et al., 2018	IL-4	Day 1, 2,3	Day 7	Mortality	-
Gong et al., 2018	IL-33	Day 1	6 months	WFNSS MFS GOS	-

Table 2-2. (Continued).

Paper	Cytokine of Interest	Sample Collection Timeframe (After aSAH)	Timeframe Patient Outcomes Were Measured	Patient Outcomes	Associated Comorbidities
Ridwan, Grote, and Simon, 2021	IL-6	Days 4 to 14	Admittance and DischardeF	FGS mRS	Delayed Cerebral Infarction
Lenski et al., 2017	IL-6	<24 hrs of EVD	-	-	Cerebral Vasospasm External Ventricular Drain-Associated Ventriculitis
Kao et al., 2015	IL-6	Days 0,1, and 2	Admittance and 1 month	GCS FGS mRS	-
Vlachogiannis et al., 2019	IL-6	Days 1, 4, and 10	-	-	Infection
Niwa et al., 2016	IL-6	Day 1-14	3 months	GOS	-
Sheng-Yin Lv et al., 2017	IL-1β, IL-18, TNF-α	Days 1-3, 4-6, and 7-9	Admittance and 6 months	HHS WFNSS FGS	Cerebral Edema Cerebral Vasospasm
Chen et al., 2017	MIF	Day 1	Admittance and 6 months	WFNSS FGS	-
Schiefecker et al., 2017	IL-6	every 24 hours	3 months	HHS	Intraparenchymal bleeding -Deranged cerebral metabolism
Höllig et al., 2015	IL-6	Days 0, 1, 4, 7, 10 and 14	Discharge and 6-months	mRS	-
Coulibaly et al., 2020	IL-1α, IL-2, IL-17, TNFα	Day 3	3 months	mRS	-
Savarraj et al., 2017	IL-6, IL-8, IL-10, and TNF-α	Day 1, 2-4, 3-5 days, and 6-8	Discharge	HHS	-
Moraes et al., 2020	IL-17A	Day 1,2,3, 3+	-	Mortality	Vasospasm
Yang et al., 2019	MIF	Day 1	Discharge	HHS	Delayed Cerebral Ischemia
Zhong et al., 2017	IL-6 and IL-10	Day 1	Discharge	GOS HHS MFS	-

Notes: HHS = Hunt Hess Scale, GOS = Glasgow Outcome Scale, FGS = Fisher Grading Scale, MFS = Modified Fisher Scale, mRS = Modified Rankin Score, WFNSS = World Federation of Neurological Surgeons Scale.

However, some limitations in the existing body of knowledge were found. There were common limitations in the number of cytokines studied as well as few investigations of the pro-/anti-inflammatory balance of said cytokines. Other gaps identified were as follows: a paucity of information on how these cytokines relate to long-term patient outcomes (with even fewer studies having clinically meaningful longitudinal data in this regard); an absence of multi-tissue studies to allow comparison of systemic and neuro-specific inflammatory responses; and methodological issues with the representative preclinical aSAH induction models, with most included studies using these animals as reservoirs to test pharmacological agents on inflammatory response regulation. We will discuss each of these threads in turn. The overall nature of the information published in these papers was compared to better understand the differences between the inflammatory nature of human and animal model research being conducted (**Figure 2-3**).

Scope of Cytokines Investigated and Pro-/Anti-Inflammatory Balance

Across species, the vast majority of studies (n=106) investigated only 4 out of all possible cytokines: IL-1α, IL-1β, IL-6, and TNF-α. These cytokines are each well-known as pro-inflammatory markers and therefore in the context of a recent aneurysm rupture would generate the recruitment of T cells and trigger further immune system response cascades (Dinarello, 2000; Kany et al., 2019). A failure to capture the full complement of inflammatory response is myopic. The literature overwhelmingly demonstrates a lack of consideration for the relative countering effects from anti-inflammatory factors (such as IL-4, IL-8, IL-10, IL-12, and IL-13), which could just as easily influence the post-aSAH environment as the pro-inflammatory markers more commonly studied. The balance of pro- and anti-inflammatory effects of these cytokines is dynamic due to continuous feedback from the immune system and localized responses in the injury area. In the context of aSAH recovery, an understanding of the relative trajectory of this balance remains important but not well-considered.

Furthermore, the balance between pro- and anti-inflammatory cytokines differs at the molecular, organ, and whole-host levels (Cicchese et al., 2018). Similar questions concerning this balance have been investigated in conditions such as generalized anxiety spectrum disorder (GAD) and Multiple myeloma (MM). For example, patients with GAD showed significantly higher ratios of TNF-α:IL10 and TNF-α: IL4 when compared to control groups (Hou et al., 2017). In MM, researchers found that an unbalanced cytokine system contributed to the condition and indicated that pro-inflammatory IL-6 and IL-1 play a role in tumor eradication (Musolino et al., 2017). This information suggests this balance is a very important phenomenon in the development of inflammatory response that creates disease phenotypes. Without full consideration of all relevant cytokines and the relative16etabolionship of each, it is unclear how best to translate this information for therapeutic benefit in patients. A better consideration of the broad range of cytokines, with the measurement of counterplaying influences from multiple cytokine classes, would more fully illustrate the inflammatory environment present, both systemically and locally.

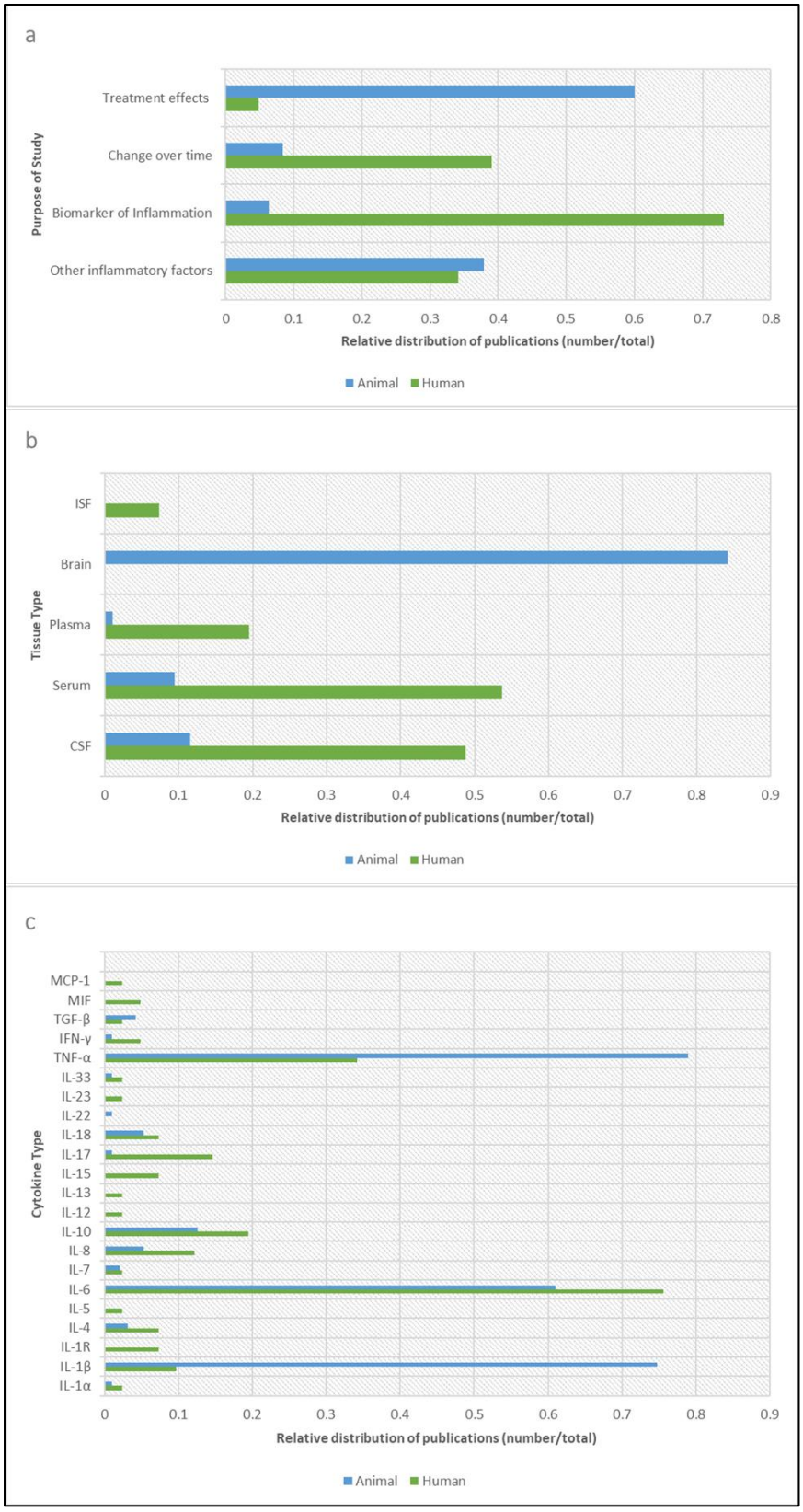

Figure 2-3. Key characteristics between human and animal studies compared.

Clinical Longitudinal Studies

Most research conducted on humans examined cytokine changes over time relative to the rupture but not relative to specific patient outcomes, a fact that also limits the translational capacity of the information (Bjerkne Wenneberg et al., 2021; Al-Tamimi et al., 2019). In our included papers, however, specific cytokines were well-considered as biomarkers of injury severity (Lenski et al., 2017; Ridwan et al., 2021; Kao et al., 2015). Increases in these specific markers (e.g., IL-1β, IL-6, and TNF- α), particularly in the acute (days) versus hyperacute (>24 hours) time period after initial injury, increased the likelihood of a poor outcome, but such effect was largely related to associations with stroke severity scores (Lv et al., 2018). This information underscores the deleterious effects that increased inflammation can have following aSAH.

Still, there is an overall dearth of the literature that demonstrates how these cytokines are associated with long-term recovery prognosis apart from just stroke severity. Most studies simply measured cytokine levels at 2 or more hyperacute timepoints (Roa et al., 2020; Gusdon et al., 2020). Long-term studies of cytokine change post-stroke are typically tested at 7, 10, or 14 days following rupture. The longest-term studies observed collected tissue 14 days after aSAH, but these studies were few (Niwa et al., 2016; Höllig et al., 2015). A better understanding of the relationship between cytokine changes and outcomes, as well as a better understanding of the longitudinal trajectory of these changes, would place this information in context for clinical use.

Tissue Considerations

In addition, there is a lack of multi-tissue studies conducted in preclinical models, so it becomes difficult to compare local neurological versus systemic response to rupture. For example, the inflammatory response as seen in the CSF may be significantly different than what is found in the brain tissue and blood following aSAH. In preclinical work, multiple tissue samples are easily overcome, a boon that is largely not available in human studies for ethical reasons. Although blood is easily collected and CSF may be collected from a ventriculostomy drain (common in standard of care for these patients), there are no studies that included post-mortem human brain tissue samples for analysis. When comparing results from preclinical and human studies the tissue of origin must always be taken into consideration, and this is one reason that a translation gap exists for preclinical and clinical work in this area.

Pitfalls of Animal Models

Further, common aSAH rupture models used may not be appropriately representative for the study of inflammatory response. The three most commonly used animal models for aSAH include blood or hemolysate injection or infusion, blood clot placement, and arterial puncture (Marbacher et al., 2019). Each model has its own pros and cons. While all are successful at showing the effect of inflammation resulting from

external forces creating a response in the brain, they are not accurate at mimicking the actual mechanical actions that take place during spontaneous vessel rupture, as what occurs during aSAH.

For example, the second most common model is the blood-injection aSAH model whereby blood is injected directly into the SAH space (Kamp et al., 2014). From an inflammatory perspective, external injection would create a different environment relative to an aneurysm rupturing from a blood vessel. Consequently, the cytokine change results generated from this model may not be fully generalizable to human subjects. This model also does not account for the ongoing perfusion of blood into the SAH space that would result in subsequent increases in intracranial pressure and further damage to the surrounding tissue.

Of the models represented in our review, the arterial puncture model may be the best for accurate recreation of what is seen in humans. While this model is still not a spontaneous rupture, it results in the destruction of a blood vessel and the ongoing perfusion of blood into the SAH space. Still, there may remain some differences in the inflammatory response due to the mechanical induction of this injury. Better and more representative models of vascular disruption should continue to be developed.

Cytokine Change as a Marker for Treatment Effectiveness

In general, most included preclinical studies utilized cytokine levels as a means to illustrate inflammatory response, with many considering the cytokine change alterations relative to pharmacologic administration (Wei et al., 2017; Zhang et al., 2016; Tu et al., 2018; Li and Han, 2018). In these studies, researchers often considered specific proteins of interest and only used cytokines for additional information regarding overall inflammatory profiles. Largely, the preclinical animal model work focused on cytokine change in response to the pharmacological action of drugs. Rolipram, rutin, and salvianolic acid administration all produced decreases in TNF-α, IL-6, and IL-1β (Hao et al., 2016; Gu et al., 2017; Peng et al., 2018). However, each therapeutic achieved this response in different ways. Rolipram worked by regulating the SIRT1 (Sirtuin 1)/NF-κB pathway, rutin by regulating the RAGE (receptor for advanced glycation end product)–-NF-κB pathway, and solvianolic acid by influencing ERK/P38/NRF2 signaling. In these studies, the three mentioned cytokines were used as markers for inflammation to better inform how these drugs work on influencing the signaling pathways involved in their production.

Behavioral assessment of outcomes and their relationship to cytokine changes were also utilized in these studies. Most animal studies relied on changes in modified neurobehavioral scores such as the modified Garcia score to measure how specific treatments improved outcomes following SAH (Tu et al., 2018; Liu et al., 2019). While others used more specialized tools, such as Morris water maze and rotarod performance tests, to see how treatments improved memory and motor function (Wang et al., 2020;

Zhang et al., 2020), the robustness and selection of these behavioral measures often depended on the exact questions the researchers were trying to answer.

The translational potential of this work is limited without first knowing the longitudinal effects of these pharmacologics on the relative pro-/anti-inflammatory balance that would produce optimal patient outcomes. In addition to these considerations, further preclinical studies specifically designed to inform how environmental factors (such as pre-stroke diet, fitness, and stress levels) affect the inflammatory pathways following aSAH would be very valuable.

Summary

Overall, there is a need for more research that focuses on cytokine changes across tissues and specifically how they can relate to outcomes and recovery following aSAH. Further, there remains a need for comprehensive research investigating the whole complement of cytokines to better understand the inflammatory environment post-aSAH. This includes considerations of the relationship between anti- and pro-inflammatory cytokines following injury. Animal models should be thoroughly considered for their limitations in translational capabilities for human subjects and for their acumen in accurately representing aneurysm rupture conditions. By conducting more comparative and controlled research, the translational gap between human and animal studies may be closed, allowing for the development of better treatments and improved outcomes for these patients.

CHAPTER 3. A COMPARATIVE STUDY OF THE INFLAMMATORY ENVIRONMENT FOLLOWING ANEURYSMAL SUBARACHNOID HEMORRHAGE

Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) is a dangerous form of stroke resulting from the rupture of an aneurysm that subsequently releases blood into the SAH space surrounding the brain. This release of blood results in inflammation and increased intracranial pressure, pressure that in turn causes further inflammatory cascades and the death of brain tissue —all major contributors to the high mortality and morbidity rates seen in this clinical population. After aSAH, the rapid increase in inflammation can lead to the development of detrimental secondary effects such as vasospasm, delayed cerebral ischemia, and secondary infarction. A better understanding of the inflammatory response, and the trajectory of that response during stroke recovery, has the promise to help guide treatment decisions and the development of future therapeutics.

In response to any injury, the immune system releases small molecules called cytokines to recruit immune cell activation. The balance of cytokines is dynamic due to continuous feedback from the immune system and localized responses in the area of the injury. Furthermore, the composition of the cytokine complement differs at the molecular, organ, and whole-host (i.e., systemic) levels (Kany, Vollrath, and Relja, 2019; Geginat et al., 2016; Cicchese et al., 2018). It is likely that unregulated balance of specific cytokines may play a role in the development of the secondary clinical sequelae post-aSAH; the trajectory of these changes may also play a role. Thus, a greater understanding of how these cytokines change over time and the relative levels of each cytokine (both systemically and locally) following aSAH are key pieces of information that are needed before these changes can be targeted in therapeutic interventions.

Pre-clinical animal models are commonly used for this type of inquiry. The two main types of animal models utilized for these experiments are the aSAH single/double-blood-injection model and the aSAH endovascular puncture model. The aSAH single-blood-injection model is generated by the direct injection of blood into the SAH space (Kamp et al., 2014; Lee et al., 2008; Jackowski et al., 1990). An advantage here is that an increase in intracranial pressure is observed; however, this does not recreate the inflammatory environment post-aneurysm rupture.

Possibly better for the measurement of the neuroinflammatory environment, the second model involves the mechanical puncturing of a blood vessel within or around the brain (Bederson et al., 1995; Höllig et al., 2015; Leclerc et al., 2018). This results in the destruction of a blood vessel that release blood into the SAH space, as is observed in humans with aSAH. It is from these models that interleukin-1β (IL-1β) has begun to be tested as a therapeutic target (Dinarello, 2018; Rothwell & Luheshi, 2000; Viana-Huete & Fuster, 2019; Lopez-Castejon & Brough, 2011). Yet, despite promise in animal models, many therapeutic trials have historically failed to jump the translational gap for

successful application in the human patient. One reason may be that these artificially created models may not fully represent the inflammatory environment that is seen in the human condition, as there is a dearth of side-by-side comparisons across species. The lack of data is especially noticeable when comparing the localized neuroinflammatory environment (i.e., CSF and brain samples) with the systemic inflammatory environment (i.e., blood) that is more easily accessible and thus may be most appropriate for measurement in clinical practice.

Here, we aimed to address this gap. Using an endovascular puncture model of aSAH in rats, we compared cytokine results in CSF and blood serum to those obtained in the same tissues taken from human aSAH patients. These direct comparisons allow a better understanding of how well this animal model represents the actual inflammatory environment observed in humans. We also explored the trajectory of cytokine change over time in our human samples. These results will help future work navigate that translational gap to improve post-aSAH outcomes.

Methods

Animal Model of aSAH

Sample

A total of 10 laboratory-grade Wistar rats (Charles rivers) were used. These animals were adult males with an average weight of 402 grams. Animals were housed in UTHSC's rodent animal facility and monitored by veterinary staff under standard conditions (temperature 21–25 °C, humidity 45–50%) on a 12:12 h light-dark cycle with ad lib access to standard food and water. All American Association for Laboratory Animal Science (AALAS) and institutional (IACUC) safety guidelines were followed throughout this experiment (UTHSC IACUC approval # 20-0138).

Experimental procedures, biospecimen collection, and handling

A sham procedure was conducted (n=4) or aSAH was experimentally induced (n=6) when rats were 12 weeks in age (equivalent to middle-aged adults, a typical time of onset of aSAH in human patients). All rats were first anesthetized (Isoflurane) and placed on a surgical platform with a heating pad to maintain body temperature. The rat's necks were then shaved, and the right internal carotid artery was surgically exposed. In the sham group, the carotid artery was then sutured closed. The aSAH group of rats underwent surgery following the procedures outlined in the Sheffield Model of Subarachnoid Hemorrhage (Veelken et al., 1995). Briefly, a nylon suture was inserted up the internal carotid artery until it reached the Circle of Willis. Once there, the suture was pushed forward, piercing a hole in the right anterior cerebral artery, resulting in a bleed in the ventral portion of the brain, a phenomenon similar to aSAH. Both sham and aSAH rats were then sutured closed and injected with 2 ml of Lactated Ringer's saline solution

subcutaneously at the scruff of the neck. All rats were then allowed to recover under a heating lamp. Upon transfer back to their cage, a nutritional gel was provided to ease recovery. After 24 hours, all animals were humanely euthanized following all institutional guidelines and the IACUC-approved protocol. Samples of CSF and plasma were then collected postmortem in both aSAH and sham groups. CSF was collected by carefully inserting an 18-gauge needle connected to surgical tubing and a syringe into the cisterna magna. A technique similar to this was previously established in mice but was adapted here for use in our rats (Lim et al., 2018). CSF samples were then centrifuged, and the supernatant was collected for further analysis. Blood was collected from the heart using a syringe, placed in an EDTA tube, and separated by centrifuge into plasma and buffy coat. All samples were then placed in long-term storage at -80°C until data analysis.

aSAH Clinical Samples

Sample and setting

This portion of the study capitalizes on the enrollment of a larger, longitudinal, and observational study of outcomes after aSAH that enrolled patients at the time of hospitalization for aSAH. Patients were screened for potential inclusion by the attending neurologist involved in their care at Methodist University Hospital. Inclusion criteria included: males and females over the age of 18, diagnosis of aSAH confirmed by angiography, Hunt/Hess grade greater than or equal to 2 and/or Fisher score greater than or equal to 3, and English-speaking. Patients were excluded for the presence of previous neurological disorders (including prior stroke), sepsis, or infectious or preexisting inflammatory disease that may affect cytokine levels and confound results. Pregnant women were excluded, as this may have confounded cytokine levels analysis through fetal circulation; patients with a history of blood transfusion within the previous 12 weeks were excluded for similar reasons. The attending neurologist involved in their care obtained verbal consent to be approached by research personnel who provided risks and benefits of participation and obtained informed consent from the patient or legally authorized representative. Demographic and clinical data were obtained from the electronic medical record, including age, race, sex, weight, height, treatment method, prior medical history, medications, comorbidities prior to aSAH, admission Glasgow Coma Scale (GCS) Score, Fisher score, and Hunt/Hess Grade. Blood samples were obtained via standard venipuncture by drawing an additional tube at the time of routine clinical blood draws. CSF was obtained noninvasively via an external ventriculostomy drain. This protocol was approved by the Institutional Review Board of the University of Tennessee Health Science Center, #15-03974-FB.

Biospecimen collection and handling

Both blood and CSF specimens were collected between 0400 and 0700 across 3 different timepoints in the acute recovery period (Days 1-3, 4-6, 7-9). After collection, the samples were placed on ice and transported to the laboratory. At the lab, the blood

and CSF were centrifuged and processed in a similar manner to the rat samples above. These components were also placed in long-term storage at -80°C until further analysis.

Measurement of Cytokine Levels

In both rat and human samples, measurement of the cytokine levels in the tissues was carried out using an ELISA-based multiplex assay run on a MESO QuickPlex SQ120. This machine is a high-quality, enzyme-linked immunosorbent assay instrument that allows multiplexing of cytokine markers with a rapid 90-second runtime on 96-well plates. Premade panels are commercially available for this instrument to multiplex for the detection of several different types of cytokines. For the rat samples, the V-PLEX Proinflammatory Panel 2 Rat Kit (Cat No. K15059D-1) was utilized. This panel analyzed levels of FN-γ, IL-1β, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-13, and TNF-α. Note that multiple studies have shown that KC/GRO, a chemoattractant for neutrophils, serves as a related protein and representative marker for IL-8 in rodent samples (Dunstan et al., 1996; Shiratori et al., 1994; Merz et al., 2003). These cross-sectional results were then compared to that of the human samples as described below.

For the human experiment, the panel was a custom UPLEX Panel developed to measure known neuroinflammatory biomarkers in human plasma and CSF. Similar assays have been successfully utilized in a range of several prior studies. These include investigations that range from the effect of cytokines in the development of Alzheimer's disease and glaucoma to the role they play in chronic traumatic encephalopathy (Cherry et al., 2017; Gupta et al., 2017). The cytokines measured on our panel included IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, IL-33, and TNF-α. For the human samples, all three timepoints were run and the results collected. The resultant cytokine levels were assessed by timepoint and then compared over time within and between subjects for associations with clinical and demographic characteristics.

All samples for this project were prepared in duplicate to allow for technical and biological replicates. Built-in quality controls are included in the assay plates for each marker.

Statistical Analysis

The software program MSD Discovery Workbench 4.0.13 was utilized to extract and analyze the raw cytokine levels collected by the MESO QuickPlex SQ 120 instrument. For clarity in the description of these studies, those cytokines that were tested, but which were not present/were undetectable in any of these samples, were not reported on in this article (this excludes FN-γ, IL-5, IL-13, IL-17A, & IL-33). After removing those cytokines, the raw data were exported for additional statistical analyses conducted using R statistical software, version R-4.1.2 (Ihaka & Gentleman, 1996; Ripley, 2001). In rats, two-sample t-tests compared results between the sham and aSAH groups. For the human group, absolute cytokine levels were measured as well as the

trajectories across the timepoints. The means, standard deviations, and univariate associations for clinical and demographic factors were considered. The cytokine levels were then modeled in robust linear regression, with demographic/clinical variables serving as predictors. All tests were two-sided, and statistical significance was set at a p value of less than or equal to .05 without any multiple testing adjustment.

Results

Animal Model Results

In the rat model of aSAH, cytokine levels between CSF and plasma were qualitatively similar. In both tissues, levels of IL-6 and KC/GRO were significantly ($p<0.05$) higher than all other cytokines. In the CSF, KC/GRO was present at a concentration of 93.89 pg/ml, while IL-6 was present at a concentration of 76.55 pg/ml. These are 4.3 and 3.5 times larger, respectively, than the next most present cytokine, IL-10 (21.72 pg/ml); the remaining cytokines were present at ~1/2 to 1/3 of this lower concentration **(Figure 3-1a)**. In rats, the inflammatory environment in CSF appears to be more diverse than that in plasma; IL-4 and IL-1β were present in CSF samples but were not detected in our plasma samples. The remaining cytokines were present in plasma, with KC/GRO at the highest concentration (95.97 pg/ml) and IL-6 present at 78.33 pg/ml. TNF-α was present at low levels in the plasma samples (3.29 pg/ml) **(Figure 3-1b)**.

When comparing the differences in cytokine levels between the sham and aSAH rats, some differences were observed. Specifically, average KC/GRO levels in the CSF of aSAH rats (93.89 pg/ml) were significantly higher than that of sham rats (43.88 pg/ml, $p=0.013$). A similar phenomenon was observed in the IL-6 CSF levels of aSAH rats (76.55 pg/ml) compared to sham rats (40.46 pg/ml), though this result was nonsignificant ($p=0.392$) (**Table 3-1**).

Human Results

Demographic and clinical information

Our 13 subjects were an average of 52 years old (IQR: 49~58), 54% African American, and 54% female with a median Hunt/Hess Grade 3 and Fisher score 4. Average BMI was 31.77, and about half were smokers (53.85%). Over half the sample had been diagnosed with cardiac disease prior to the aSAH (53.85%); nearly half had some form of pulmonary disease (46.15%); and roughly a third had a prior history of other neurological disease (30.77%), diabetes (30.77%), or gastrointestinal disease (38.46%) (**Table 3-2**).

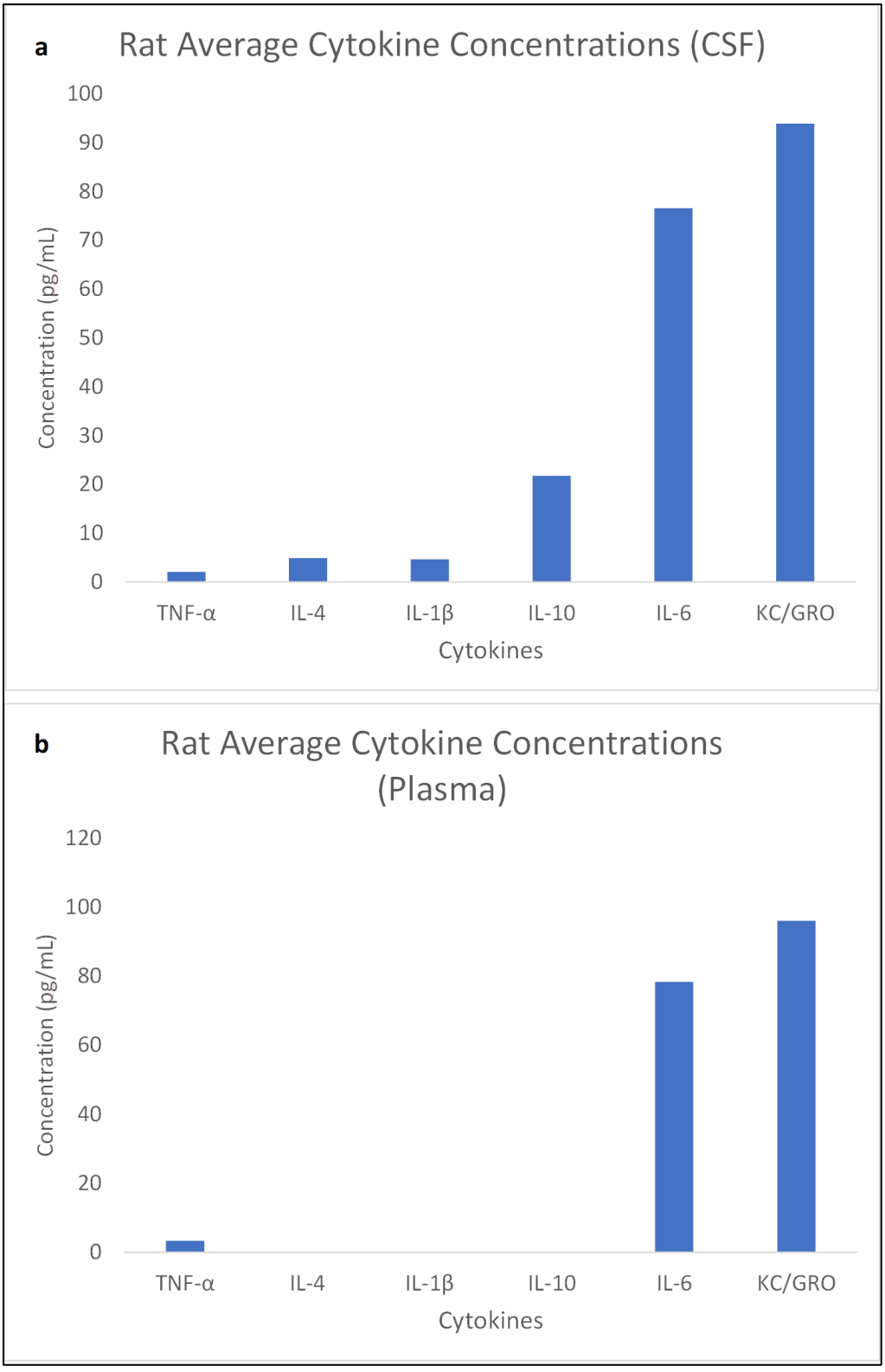

Figure 3-1. The average cytokine concentrations in rats following aSAH surgery.

Table 3-1. Comparison of SHAM and SAH model cytokine concentrations.

Sample	Cytokine	Mean_ SAH	Mean_ Sham	t	d.f.	P Value
CSF	IL-6	76.55	40.46	1.01	2.83	0.3928
	KC/GRO	93.89	43.88	3.74	5	0.0134
	IL-1ß	3.95	4.98	-0.78	1.86	0.5214
Plasma	IL-6	78.33	129.05	-1.83	5.99	0.1168
	KC/GRO	95.97	101.42	-0.06	6.41	0.9533
	TNF-a	3.25	2.96	0.39	6.66	0.7103

Notes: CSF= Cerebrospinal fluid, SAH= Subarachnoid Hemorrhage, d.f.= degrees of freedom.

Table 3-2. Demographics of the study cohort.

Characteristic	Levels	N (%) or Median (IQR)
Age		52 (49~58)
Sex	Female	7 (53.85%)
	Male	6 (46.15%)
BMI		31.77 (19.13~39.54)
BMI category	Normal Weight	3 (23.08%)
	Obese	7 (53.85%)
	Underweight	3 (23.08%)
Race	African American	7 (53.85%)
	Caucasian	6 (46.15%)
Fisher Score	3	4 (30.77%)
	4	9 (69.23%)
Hunt Hess Grade	2	5 (38.46%)
	3	7 (53.85%)
	4	1 (7.69%)
Prior history of cardiac disease	No	6 (46.15%)
	Yes	7 (53.85%)
Prior history of pulmonary disease	No	7 (53.85%)
	Yes	6 (46.15%)
Prior history of neurological disease	No	9 (69.23%)
	Yes	4 (30.77%)
Diabetes	No	9 (69.23%)
	Yes	4 (30.77%)
Gastrointestinal disease	No	8 (61.54%)
	Yes	5 (38.46%)
Smoking history	No	6 (46.15%)
	Yes	7 (53.85%)

Notes: IQR= Interquartile range, BMI = body mass index.

Human cytokine levels

As in rats, IL-6 and IL-8 levels were significantly higher than all other cytokines in both tissues ($p<0.05$). In CSF, IL-8 was the most present in our sample (1022.89 pg/ml) followed by IL-6 (745.87 pg/ml) **(Figure 3-2a)**. The next most present cytokines in our samples were at much lower concentrations (IL-1β=3.97 pg/ml and TNF-α=2.29 pg/ml). Similar average relative concentrations were also seen in plasma (IL-6=8.65 pg/ml, IL-8=7.86 pg/ml, TNF-α=1.47 pg/ml; **Figure 3-2b**). When compared for concentrations across tissues, the concentration levels of IL-1β, IL-6, and IL-8 were significantly higher in CSF as compared to plasma ($p<0.001$). IL-10, IL-4, and TNF-α were not significantly different across tissues ($p >0.05$) (**Table 3-3**).

Trajectory of human cytokine levels following aSAH

Cytokine levels were also assessed for changes over time across three timepoints in both tissues. IL-10 showed significant decreases in levels over time in CSF (β=-0.57, $p= 0.007$). IL-6 and TNF-α showed a similar downward trend, but this was not statistically significant (**Table 3-4**). Conversely, in plasma, levels of TNF-a ($p= 0.008$) were found to increase over time, with the observed differences between timepoint 1 to 3 being quite marked ($p= 0.017$) (**Table 3-5**).

Across both tissues, IL-8 appears to have the largest and most consistent increase following aSAH **(Figure 3-2)**. However, this trajectory did not reach significance for either tissue. Still, in plasma, comparisons of timepoint 1 to 2 and timepoint 1 to 3 both reached significance ($p=0.037$ and $p= 0.008$, respectively).

Association of cytokine levels with demographic and clinical factors

The association of various cytokines with patient demographics and clinical factors was then analyzed. In CSF, cytokine change was associated with the demographic factors of age, race, and sex **(Table 3-6)**. Older age was associated with cytokines IL-1β ($p= 0.035$), IL-6 ($p= 0.063$), and IL-8 ($p= 0.041$). TNF-α ($p= 0.039$) and IL-4 ($p= 0.036$) cytokine levels were associated with male sex, while stroke severity as measured by Fisher Scale was found to be associated with IL-1β ($p= 0.021$) and TNF-α ($p= 0.009$) levels. Lastly, higher levels of IL-1β ($p= 0.029$) were seen in Caucasian patients.

Interestingly, in plasma, the tested cytokines were often associated with prior medical history such as: diabetes (IL-8, $p= 0.040$), smoking (IL-8 $p= 0.027$), history of pulmonary disease (IL-4, $p= 0.013$; and IL-6, $p= 0.061$), and history of gastrointestinal disease (TNF-α, $p= 0.016$) **(Table 3-7)**. In plasma, higher BMI was also found to be associated with IL-10 ($p= 0.0066$).

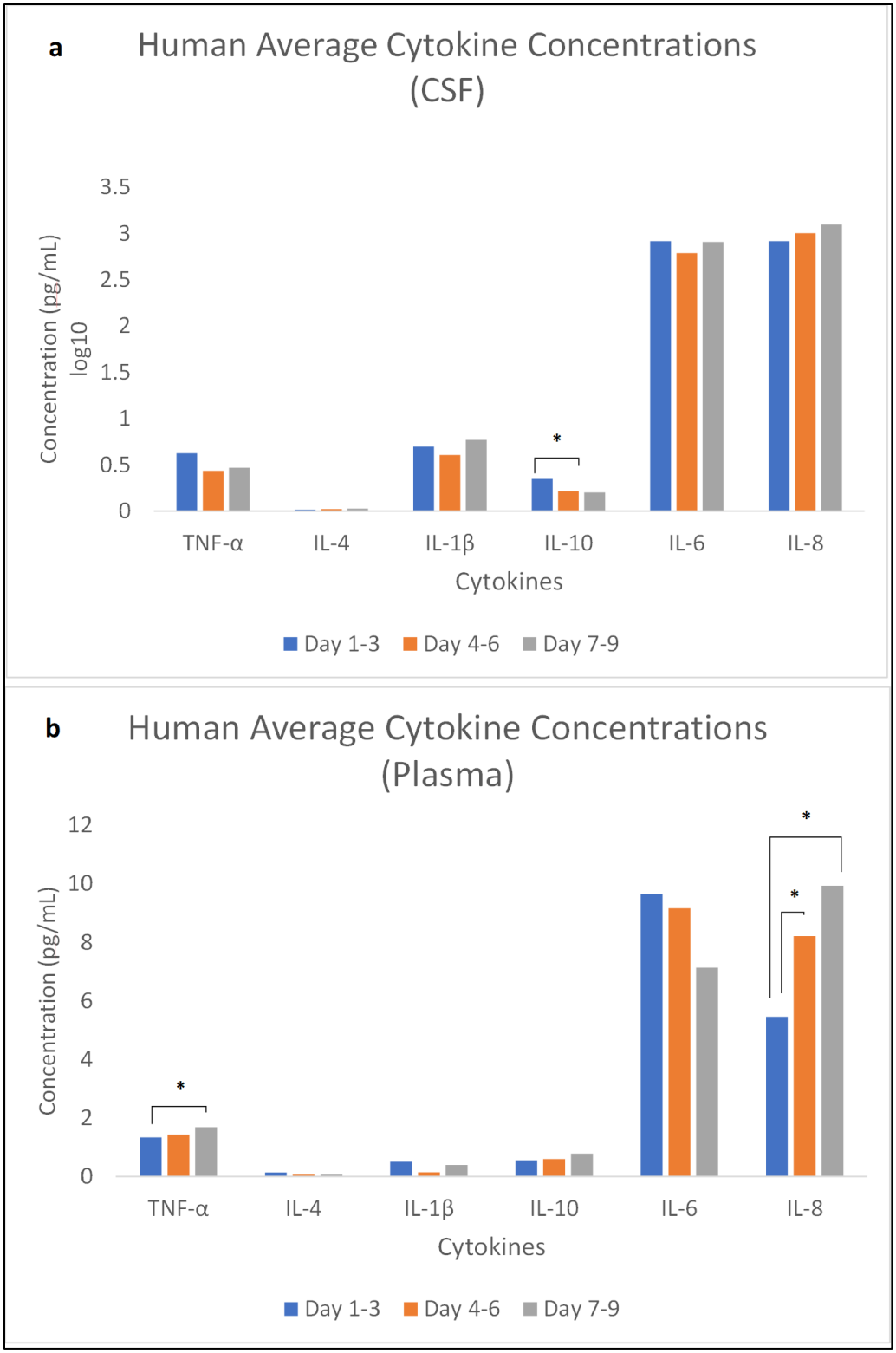

Figure 3-2. The average cytokine concentrations over time in patients following aSAH.

Table 3-3. Comparison of cytokine levels of plasma versus CSF in patients after aSAH.

Cytokine	Estimate	CI95	P Value
IL-10	0.274	-0.21 ~ 0.757	0.2714
IL-1ß	-2.523	-2.989 ~ -2.058	0.00001
IL-4	0.044	-0.361 ~ 0.45	0.8309
IL-6	-3.866	-4.491 ~ -3.241	0.00001
IL-8	-4.464	-4.89 ~ -4.038	0.00001
TNF-a	0.089	-0.256 ~ 0.433	0.6157

Table 3-4. Time trend changes after aSAH in CSF.

Cytokine	Estimate	CI95	P Value
IL-10	-0.574	-0.952 ~ -0.195	0.0066
IL-1ß	0.137	-0.264 ~ 0.537	0.5095
IL-6	-0.210	-0.775 ~ 0.356	0.4744
IL-8	0.199	-0.247 ~ 0.645	0.3903
TNF-a	-0.353	-0.736 ~ 0.03	0.0828
IL-4	0.215	-0.109 ~ 0.54	0.2073

Table 3-5. Time trend changes after aSAH in plasma.

Cytokine	Estimate	CI95	P Value
IL-10	0.179	-0.036 ~ 0.393	0.1152
IL-1ß	0.048	-0.292 ~ 0.389	0.7830
IL-4	0.000	-0.353 ~ 0.352	0.7830
IL-6	-0.222	-0.516 ~ 0.072	0.9979
IL-8	0.255	0.082 ~ 0.427	0.1509
TNF-a	0.113	0.026 ~ 0.199	0.0079

Table 3-6. Association of patient cytokine levels with clinical factors in CSF.

Cytokine	Clinical Factor	Change	CI95	P Value
IL-1β	Age	0.034	0.003 ~ 0.064	0.0354
IL-1β	Race	0.721	0.097 ~ 1.344	0.0294
IL-1β	Fisher	-0.824	-1.492 ~ -0.156	0.0206
IL-6	Age	0.075	0.004 ~ 0.145	0.0631
IL-8	Age	0.05	0.008 ~ 0.093	0.0408
TNF-α	Sex	-0.685	-1.314 ~ -0.057	0.0391
TNF-α	Fisher	-0.916	-1.572 ~ -0.261	0.0094
IL-4	Sex	-0.579	-1.084 ~ -0.074	0.0357

Table 3-7. Association of patient cytokine levels to clinical factors in plasma.

Cytokine	Clinical Factor	Change	CI95	P Value
TNF-α	GI	-0.462	-0.782 ~ -0.142	0.0163
IL-8	Smoking	0.436	0.102 ~ 0.77	0.0265
IL-8	Diabetes	-0.444	-0.816 ~ -0.071	0.0395
IL-6	Pulmonary	0.905	0.054 ~ 1.757	0.0612
IL-4	Pulmonary	-0.727	-1.271 ~ -0.184	0.0137
IL-10	BMI	0.04	0.017 ~ 0.064	0.0066

Notes: GI = gastrointestinal, BMI = body mass index.

Discussion

The overall goal of our research was to better understand the inflammatory response that takes place following aSAH. In this project at hand, we elected to investigate cytokine change due to these molecules' important role in immune cell recruitment and signaling. Along the same lines, we utilized two different tissue types, CSF and plasma, to better characterize the inflammatory response locally and systemically. Overall, our results suggest that the aSAH inflammatory response in rats is similar both systemically and localized. Humans show a diverse cytokine environment in both tissues, with the largest concentration of cytokines being found in the localized environment obtained from CSF.

We conducted our investigations in both rat models and human subjects to better understand the differences between these species and how they may impact the translation of results found in animal models for improving treatment for human patients. Across species, the two cytokines with the highest concentrations were IL-6 and IL-8 (KC/GRO in rats). An interesting result in human samples was that while the CSF levels of IL-6 and IL-8 were significantly higher than what was found in plasma, the levels of TNF-a, IL-1β, IL-4, and IL-10 were similar across both tissues. This suggests the cytokines IL-6 and IL-8 could play a greater role in regulating the localized inflammatory response, relative to the other cytokines.

Role of IL-6

IL-6 is a well-established, pro-inflammatory cytokine. It is secreted by osteoblasts, serves as a myokine, and is secreted by macrophages (Nara & Watanabe, 2021). Unsurprisingly, it has been found to stimulate the inflammatory response in conditions such as diabetes, Alzheimer's disease, atherosclerosis, and intracerebral hemorrhage (Dayhoff-Brannigan et al., 2008; Wright et al., 2006; Leasure et al., 2021). However, IL-6 has also been shown to control local and systemic anti-inflammatory responses, as it has been found to stimulate the production of IL-1 receptor antagonists and activate STAT3 (in models of cancer), both of which are anti-inflammatory mediators (Gabay, 2006; Scheller et al., 2011). Here, this cytokine may play a multifaceted role. After aSAH, several other organ systems are affected in the aftermath of rupture, requiring a system-wide inflammatory response. This generalized oxidative stress may be one of the reasons why we see such an increase in this particular cytokine.

Role of IL-8 (KC/GRO)

The cytokine IL-8 also plays an important role in the inflammatory response by inducing a local, large, and long-lasting accumulation of neutrophils to the site of damage (Bernhard et al., 2021; Harada et al., 1994). These neutrophils play an important role in stroke recovery following ischemic stroke where they can result in the elevation of cerebral edema, brain injury, and blood-brain barrier disruption (Jickling et al., 2015).

Here, our results indicate that elevations in IL-8 may serve a similar role in aSAH. Additionally, it has been found that elevated levels of IL-8 are seen in patients with diabetes and in those who have a history of smoking (Cimini et al., 2017; Mio et al., 1997). Our results here are consistent with this previous work. These risk factors that contribute to aSAH onset, in turn may also contribute to the dysregulated inflammatory response that is observed after aneurysm rupture. Lastly, our study was able to show that there was a significant difference for KC/GRO levels in CSF between the sham and aSAH surgery groups of rats. This illustrates, that the surgery does produce a similar inflammatory response to aSAH in vivo.

Change in Cytokine Levels Over Time

Three of our investigated cytokines (IL-10, IL-8, and TNF-a) demonstrated change over time, with all fluctuating over the course of the first nine days post-rupture in aSAH patients. It is known from the literature that IL-10 is an anti-inflammatory cytokine that is important for controlling the inflammatory response. In our human CSF samples, we observed a significant decrease of IL-10 over time (**Figure 3-2a**). This illustrates that after aSAH, there is most likely an increase in localized inflammatory response that can be measured in the CSF. In other words, in response to the hemodynamic disturbances and tissue damage taking place, the immune system responds by decreasing the levels of IL-10 in the localized area of rupture. Supporting this thought, it has been found that increased levels of IL-10 can negatively affect the response to associated hemodynamic disturbances and tissue damage (Iyer & Cheng, 2012).

In plasma, we observed a consistent increase of IL-8 and TNF-a over time. As previously discussed, IL-8 is responsible for neutrophil response and recruitment. So, because of aSAH disturbance, pro-inflammatory IL-8 levels increased over time, compensating for the dysregulation. On the other hand, TNF-a is released by macrophages and plays an important role in systemic inflammatory response that involves pro-apoptotic and pro-inflammatory signaling cascades (Parameswaran & Patial, 2010). In our patients, this release takes place system-wide in the plasma in response to aSAH onset in order to induce appropriate inflammatory response. IL-8 and TNF-a are both proinflammatory and increase over time. This increase is appropriate and expected, given the roles we know these two cytokines play in inflammatory regulation. The specific cytokine changes that we have observed in our tissues are consistent with the roles we know these cytokines play in other conditions. Likewise, the change trajectory we have observed following aSAH is consistent with the role we know they play in systemic circulation.

Cytokine Change Associated with Clinical and Demographic Factors

Our results also show that cytokine levels and inflammatory response are associated with several other demographic and clinical factors. Past medical conditions, age, sex, and race can all influence the inflammatory response. Inflammatory conditions,

such as a history of smoking, pulmonary disease, gastrointestinal disease, obesity, and diabetes were associated with both absolute cytokine levels and change over time in our human samples, both in CSF and in plasma. These results are consistent with the other literature on the topic and may not reflect associations with only the aSAH condition itself. For example, TNF-a has been found to play an important role in gut-related diseases and intestinal homeostasis (Ruder et al., 2019) while low IL-10 levels have been found to be associated with metabolic syndrome in obese women (Esposito et al., 2003). Such preexisting conditions may alter cytokine levels and change, both within the context of aSAH and independent of its effects.

Alternatively, we found that cytokine levels in CSF were relative to demographic factors such as age, sex, and race. This association may be one possible biological correlate of the demographic associations with mortality and morbidity rates for this clinical group. For example, both IL-1ß and IL-6 levels have been previously shown to be predictors of poor outcomes following ischemic stroke (Shaafi et al., 2014; Sobowale et al., 2016). Taken together, the specific cytokines and their levels, along with other clinical and biological factors, may someday serve as predictive markers for recovery outcomes post-aSAH.

Translational Implications

Taken together, our results paint an interesting picture for the translational implications of this work. First, they illustrate that shared cytokines may be drivers of the inflammatory response following aSAH. In both species, IL-6 and IL-8 (KC/GRO) were shown to be the principal cytokines present. However, there appears to be a difference in the levels and characteristics of inflammatory response in the different tissues. In humans, the response seems to be more localized to the CSF, while in the rat models, the response was more mixed in nature. This could be due to unique genetic differences, differences in leukocyte composition, difference in size between rats and humans, or the surgical aSAH model itself.

Lastly, the difference could also be due to any anti-inflammatory treatments given to patients during hospitalization. Many of these treatments are not able to cross the BBB, and most are not given via the intrathecal route. As a result, systemic inflammation is controlled while localized inflammation continues. This could potentially have altered our results.

However, our results have interesting translational implications overall. It appears that IL-6 and IL-8 (KC/GRO) behave similarly in both species and can be possibly targeted for future treatment development. For example, one animal study showed that administration of a novel selective proinflammatory cytokine inhibitor (CNI-1493) decreased IL-6 levels and reduced the likelihood of vasospasm post-aSAH (Bowman et al., 2006). It reasons that such inhibitors may also be used to reduce other secondary sequelae, and these may improve recovery outcomes. However, such therapies may have marked off-target effects, and much research remains to be done.

Conclusions

Our study illustrates that the inflammatory environment present following aSAH is primarily modulated by cytokines IL-6 and IL-8, though other several other cytokines are also present in lower levels. It appears that the inflammatory environment of both rats and humans is similar while there are specific differences that arise across the two tissues examined here.

Despite our interesting results, there remains a need for more research in this area. Specific areas for inquiry include further information on cytokine changes across tissues along with more detailed information about how these changes relate patient outcomes and recovery following aSAH. This includes considerations of the relationship between and balance of anti- and pro-inflammatory cytokines following aneurysm rupture. In such work, animal models should be critically examined for their limitations in translational capabilities for human subjects and for their acumen in accurately representing aneurysm rupture conditions.

CHAPTER 4. VERAPAMIL INHIBITS TXNIP-NLRP3 INFLAMMASOME ACTIVATION AND PRESERVES FUNCTIONAL RECOVERY AFTER INTRACEREBRAL HEMORRHAGE IN MICE[2]

Introduction

While intracerebral hemorrhage (ICH) accounts for only 10-15% of all strokes, mortality rates after ICH can reach >50%, and approximately 74% of those who do survive experience severe disabilities and post-stroke cognitive impairment (van Asch et al., 2010). Unfortunately, surgical intervention is the only option, as there are no effective therapeutic treatments for ICH (Broderick et al., 2007; de Oliveira Manoel, 2020). The resulting hematoma within brain parenchyma induces a series of adverse events, causing primary and secondary brain injury. Primary brain damage includes edema, which induces mass effects and increased intracranial pressure that can lead to herniation and death. Secondary brain damage results from activation of microglia, which drives pro-inflammatory cytokines as well as oxidative and cytotoxic cascades that lead to cell death and functional impairment (Shao et al., 2019). These inflammatory and oxidative factors also stimulate expression of matrix metalloproteinases, a gene family of extracellular matrix enzymes, which degrades junction proteins like zonula occludens-1 (*ZO-1)* and increases the blood-brain barrier (BBB) permeability after stroke (van Asch et al., 2010) and ICH (Keep et al., 2008) to create further damage and worsen prognosis. Hence, there is an urgent need to better understand the pathways/targets that are active after ICH to develop effective therapies.

Although the precise mechanisms underlying secondary brain injury after ICH are complex and poorly understood, several studies have suggested that inflammatory responses are likely to be a prominent early feature in the pathogenesis of ICH (Broderick et al., 2007; Shao, Tu, and Shao, 2019). Microglial cells play critical roles in the pathophysiology of ICH by regulating inflammation, innate immune response, and hematoma resolution, the key determinants of secondary brain damage and functional recovery after ICH (Broderick et al., 2007). Thioredoxin-interacting protein (TXNIP) is a binding partner of thioredoxin (TRX) and functions as a negative regulator of the TRX reductase activity (Nasoohi et al., 2018). TXNIP is an intriguing candidate molecule that links glucose/oxidative stress and inflammation to cellular injury, making it a multifaceted target for neurovascular degeneration (Ishrat et al., 2015;Mohamed et al., 2015; Nasoohi, Ismael, and Ishrat, 2018; Nasoohi, Parveen, and Ishrat, 2018). Recently, we and others demonstrated that ischemic stroke-induced TXNIP upregulation is associated with increased brain damage and a higher incidence of hemorrhagic transformation (HT) in hyperglycemic conditions, an important comorbidity associated with increased risk of hemorrhage and poor stroke recovery (Capes et al., 2001; Hafez et al., 2014; Ismael et al, 2020). TXNIP also participates in early brain injury after subarachnoid hemorrhage

[2] Reproduced from initial submission to a journal with the authors' approval. Devlin P,* Ismael S,* Ishrat T, Stanfill AG. Verapamil inhibits TXNIP-NLRP3 inflammasome activation and preserves functional recovery after intracerebral hemorrhage in mice. *Co-first authors.

(SAH) by mediating inflammation (Tsubaki et al., 2020; Zhao et al., 2017). Moreover, TXNIP is required for activation of the NOD-like receptor protein 3 (NLRP3)-inflammasome, a multi-protein complex involved in instigating inflammation and immune regulation in several neurovascular injury models including ICH (El-Azab et al., 2014; Ishrat et al., 2015; Mohamed et al., 2014; Mohamed et al., 2015; Nasoohi et al., 2018). The NLRP3 inflammasome is composed of oligomers of the pattern recognition receptor NLRP3, apoptosis-associated speck-like (ASC) adapter protein, and the downstream effector enzyme pro-caspase-1. Following NLRP3-inflammasome activation, NLRP3 responds by recruiting and assembling with the ASC, which in turn recruits pro-caspase-1, causing its cleavage and activation into active cleaved caspase-1 (Cl-Cas-1). Activated cleaved caspase-1 then cleaves pro-IL-1β into the active cleaved IL-1β (Cl-Cas-1) form, which exerts its well-founded detrimental pro-inflammatory and pro-apoptotic effects upon release into the extracellular environment (Martinon et al., 2002). Moreover, NLRP3 activation is mainly located in microglia (Yang et al., 2014; Ye et al., 2017), which are among the first non-neuronal cells on the scene during the innate immune response to ICH. Together, these findings suggest that TXNIP is a promising new therapeutic target, serving as an upstream molecular link between inflammation and neurovascular damage.

Although TXNIP lacks a specific pharmacological inhibitor, we and others have demonstrated that verapamil is an effective inhibitor of TXNIP in vivo (Al-Gayyar et al., 2011; Chen et al., 2009). Verapamil is a commonly prescribed phenylalkylamine L-type calcium (Ca^{2+}) channel blocker (CCB) with nonspecific TXNIP inhibitor properties and has been approved by the FDA for treatment of angina, hypertension, and arrhythmias (Popović et al., 2020). It is also used as the first line in the prevention of cluster headaches (Petersen et al., 2019). Pharmacological studies have shown that verapamil has a wide therapeutic spectrum, including antihypertensive, anti-inflammatory, and antioxidative effects and regulation of the BBB function, due to its effect on P-glycoprotein as well as adjustment of cellular Ca^{2+} homeostasis (Ismael et al., 2021a; Ismael et al., 2021b; van Assema et al., 2012). Recent studies from our group and others have indicated a potential positive effect of verapamil in neurological disorders, including cognitive deficits and ischemic stroke (Ismael et al., 2021a; Ismael et al, 2021b; Jackson et al., 2018; Melone et al., 2018; Popović et al., 2020).

Our recent study supports the use of verapamil as a safe adjunct therapy in thrombolytic approaches for neurovascular complications (such as HT) associated with hyperglycemic conditions after ischemic stroke (Ismael et al., 2021). It reasons that similar benefits may be observed during the administration for ICH. Therefore, this study will be the first to determine whether verapamil affects the TXNIP-NLRP3 axis' contribution to neuroinflammation and long-term functional recovery after ICH, which will lead to the development of new therapeutic strategies for hemorrhagic stroke.

Methods and Materials

Experimental Procedure and Treatment Regimen

All animal experiments conformed to standard procedures approved by the Institutional Animal Care and Use Committee (IACUC) at UTHSC. The studies were reported in accordance with ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines (Kilkenny et al., 2010). Adult (10–12 weeks old) male C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed in standard conditions of humidity (45–50%), temperature (21–25 °C), and a 12-h light/dark cycle with ad lib access to food and water. Animals were assigned to three experimental groups: 1) Sham + saline, 2) ICH + saline, and 3) ICH + verapamil, n=5-9/group. Verapamil (Sigma, USA) was dissolved in sterile saline and acutely administered (0.15 mg/kg, intravenously) 1 h post-ICH followed by oral administration (1mg/kg/day) in drinking water. The verapamil dose was determined based on previous publications (Fraser et al., 2017; Maniskas et al, 2016). The animals were evaluated for two endpoints: (1) 72 hours, to study the acute effect of TXNIP modulation in ICH (n=7/group); and (2) 30 days, to evaluate long-term functional outcome (n= 5-8/group). The experimental design is depicted in **Figure 4-1**.

Induction of ICH in Mice

ICH was induced in adult male C57BL/6 mice, as reported previously (Ahmed et al., 2018). Briefly, experimental animals were anesthetized using 3% isoflurane and maintained with 1.5–2% during surgery. Animals were then placed on a stereotaxic head frame, and a burr hole (0.5 mm) was made on the skull 2.2 mm lateral to bregma. Intrastriatal injection of bacterial type IV collagenase (0.045 U in 1 μL PBS, Sigma, St. Louis, MO) was completed with stereotaxic guidance for 3.0 mm into the right striatum by using a 26-G Hamilton Syringe. The temperature was monitored and maintained at 37±2°C during surgery using a homeothermic heating blanket (Kent Scientific). The animals were allowed to recover from anesthesia on the heating pad and then returned to their home cages. Sham-operated animals were subjected to a similar surgery procedure without collagenase injection.

Rotarod

Motor impairment was evaluated using an accelerating rotarod (Med Associates, Inc., VT, USA). Rotarod tests were performed according to our previous studies (Ishrat et al., 2009). All animals were given 3 training sessions 10 minutes apart before surgery to establish baseline performance. Animals were first habituated to the stationary rod and then exposed to the rotating rod at basal speed. The rod was started at 3 revolutions per minute (rpm) and accelerated linearly to 30 rpm within 300 seconds. Latency to fall off the rotarod was then determined before ICH (pre-surgery) and at 72 hours post-ICH. To

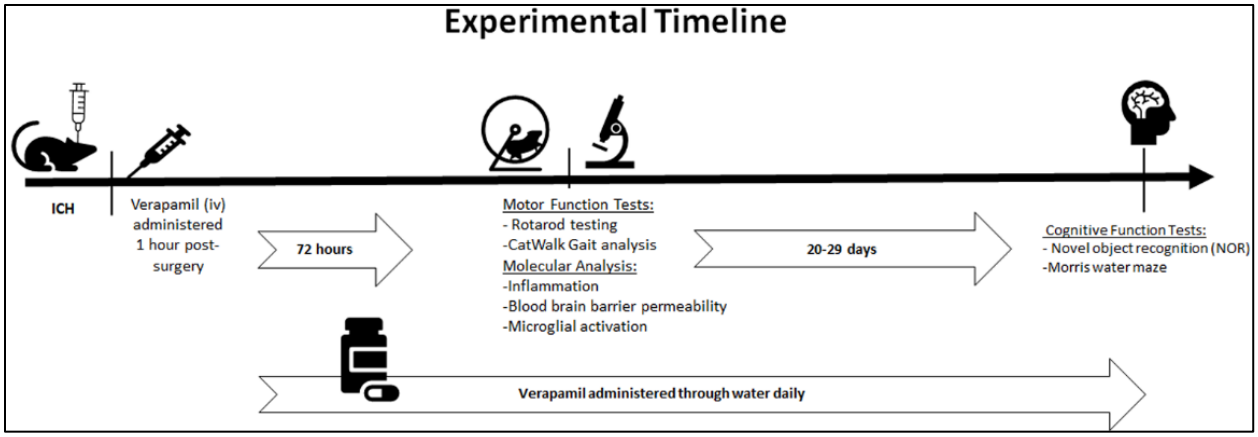

Figure 4-1. Schematic representation of experimental design.

complete testing, animals were required to stay on the accelerating rod for a minimum of 30 seconds. If they were unable to reach this criterion, the trial was repeated a maximum of three times. The two best (largest) fall latency values an animal could achieve were then averaged and used for data analysis. Animals not falling off within 5 min were given a maximum score of 300 seconds.

CatWalk Test

The Catwalk test was performed to assess gait control parameters in animals following ICH, which is intimately affected by sensorimotor frailty (Herbin et al., 2007). The Catwalk XT is an advanced video-based gait analysis system that dynamically measures the footprints of voluntarily moving animals. The system records animals' footprint images, and foot force profiles are captured by a camera mounted underneath a glass walkway (Ismael et al., 2018). These initial recordings work as inputs into the CatWalk XT software package (Noldus Information Technology, Wageningen, The Netherlands) to calculate the specified indices.

Basic measures of gait cycles were obtained by the Catwalk system to automatically calculate several variables including "Gait/Step cycle," "Paw intensity," and "Inter-paw coordination," each detailed in the supplementary materials. Step cycle indicates the time needed to complete a single cycle of a step, with the duty cycle being the percentage of the whole step cycle covered by the stance phase. Duty cycle is described by percentage of the step cycle. Paw intensity reflects the maximal weight over the paws and mirrors any gait shift during the gait cycle. Finally, "Inter-paw coordination," mostly addressed by phase dispersion, reflects gait shift derangement, galloping, or limping by comparing anchor paws' step cycles.

Novel Object Recognition (NOR) Test

Based on the spontaneous tendency of rodents to explore and interact with a new object, the novel object recognition test (NOR) was utilized to evaluate animals' non-spatial, short-term working memory (Ishrat et al., 2006). Through a standard test procedure, animals were allowed to explore two identical objects (acquisition trial), one of which was replaced in the next phase (preference trial). In the habituation phase (10 minutes) conducted 1 day before the start of the test, animals were allowed to acclimate to an empty standard-sized box (45x45x35cm). On the designated test day, animals were first presented with 2 identical objects and for 5 minutes were allowed to explore the objects that were placed equidistant from the walls of the box, in the center, and spaced 20 cm apart (acquisition trial).

Following sample object exposure, the animals were returned to their home cage for a 90minute retention period. The ^{2n}d preference/test trial (5 minutes) was conducted in the same manner as the first trial except that a new/novel object replaced one of the familiar/sample objects (preference trial). The interval between sample and test trials was

adjusted to 5 minutes for selective testing of short-term working memory. The time animals spent exploring familiar and new objects were recorded to calculate recognition index (RI) as indicators of working memory using the following formula (**Equation 4-1**):

$$\text{Recognition index (RI)} = T_N / (T_N + T_F) \qquad \textbf{(Eq. 4-1)}$$

where T_F and T_N are the times spent interacting with the familiar and novel object, respectively.

Morris Water Maze (MWM) Test

The Morris water maze (MWM) was used to assess animals' spatial learning and memory through a combination of training and probe trials (Ahmed et al., 2018). The initial learning/training phase consisted of 28 total trials. These were conducted as single daily sessions of 4 trials, 60 seconds each, and a trial interval of approximately 30 seconds for 7 consecutive days. In brief, training trial animals were allowed to learn the location of the hidden escape platform submerged 2 cm below the surface of the water (25 ± 2 °C) in a circular water tank (132 cm diameter and 60 cm height) divided into 4 virtual quadrants. On each trial day, animals were allowed to swim freely on four trials (once from each starting position). If the animal failed to reach the escape platform within the maximal allowed time of 60 seconds, it was gently placed on the platform and allowed to remain there for 30 seconds. Fixed flags on particular quadrants were used as spatial cues for the animals. The time to reach the hidden platform underneath the water (escape latency) was recorded as the index of learning. Spatial reference memory and memory consolidation were assessed with a standard place task/probe test conducted 24 hours (on day 8) after the last daily training session. During the probe test, the platform was removed and animals allowed to swim freely in the pool for 60 seconds. The time spent and distance moved in the target quadrant indicated the degree of memory consolidation that had taken place 24 hours after learning.

Determination of Hematoma Volume

The whole brain of each mouse (n =4/group) was sliced into 1 mm serial coronal sections with the help of brain matrix. Hematoma volume was determined using Image J software (NIH, USA) wherein the area of the hematoma was quantified.

Western Blot Analysis

After sacrifice at 72 hours post-ICH induction, whole brains were rapidly dissected into 4.0 mm coronal sections (≈ 0.5 and −3.5 mm from bregma) using a matrix. Perihematomal regions were extracted for Western blot analysis, as previously described (Ismael et al., 2018). Briefly, brain tissues were homogenized with a RIPA buffer containing protease and phosphatase inhibitor cocktails. Protein concentrations were determined using the BCA Method (Thermo Scientific USA.). A total of 30 µg protein

was loaded into each lane and resolved, followed by transfer to PVDF membranes. The membranes were blocked for non-specific binding with 5% skimmed milk and probed with primary antibodies against NLRP3, caspase-1, ASC (1:1000; AG-20B-0014; AG-20B-0042; AG-25B-0006; Adipogen Life Sciences), TXNIP (1:1000; NBP1-54578SS; Novus Biologicals), ZO-1 (1:1000; SC7273; Cruz Biotechnology), cleaved IL-1β, Phospho-NFκBp65 (ser536), NFκBp65, TRX, GLUT1(1:1000; 11948; 3033; 3034; 2429; 12939; Cell Signaling Technology), and β-Actin antibody (1:10,000; A5316 Sigma) at 4ºC overnight. Following TBS-T washes, the membranes were incubated with horseradish peroxidase (HRP)-conjugated, anti-mouse IgG antibody and an anti-rabbit antibody (1:10000; A9044; A9169; Sigma USA) for 1 hour at room temperature. The bands were then visualized using an enhanced chemiluminescent substrate system (Thermo Fisher Scientific) and analyzed by densitometry using Image-J 1.51J8 software. These were normalized to loading controls and expressed as fold change.

Slot Blot Analysis

Nitrotyrosine (NT) immunoreactivity was measured by slot blot analysis. In brief, brain tissue homogenate was prepared in a lysis buffer, and 25 μg protein were immobilized onto a nitrocellulose membrane from each group using a slot blot micro-filtration unit. After blocking with 5 % non-fat milk, the membrane was incubated against an anti-nitro tyrosine antibody and visualized by enhanced chemiluminescent substrate system (Thermo Fisher Scientific). The optical density was quantified using ImageJ software.

Immunoglobulin Extravasation

Extravasation of endogenous Immunoglobulin G (IgG) was performed to assess BBB permeability following stroke. Penumbral proteins (30 μg/well) were size-fractionated on SDS-Page gels and electroblotted on a PVDF membrane. Blots were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:10000; A9044; Sigma USA) for 1 hour at room temperature and processed for visualizing the immunoreactive signal. The protein bands were quantified using Image J software.

Immunofluorescence Staining

At 72 hours after ICH, mice were anesthetized with ketamine/xylazine and transcardially perfused with ice cold PBS (30 ml). Brains were then removed and post-fixed in 10% buffered formalin overnight at 4 °C and then sequentially immersed in 30% sucrose in PBS solution for 72 hours. The brains were sectioned in the coronal plane at a thickness of 10 microns and blocked with Serum-Free Protein Block (X0909, DAKO) followed by incubation with primary antibodies against TXNIP (1:100; NBP1-54578SS; Novus biologicals) at 4°C, overnight in a humid chamber. Sections were washed and

incubated with fluorescent anti-mouse secondary antibodies (1:200; 072-04-18-03; Dylight-549, KPL) for 1 h at room temperature. For double staining, the process was repeated with primary antibodies against Neu-N (1:250; ABN78, Millipore), Iba-1 (1:250; WDF6884; WAKO) or GFAP (1:500; 20334, Dako). After single or double-staining, sections were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen) and viewed using a Zeiss 710 confocal laser-scanning microscope. Negative controls were prepared by omitting the primary antibodies.

Statistical Analysis

All results were expressed as mean ±SE and calculations were obtained using GraphPad Prism software. Studen''s t-test or one- or two-way ANOVA was performed followed by Tuke''s test for individual comparisons. The criterion for statistical significance was set at $p<0.05$.

Results

Verapamil Treatment Reduced Hematoma Volume, Oxidative Stress, and Inhibited ICH-Induced TXNIP-NLRP3 Inflammasome Activation

To determine the molecular changes associated with TXNIP modulation with verapamil treatment, an acute (72 hours) study was performed (**Figure 4-2**). ICH was induced as previously described, and animals were treated with verapamil. Hematoma volume was calculated from serial coronal sections. The data showed qualitatively that verapamil reduced the hematoma volume in ICH animals, although this result did not achieve statistical significance (**Figure 4-2A**). Further, TXNIP and components of NLRP3 expression were analyzed by Western blot in the perihematomal area at 72 hours after ICH (**Figure 4-2B, 4-2F-H**). The expression of TXNIP was significantly ($p=0.017$) increased in the ICH group compared to shams along with reciprocal down-regulation of antioxidant thioredoxin TRX ($p=0.001$) (**Figure 4-2B, C**), suggesting that ICH induces the activation of TXNIP. Interestingly, verapamil administration significantly ($p=0.049$) inhibited the expression of TXNIP compared to the ICH-vehicle group (**Figure 4-2B**). Cell-specific expression of TXNIP was further examined either in ICH cortical sections by immunohistochemistry. Slot blot analysis has shown that ICH induced oxidative stress, which is evident by increased nitrotyrosine (NT) level (**Figure 4-2D**), an indicator of super oxide-dependent peroxynitrite formation (Ismael et al., 2021). Treatment with verapamil significantly attenuated NT levels. Our result showed that TXNIP is colocalized with NeuN, GFAP, and Iba-1, suggesting that TXNIP overexpressed in neurons and glial cells such as astrocytes and microglia (**Figure 4-2E**). Further, we examined the effect of TXNIP inhibition on the expression of TRX. However, verapamil did not modulate the TRX expression in ICH animals (**Figure 4-2C**).

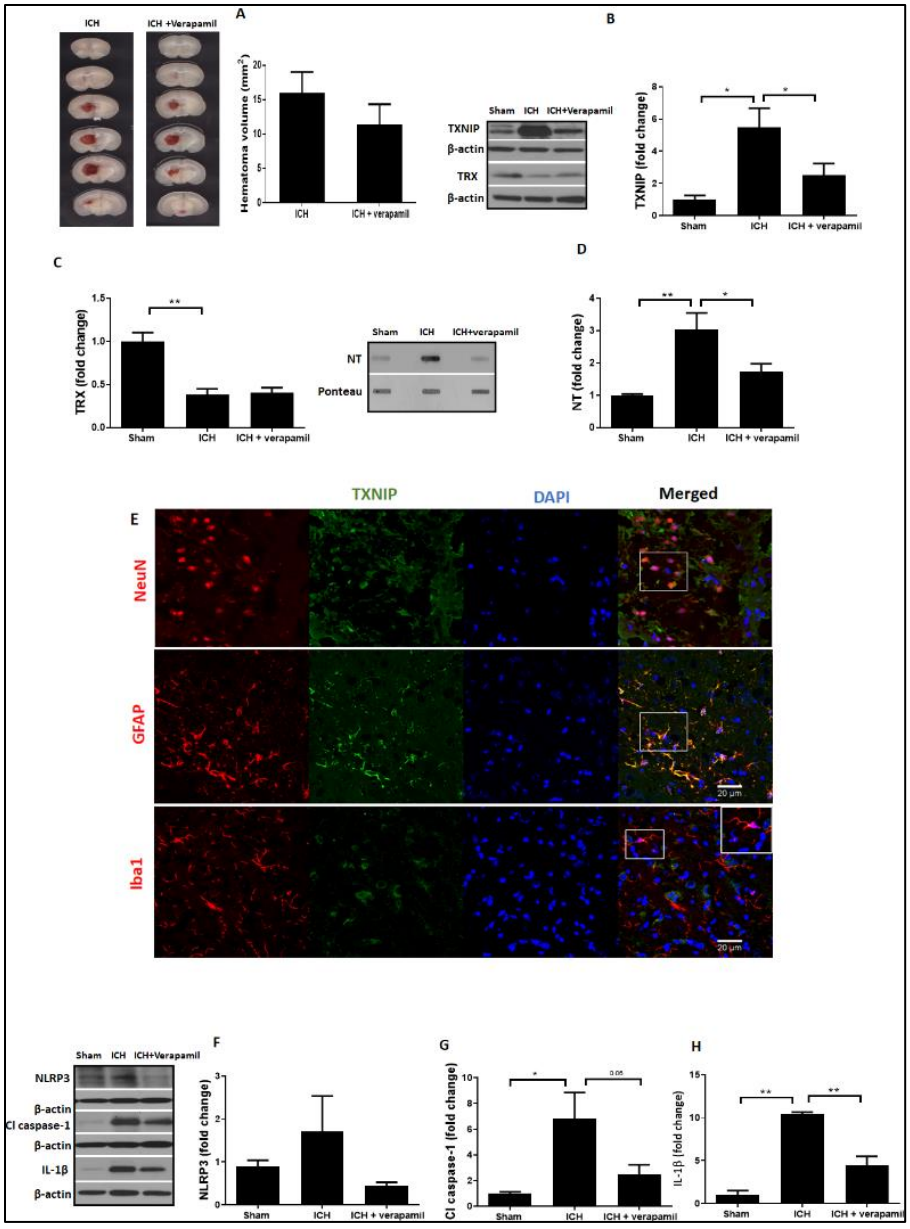

Figure 4-2. Administration of verapamil-ameliorated hematoma volume and TXNIP-associated NLRP3 inflammasome activation in mice after ICH.

One of the consequences of TXNIP activation is the direct activation of the NLRP3 inflammasome (Ismael et al., 2021a; Ismael et al, 2021b). The principal constituents of NLRP3 inflammasome assembly, as well as the consequent caspase-1 and IL-1β synthesis, were analyzed to determine the inflammasome activation in perihematomal regions. Our results showed that the expression of NLRP3 inflammasome products, such as cleaved caspase-1 (p=0.05) and cleaved IL-1β, were significantly (p=0.0001) elevated at 72 hours after ICH when compared to shams, although NLRP3 itself did not show significant elevation (**Figure 4-2F-H**). Further, treatment with verapamil inhibited ICH-induced NLRP3 inflammasome activation, as evidenced by reduced expression of cleaved caspase-1 (p=0.06) and cleaved IL-1β (p=0.001); however, verapamil administration did not provide a discernible effect on NLRP3 protein level (**Figure 4-2F-H**). The contribution of NLRP3 inflammasome activation following stroke is established in earlier studies to contribute to stroke injury through the pro-inflammatory cytokines as well as the pleiotropic effects of cleaved caspase-1 in mediating pyroptosis and apoptosis (Melone et al., 2018).

Verapamil Treatment Attenuated Inflammatory Priming and Pro-Inflammatory Microglial Markers After ICH

We further elucidated the effect of verapamil on phosphor- NFκBp65/NFκBp65 as an index for activation of NFκB, the transcriptional regulator of inflammatory genes in the perihematomal area at 72 hours after ICH (**Figure 4-3A**). There was higher expression (p=0.08) of45etabolio-NFκBp65/NFκBp65 in the ICH-vehicle group as compared to shams, which was marginally inhibited by verapamil treatment. To examine the effect of verapamil on microglial activation, we next determined the expression of the pro-inflammatory microglial markers Iba-1 and EZH2 at 72 hours after ICH (**Figure 4-3B, C**). The expression of Iba-1 and enhancer of zeste homolog-2 (EZH2) were increased after ICH compared to shams. EZH2 histone methyltransferase plays a critical role in microglial activation (Chen et al., 2019).

Treatment with verapamil significantly (p=0.03) reduced expression of these pro-inflammatory microglial markers in the ICH-verapamil group compared to the ICH-vehicle group (**Figure 4-3B, C**). Consistently, immunohistochemical analysis demonstrated elevated immunoreactivity to Iba-1, which is ameliorated by treatment with verapamil (**Figure 4-3D, E**).

Verapamil Alleviated Blood Brain Barrier Permeability After ICH

BBB disruption is a key pathophysiological feature of ICH. ZO-1 level has been closely associated with the degree of BBB damage and has been established as an indicator of BBB destruction. Next, we examined the effects of verapamil on the level of BBB junction protein ZO-1 at 72 hours after ICH (**Figure 4-4A**). The level of ZO-1 was significantly (p=0.01) decreased in the ICH-vehicle group compared to shams. Administration of verapamil marginally attenuated ZO-1 expression in ICH-verapamil

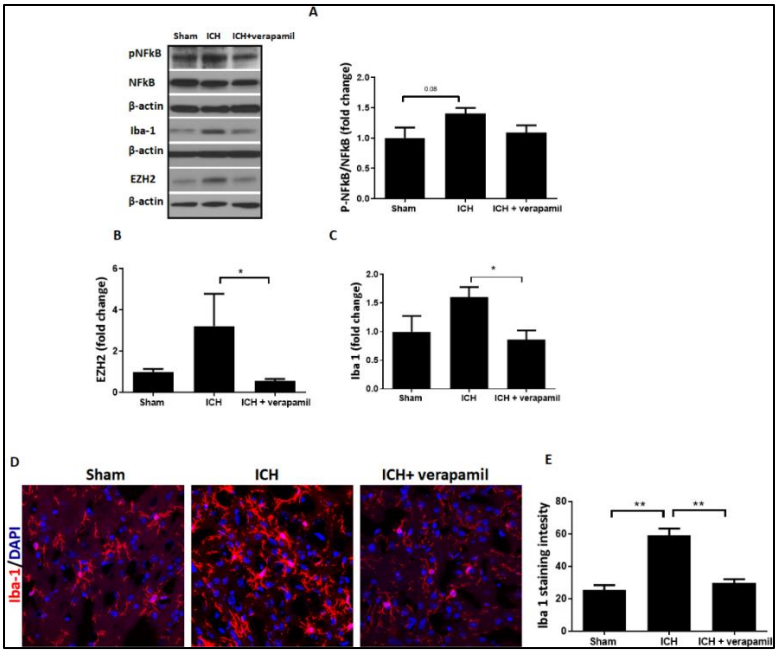

Figure 4-3. Inhibition of TXNIP with administration of verapamil ameliorated NFkB activation and microglial activation in mice after ICH.

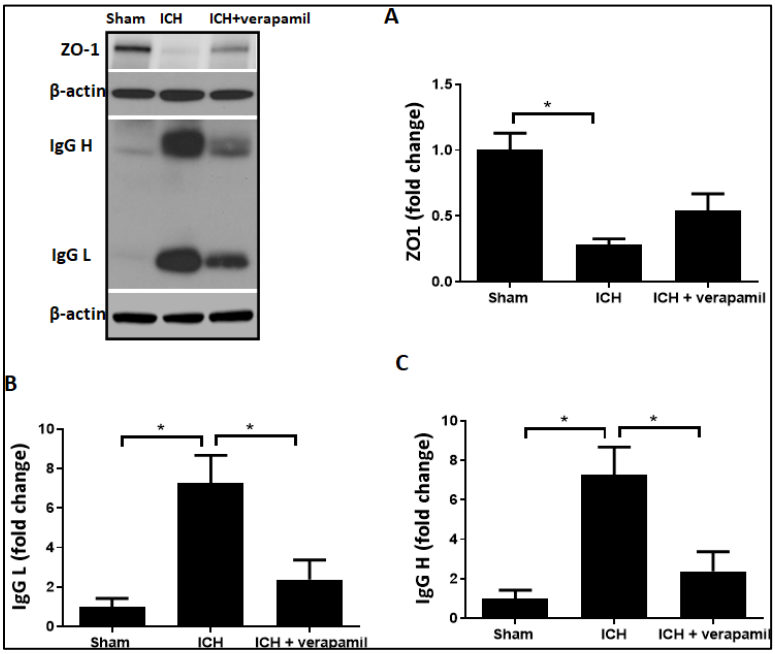

Figure 4-4. Administration of verapamil-attenuated, ICH-induced BBB breakdown in mice after ICH.

compared to the ICH-vehicle group (**Figure 4-4A**). Also, we examined the effect of verapamil on IgG extravasation, a marker of BBB disruption after ICH, by using the immunoblotting of endogenous IgG heavy and light chains at 72 hours after ICH. In the final analysis, we found a significant increase in expression of IgG heavy chain ($p=0.008$) and light chain ($p=0.008$) in the perihematomal regions of the brain (**Figure 4-4B, C**). Verapamil significantly ($p=0.014$) attenuated the brain level of IgG light and heavy chain.

Effect of Verapamil Treatment on Rotarod Performance After ICH in Mice

Rotarod performance is expressed as a percentage of pre-surgery control value. The rotarod tests showed significant ($p<0.05$) deficits in motor performance (time spent on the rotarod) in the ICH-vehicle group at 72 hours post-ICH compared to shams. Treatment with verapamil showed a trend ($p=0.06$) toward improved rotarod performance in the ICH-verapamil group compared to the ICH-vehicle group (**Figure 4-5A**).

Verapamil Administration Improved Gait Control Indices Post-ICH in Mice

The CatWalk measured several different gait indices across all limbs and the body. Those mice treated with verapamil demonstrated improvements across many of these indices when compared with saline groups. The ICH-vehicle groups showed a significant increase in body speed variation ($p=0.047$) and run variation ($p=0.0301$) when compared to the sham group. Interestingly, treatment with verapamil showed no significant improvement from the vehicle group. Paw intensity for both the left hind ($p=0.049$) and right hind ($p=0.042$) paws showed a significant increase in the ICH-vehicle group compared to the sham group. The verapamil treatment significantly ($p=0.033$) decreased these intensities like that observed in the sham group. The inter-paw coordination was measured by paw coupling the mean coupling of the left forepaw with the left hind paw and showed a significant ($p=0.05$) decrease in the ICH-vehicle group when compared to the sham group. However, the ICH-verapamil group showed a significant ($p=0.02$) increase in the coupling, when compared to the vehicle group, that was on par with the sham. The reverse was shown when looking at the pairing of the left forepaw with the left forepaw. The ICH-vehicle group showed a significant ($p=0.05$) increase in the vehicle group when compared to the sham. The verapamil group showed a significant ($p=0.043$) decrease in mean couplings when compared to the ICH-vehicle group. The single stance phase of the right hind paw showed a significant ($p=0.031$) mean decrease in the level ICH-vehicle group compared to the sham. The ICH-verapamil group showed a significant ($p=0.029$) increase in the single stance phase when compared to the vehicle group, further demonstrating the effect verapamil has in improving coordination. Lastly, the mean duty cycle of the left hindlimb showed a significant ($p=0.05$) decrease in the ICH-vehicle group when compared to the sham group. The verapamil group showed a strong decreasing trend ($p=0.08$) toward significance when compared to the ICH-vehicle group (**Figure 4-5B, C**).

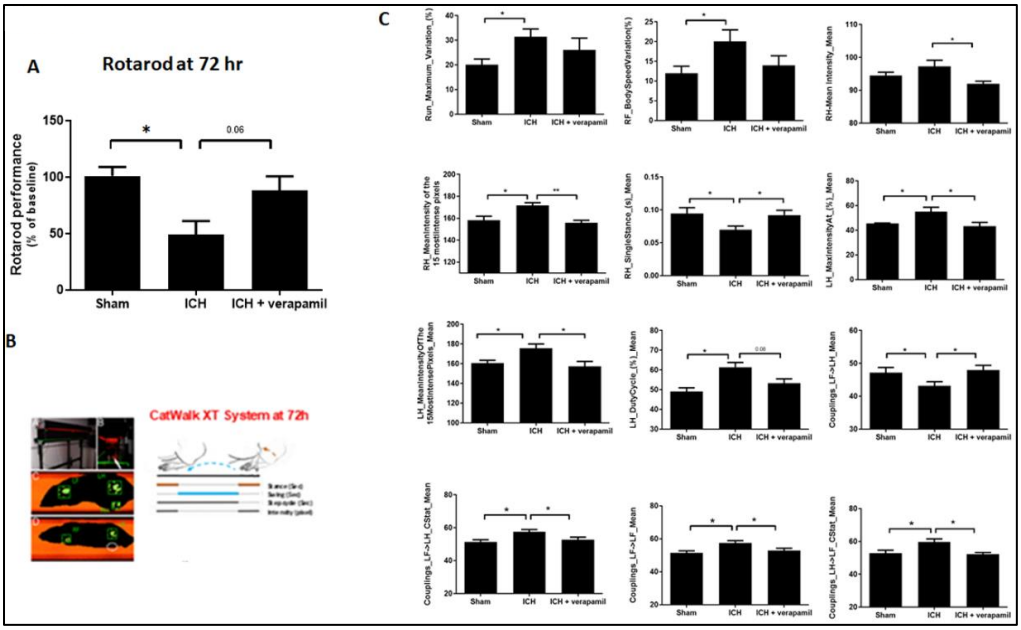

Figure 4-5. Rotarod and CatWalk XT system was used to evaluate the motor and gait control indices.

Verapamil Improved Non-spatial Working Memory Post-ICH in Mice

Novel object recognition was used to assess short-term recognition memory of the mice following ICH (Ishrat et al., 2006). A Recognition index (RI) was calculated for each mouse in each group, and the average score was compared amongst groups. The RI was calculated from the ratio of time spent on a novel object to total exploration time. A significant ($p=0.019$) decrease in recognition index was observed in ICH-mice at 21 days post-ICH compared to sham animals (**Figure 4-6A, B**). Interestingly, verapamil treatment improved preference towards the novel object in ICH mice, represented by improved recognition indices. However, the improvement was not statistically significant.

Long-Term Verapamil Treatment Improved Spatial Reference Memory Post-ICH

The Morris water maze (MWM) was used to determine the spatial learning and spatial memory of the mice following ICH, as previously described (Ahmed et al., 2018). Escape latency was calculated for 7 days of hidden platform as an average of four trials per day and considered as the measure of spatial learning. Over the seven days of hidden platform training, there was an increased mean escape latency in ICH animals (regardless of verapamil administration) when compared to sham animals (**Figure 4-6C**); this difference was significant for day 6 ($p=0.0051$) and day 7 ($p=0.0064$), showing that ICH impaired spatial learning. However, we did not find a significant difference in spatial learning between the ICH-verapamil group and the ICH-vehicle group over the seven days of hidden platform training, indicating that verapamil did not improve spatial learning in ICH animals (**Figure 4-6C**).

A probe trial was performed to evaluate reference memory consolidation in ICH animals after 24 hours of hidden platform training. The ICH-vehicle animals failed to remember the location of the platform and spent significantly less time ($p=0.042$) in the target quadrant than sham animals (**Figure 4-6D**). Verapamil treatment improved the spatial reference memory indicated by a strong trend ($p=0.06$) toward the retention of spatial memory with an increase in time spent in the target quadrant compared to ICH mice that were not given verapamil (**Figure 4-6D**). Additionally, the average number of times the mouse entered the target quadrant, the average distance (m) traveled in the target quadrant, and the average speed (m/s^2) in the target quadrant was also used to test different aspects of spatial memory. The ICH-vehicle group showed a significant decrease ($p=0.049$) in the average distance traveled in the target quadrant and a decrease in the average number (frequency) of target quadrant entries ($p=0.0155$) when compared to the sham group (**Figure 4-6E, D**). The ICH-verapamil group showed significant retention of spatial memory, illustrated by an incremental trend in the number of target quadrant entries (frequency) and a significant decrease ($p=0.05$) in the average distance traveled in the target quadrant. There was no significant difference in the average speed of mice between any of the three groups (**Figure 4-6H**).

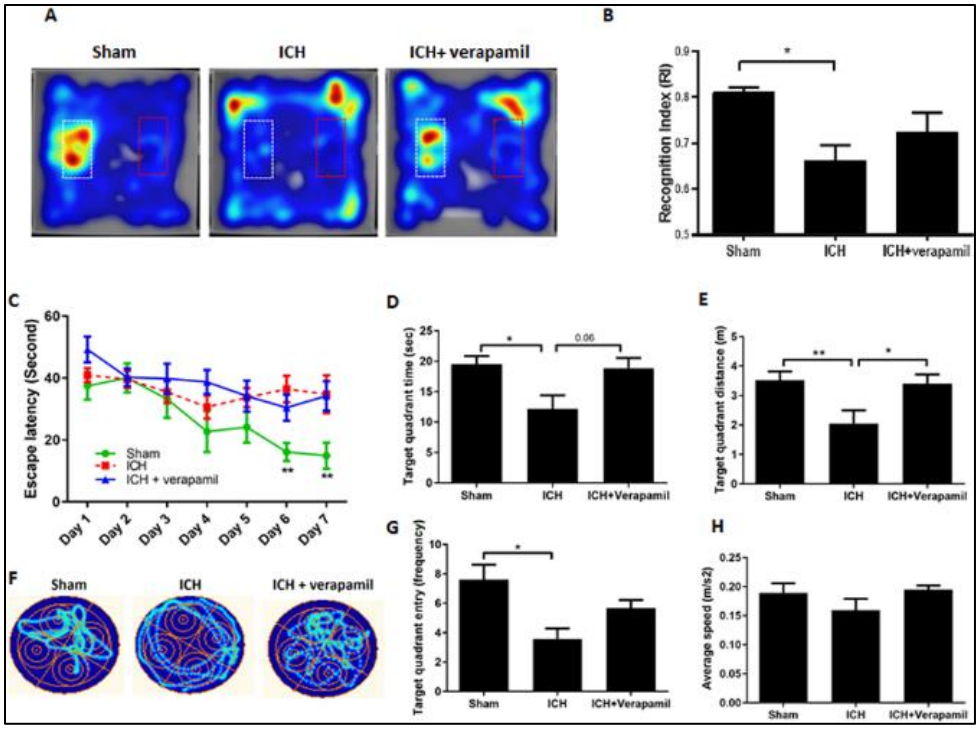

Figure 4-6. Effect of long-term TXNIP inhibition with verapamil on cognitive decline in ICH animals.

Discussion

Here, we report for the first time that inhibition of TXNIP with the administration of verapamil improved neuroinflammation, BBB damage, and acute neurological outcome after ICH, implicating the contributory role in TXNIP in the pathophysiology of ICH. Through treatment, we were able to illustrate a decrease in cellular damage in the form of bleeding and hematoma size similar to treatments utilized in previous studies (Bonsack & Sukumari-Ramesh, 2021; Hanley, 2009; King et al., 2011; Wagner et al., 2003). Verapamil has demonstrated its potential in a multitude of neurovascular (Jangholi et al., 2020; Popović et al., 2020), neurodegenerative, and cognitive conditions (Ismael et al., 2021; Kumar et al, 2016; Melone et al., 2018; Popović et al., 1997). Furthermore, our most recent studies have demonstrated that verapamil inhibits TXNIP in order to protect against brain damage in hyperglycemia-associated ischemic stroke and sporadic AD mouse models (Ahmed et al., 2021; Ismael et al., 2021). As a result of this, our findings support that verapamil inhibits TXNIP as well as improves outcome in a murine model of ICH. However, verapamil did not affect the expression of TRX after ICH.

Although the pathophysiologic mechanisms of secondary brain injury are not fully understood, several recent studies have reinforced the observation that neuroinflammation plays a critical role in the secondary brain damage after ICH (Aronowski & Zhao, 2011; Wang & Doré, 2007). The inflammatory pathways are rapidly activated in response to the presence of blood components in the tissue and are characterized by activation of inflammatory cells such as microglia and astrocytes and secretion of inflammatory cytokines (Shao et al., 2019; Tschoe et al., 2020).

The damaged and dead neuronal cells51etaboate the release and synthesis of damage-associated molecular patterns (DAMPs), which further increases microglial activation and proinflammatory responses (Zhou et al., 2014). Here, the verapamil-mediated improvement in neurological outcome after ICH was associated with an inhibition in neuroinflammatory responses. Our immunoblotting analysis showed elevated TXNIP expression is associated with increased activation of NLRP3 inflammasome components, such as cleaved caspase-1 and IL-1 β. Importantly, TXNIP is required for NLRP3 inflammasome activation, a recently discovered mechanism mediating inflammatory response in several diseases including ICH (El-Azab et al., 2014; Ishrat et al., 2015; Mohamed et al., 2014; Mohamed et al., 2015). Furthermore, genetic deletion or pharmacological inhibition of NLRP3 signaling rescued brain damage after ICH (Chen et al., 2021; Ren et al., 2018). In our study, verapamil treatment significantly attenuated NLPR3 activation by inhibiting cleaved caspase-1 and IL-1β despite no change in NLRP3 itself. This is consistent with the anti-inflammatory potential of verapamil (Ismael et al., 2021), implicating that attenuation of inflammasome activation could partly be responsible for verapamil-mediated neuroprotective effects after ICH. In addition, verapamil also marginally decreased the hematoma volume, clinically a major determinant of adverse outcome following ICH (LoPresti et al., 2014).

In addition to NLRP3 inflammasome activation, ICH induced upregulation of NF-kB. NF-kB is critical regulator of neuroinflammation following ICH, which mediates the

priming of proinflammatory cytokines such as TNF-α and IL-1β (Wagner, 2007). Inhibition of TXNIP with verapamil marginally attenuated elevated activation of NF-kB after ICH. When the NF-kB pathway is pharmacologically inhibited, there has been a demonstrated attenuation of ICH brain injury in rats (Song et al., 2020). This attenuation of injury is consistent with what we found in our experiment.

Our results here also demonstrated that ICH induced expression of EZH2, a histone-lysine N-methyltransferase involved in microglial activation and neuroinflammation through NFkB-mediated mechanism (Luo et al., 2020), was reduced by administration of verapamil. This observation is consistent with previous reports that inhibition of EZH2 attenuated microglial activation and the secretion of proinflammatory cytokines after ischemic stroke and SAH (Chen et al., 2019; Luo et al., 2020). Microglia are the first non-neuronal cells activated in response to ICH and aggravate ICH-associated brain injury by secreting various chemokines and cytokines (Aronowski & Hall, 2005). Furthermore, ICH elevated Iba-1 positive microglia with dense cytoplasm and dense processes in the perihematomal region, which was also attenuated by verapamil administration.

One of the consequences of inflammatory responses following ICH is elevated BBB disruption and permeability (Wang & Doré, 2007), which significantly contributes to the development of secondary brain injury and subsequent mortality and neurological impairment (de Oliveira Manoel & Macdonald, 2018). A loss of BBB integrity aggravates the inflammatory response by leukocyte influx and brain edema formation (Keep et al., 2008). In addition to elevated neuroinflammation, our ICH animal model showed significantly elevated extravasation of immunoglobulin G light chain and heavy chain and down-regulation of ZO-1 junctional protein, demonstrating increased BBB permeability. Verapamil administration not only significantly attenuated extravasation of immunoglobulins; it also revealed a strong trend toward the modulation of ZO-1. We have previously reported the potential benefit of verapamil in attenuating BBB permeability in a mouse model of ischemic stroke through inhibition of TXNIP (Ismael et al., 2021).

Neurobehavioral analysis highlighted many important revelations concerning ICH and verapamil's role in the treatment of ICH. We found a significant motor deficit in our animal model at 72 hours following ICH, evidenced by lower rotarod performance and sensory-motor abnormalities, illustrating the model has applicability for the clinical scenario. In our animal model, ICH affects gross motor function at 72 hours after injury and that ICH has long-term cognitive effects. The strong trend toward improved rotarod performance in the ICH-treatment group suggests there may be a reduction of motor function deficits when verapamil treatment is given after hemorrhage. The CatWalk analysis showed verapamil is effective in improving the gait control indices (e.g., duty cycle and intensity of hind limb use) and inter-paw coordination following stroke. While these findings seem promising, many translational hurdles between humans and animal models still need to be addressed before this treatment can be applied to stroke patients. In this present study, ICH induced significant impairment in cognitive function, with decreased performance seen in NOR and MWM. The recognition index was used as the

measure of non-spatial working memory, and MWM was used to evaluate the spatial learning and reference memory. Long-term verapamil administration has beneficial effects on cognitive function, including non-spatial working memory and spatial reference memory despite no change in spatial learning. This is consistent with our previous report that verapamil attenuated age-associated senile dementia and cognitive impairment in sporadic AD (Ahmed et al., 2021; Ismael et al., 2021; Popović et al., 1997).

Given the efficacy of verapamil in improving neurological outcomes after ischemic stroke and AD (Ahmed et al., 2021; Ismael et al., 2021), this study was designed as a proof-of-concept study to test the potential of verapamil in attenuating neurological outcome ICH outcomes. Our findings demonstrated that low doses of verapamil attenuated brain injury, neuroinflammation, and blood BBB dysfunction in a murine model of ICH. Further long-term treatment with verapamil improved motor function and cognitive impairment through inhibition of TXNIP. Our findings show the possible therapeutic effects of verapamil treatment for ICH and the biological pathways influenced by this treatment. Future studies should be done to focus on identifying most efficacious dose of verapamil for long-term neuroprotection and improvement of neurological outcomes after ICH in an age- and sex-independent manner. Future studies are also necessary to further elucidate the contributions of TXNIP in ICH outcomes, particularly through experiments using more potent, specific inhibitors such as TXNIP-IN- (Liu et al., 2021) and SRI-37330 (Thielen et al., 2020).

CHAPTER 5. CONCLUSION

Summary

The aim of this dissertation research was to better characterize the inflammatory environment following hemorrhagic stroke. To achieve this, we focused on two different forms of hemorrhagic stroke: aneurysmal subarachnoid hemorrhage (aSAH) and intracerebral hemorrhage (ICH), and investigated the role of several inflammatory molecules, including various cytokines, NLRP3, and TXNIP, in severity and recovery trajectories. We also included relevant clinical and behavioral data for these inquiries and utilized both patient samples and animal models to address the translational implications of our research questions.

This project was broken up into three portions. In Chapter 2, we conducted a literature review to better understand the scope of the research that has taken place regarding inflammatory cytokines involved in SAH. We aimed to identify the gaps present in the research and the nature of the research being done in both animal models and in human patients. We found that three main pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) demonstrate reliable increases following aSAH across the included studies. While this is a promising area of research for potential therapeutics, there are gaps in the knowledge base that remain and which bar progress for clinical translation of this information from animal models to human patients. In particular, there is a need for investigations that explore the systemic inflammatory response following injury in a more diverse number of cytokines, the balance of specific pro-/anti-inflammatory cytokines, and how these biomarkers relate to patient outcomes and recovery over time.

In Chapter 3, we aimed to fill one of these gaps by comparing the inflammatory cytokine environment across rat and human subjects and in CSF and plasma samples following aSAH. We found that in both humans and rats, the absolute cytokine levels of IL-6 and IL-8 were significantly higher than all other cytokines. In humans, the localized cerebral environment demonstrated a greater cytokine-mediated inflammatory response, though this difference was not observed in the rodents. Several cytokines were associated with age, sex, diabetes, smoking, and Fisher score; IL-6 and IL-8 again demonstrated the most robust associations ($p<0.05$). With further clinical correlation in individual patients, it is possible that these changes could possibly serve as targets for improved treatment options following aSAH.

Lastly, in Chapter 4 we utilized a mouse model of ICH to better characterize the inflammasome and other major proteins that are involved in inflammatory response post-stroke. The antihypertensive drug verapamil was administered to ascertain its capabilities in managing the inflammatory response following ICH and how this intervention impacts behavioral recovery. We found that verapamil treatment reduced expression of TXNIP and NOD-like receptor pyrin domain-containing-3 inflammasome activation in the perihematomal area. These protective effects were associated with decreased proinflammatory mediators, microglial activation, and blood-brain barrier (BBB)

permeability markers. Our findings also demonstrated that long-term, low-dose verapamil effectively attenuated motor and cognitive impairments. Taken together, these data indicate that verapamil has therapeutic potential in improving acute motor function after ICH. Further investigations are needed to confirm whether verapamil treatment could be a promising candidate for future clinical trials.

Implications for Hemorrhagic Stroke

When these results are taken together, this research answers some interesting questions regarding the inflammatory response following hemorrhagic stroke. Much of the research conducted to date only investigates specific cytokine change at a single timepoint in relationship to recovery or severity of stroke. While this is appropriate in initial inquiry, the multifaceted effects of stroke indicate that there are likely many complex inflammatory checks and balances. An expansion of research into uncovering the entire cytokine constellation, and into the change of individual trajectories over time, should be considered in future work.

Next, our research was able to show that the animal models we have are grossly representative of the human condition is some ways while being different in others. We confirmed that IL-6 and IL-8 (KC/GRO in rats) are the principal cytokines released following aSAH. However, we also demonstrated that that the local inflammatory response in CSF is much more robust in humans as compared to rats. There may be several factors such as genetic differences, leukocyte distribution differences, and clinical treatment protocols in humans that may be responsible for these differences. Further investigations into understanding these similarities and differences will serve to validate what has been done so far and also better inform translational implications of work conducted in animal models for future therapeutics.

The work here also better illustrated the role of NLRP3 and TXNIP following ICH and the effect of verapamil treatment. Following ICH, we observed elevated TXNIP and NLRP3 products such as cleaved caspase-1 and cleaved IL-1β, results that indicate that the TXNIP-NLRP3 inflammasome is responsible for the primary cytokine response following hemorrhagic stroke. Following treatment with verapamil, we were able to observe a decrease in these products and TXNIP, an observation that was similar to the levels seen in the sham group. Similar improvements were also observed in memory and motor metrics following verapamil administration, indicating that verapamil could serve as a possible therapeutic following hemorrhagic stroke.

Limitations

There were some limitations we encountered when conducting this research. One of the primary limitations was the size of our sample, which limited power for further, more granular analyses. This was especially true in our human subjects. Collecting CSF and any other samples from humans is limited due to the nature of research on humans,

but a longer-term study could accommodate for the needed increase in the study sample size. Another limitation concerning our patient population is the absence of a control population. A control population would be possible for our plasma samples in a longer-term study but would be more difficult to obtain for our CSF samples due to the more invasive nature of its collection. With a larger scale study, we could possibly compare the inflammatory environment from aSAH patients to populations of hydrocephalus patients, TBI patients, or some alternative neurological group that also require spinal fluid drainage on a serial basis.

Another limitation is the direct comparison of ICH and aSAH results for a generalized conclusion of hemorrhagic stroke. While both conditions are a form of hemorrhagic stroke, they occur in different areas of the brain; aSAH occurs from the bursting of a blood vessel in the subarachnoid space, while ICH most commonly occurs within the brain parenchyma. The differences in the location results in interactions of different tissue types and anatomical structures with leukocytes, which may result in unique inflammatory responses. Thus, with increased time and resources, it would be valuable to run similar investigations in both types of stroke to obtain these comparisons.

Future Directions

There are countless scientifically exciting questions that rise from this work. Perhaps the most obvious first direction is to investigate the possible relationship between the cytokines IL-6 and IL-8 and the TXNIP-NLRP3 inflammasome. It is known that IL-6 may be tangentially related to the NLRP3 inflammasome. Indeed, IL-6 levels are regularly increased in NLRP3 inflammasome-mediated conditions and are known to be a downstream target of IL-1β. Thus, IL-1β-driven inflammation and NLRP3 activation was shown to increase IL-6 (McGeough et al., 2012). This suggests that IL-6 does not have a direct role in the inflammasome-mediated response but rather is a marker for the response. It would be valuable to better investigate this relationship and how it may relate to hemorrhagic stroke.

It would also be valuable to expand the use of verapamil treatment to a model of aSAH. Running an experiment investigating the effect of verapamil in the aSAH model will illuminate important similarities and differences between ICH and aSAH. The mechanical differences between ICH and aSAH, such as site of action, are understood, but the specific inflammatory cytokine response is not deeply understood. Investigating the effect of verapamil on cytokine levels and the TXNIP-NLRP3 inflammasome will highlight these differences. These experiments will allow us to understand if verapamil's success in improving outcomes following ICH is based more so on the anatomical nature of ICH and the drug's anti-hypertensive effects or if it is specifically due to gross inflammasome interactions. This will also allow for the ability to understand the primary mechanism by which verapamil acts to improve outcomes following hemorrhagic stroke generally.

Lastly and possibly also most importantly (for potential translational implications), we should investigate the inflammatory response following aSAH and the effect of drugs more commonly used in humans as adjunct therapies. For instance, nimodipine is a calcium channel antagonist that increases cerebral blood flow through the dilation of cerebral arterioles. Pharmacological studies in both animal models and humans have shown improvements in mortality, rated alertness, and recovery (Langley & Sorkin, 1989; Scriabine & Van den Kerckhoff, 1988; Wadworth & McTavish, 1992). While this pharmacologic is widely used in practice, data are still needed on control and dosing effects relative to the inflammatory effects of hemorrhagic stroke. Running similar experiments to ours presented in Chapter 4, but instead utilizing this drug, will better inform the mechanisms of action and better inform treatment timescale, dosage, and administration for clinical practice in an accurate animal model of neuroinflammation.

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APPENDIX. PRO- AND ANTI-INFLAMMATORY CYTOKINE INVESTIGATIONS OUTLINED

Table A-1. Summary of cytokines, tissue types, and species utilized in selected research papers.

	Tissue Type					Species				
Cytokine of Interest	**Brain**	**CSF**	**Plasma**	**Serum**	**ISF**	**Human**	**Mouse**	**Rat**	**Rabbit**	**Study**
Interleukin-1α	X	X	X			X				Al-Tamimi et al., 2019
	X							X		Huang et al., 2017
			X			X				Savarraj et al., 2018
Interleukin-1β	X						X			Wu et al., 2016 Xie et al., 2020 Xu et al., 2021 Dong et al., 2016 Zeng et al., 2020 Lu et al., 2018 Tu et al., 2018 Mitsui et al., 2020 Li et al., 2020 Chen et al., 2021 Wei et al., 2017 Wu et al., 2018 Wu et al., 2015 Zhang et al., 2015 Aydin et al., 2017 Fang et al., 2016 Xu et al., 2017 Hu et al., 2018 Du et al., 2020 Luo et al., 2020 Hu et al., 2021
	X							X		Guo et al., 2016 Liu et al., 2019 Liu et al., 2016 Fan et al., 2017 Han et al., 2021 Liu et al., 2019 Liu et al., 2015 Li et al., 2016 Wang et al., 2019 Wang et al., 2017 Chen et al., 2016 Xia et al., 2017 Zhong et al., 2016

Table A-1. (Continued).

Cytokine of Interest	Tissue Type					Species				Study
	Brain	CSF	Plasma	Serum	ISF	Human	Mouse	Rat	Rabbit	
Interleukin-1β (Continued)										Li and Han, 2018
										Zhang et al., 2016
										Peng et al., 2018
										Hao et al., 2016
										Gu et al., 2017
										Miyamoto et al., 2017
	X							X		Xu et al., 2021
										Wang et al., 2021
										Chen et al., 2019
										Guo et al., 2020
										Zhou et al., 2015
										Shi et al., 2015
										Zhang et al., 2019
	X								X	Shi et al., 2019
										Zhou et al., 2015
		X				X				Al-Tamimi et al., 2019
										Lv et al., 2018
										Schallner et al., 2015
			X			X				Al-Tamimi et al., 2019
										Savarraj et al., 2018
										Matas et al., 2019
				X		X				Zhong et al., 2017
				X				X		Duris et al., 2019
IL-1Ra				X		X				Wenneberg et al., 2021
										Savarraj et al., 2018
Interleukin-2		X				X	X			Coulibaly et al., 2020
				X		X				Zhong et al., 2017
			X	X		X				Zhou et al., 2017
Interleukin-4	X							X		Li et al. 2020
										Wei et al., 2017
		X				X				Al-Tamimi et al., 2019
										Righy et al., 2018
				X		X				Al-Tamimi et al., 2019
										Zhou et al., 2017
			X			X				Zhou et al., 2017
Interleukin-6	X						X			Wu et al., 2016
										Xie et al., 2020
										Wang et al., 2020
										Blecharz-Lang et al., 2018
										Xu et al., 2021
										Dong et al., 2016

Table A-1. (Continued).

Cytokine of Interest	Tissue Type					Species				Study
	Brain	CSF	Plasma	Serum	ISF	Human	Mouse	Rat	Rabbit	
Interleukin-6 (Continued)										Zeng et al., 2020
										Huang et al., 2017
	X						X			Lu et al., 2018
										Tu et al., 2018
										Mitsui et al., 2020
										Huang et al., 2017
										Zhao et al., 2016
										Wei et al., 2017
										Zhang et al., 2015
										Aydin et al., 2017
										Fang et al., 2016
										Xu et al., 2021
										Hu et al., 2018
										Luo et al., 2020
										Hu et al., 2021
										Guo et al., 2016
										Liu et al., 2019
										Fan et al., 2017
	X							X		Liu et al., 2019
										Xu et al., 2017
										Li et al., 2016
										Wang et al., 2019
										Yin et al., 2018
										Wang et al., 2017
										Chen et al., 2016
										Li and Han, 2018
										Zhang et al., 2016
										Peng et al., 2018
										Hao et al., 2016
										Gu et al., 2017
										Dang et al., 2017
										Chen et al., 2019
		X		X					X	Croci et al., 2020
	X								X	Croci et al., 2019
										Al-Tamim et al., 2019
										Helbok et al., 2015
										Wu et al., 2016
		X				X				Matsumoto et al., 2019
										Ridwan et al., 2021
										Lenski et al., 2017
										Kao et al., 2015

Table A-1. (Continued).

Cytokine of Interest	Tissue Type					Species				Study
	Brain	CSF	Plasma	Serum	ISF	Human	Mouse	Rat	Rabbit	
Interleukin-6 (Continued)		X				X				Vlachogiannis et al., 2019 Niwa et al., 2016 Schallner et al., 2015 Schiefecker et al., 2017
			X			X				Al-Tamimi et al., 2019 Savarraj et al., 2018 Matas et al., 2019 Wenneberg et al., 2020
										Savarraj et al., 2018 Chaudhry et al., 2017 Matsumoto et al., 2019
				X		X				Ahn et al., 2019 Lenski et al., 2017 Chen et al., 2017 Savarraj et al., 2017 Yang et al., 2020 Zhong et al., 2017
Interleukin-8	X							X		Yin et al., 2018 Gu et al., 2017
		X				X				Al-Tamimi et al., 2019 Schallner et al., 2015
			X			X				Al-Tamimi et al., 2019 Savarraj et al., 2018
				X		X				Savarraj et al., 2017 Zhong et al., 2017
				X		X				Gusdon et al., 2020
Interleukin-10	X						X			Zeng et al., 2020
				X			X			Wang et al., 2020 Li et al., 2020
	X							X		Liu et al., 2018 Xu et al., 2017 Xu et al., 2021 Wang et al., 2021
		X				X				Al-Tamimi et al., 2019 Schallner et al., 2015

Table A-1. (Continued).

Cytokine of Interest	Tissue Type					Species				Study
	Brain	CSF	Plasma	Serum	ISF	Human	Mouse	Rat	Rabbit	
Interleukin-10 (Continued)			X			X				Al-Tamimi et al., 2019 Zhoe et al., 2017
				X		X				Savarraj et al., 2018 Chaudhry et al., 2020 Savarraj et al., 2018 Zhong et al., 2017
				X		X				Zhoe et al., 2017
Interleukin-13				X		X				Savarraj et al., 2018
Interleukin-15			X			X				Al-Tamimi et al., 2019 Savarraj et al., 2018
				X		X				Savarraj et al., 2018
Interleukin-17	X							X		Guo et al., 2020
		X				X				Al-Tamimi et al., 2019 Coulibaly et al., 2020 Moraes et al., 2020
			X			X				Al-Tamimi et al., 2019
				X		X				Chaudhry et al., 2017
Interleukin-18	X							X		Du et al., 2020 Wang et al., 2017 Guo et al., 2020
		X				X				Al-Tamimi et al., 2019 Lv et al., 2018
Interleukin-23				X		X				Chaudhry et al., 2017
Interleukin-33	X							X		Huang et al., 2015 Gong et al., 2018
Tumor necrosis factor alpha	X						X			Wu et al., 2016 Xie et al., 2020 Xu et al., 2021 Zeng et al., 2020 Yagi et al., 2015 Lu et al., 2018 Tu et al., 2018 Mitsui et al., 2020
				X			X			Wang et al., 2020 Li et al., 2020 Zhang et al., 2015 Chen et al., 2021 Zhao et al., 2016

Table A-1. (Continued).

Cytokine of Interest	Tissue Type					Species				Study
	Brain	CSF	Plasma	Serum	ISF	Human	Mouse	Rat	Rabbit	
										Wei et al., 2017
										Wu et al., 2018
										Wu et al., 2015
										Zhang et al., 2015
										Cai et al., 2017
										Aydin et al., 2017
										Fang et al., 2016
										Xu et al., 2017
										Hu et al., 2018
										Luo et al., 2020
										Hu et al., 2021
										Wu et al., 2015
										Liu et al., 2019
										Liu et al., 2016
										Fan et al., 2017
										Xu et al., 2017
										Li et al., 2016
										Wang et al., 2019
										Yin et al., 2018
Tumor necrosis factor alpha (Continued)										Wang et al., 2017
										Chen et al., 2016
										Xia et al., 2017
	X									Shi et al., 2015
										Zhong et al., 2016
										Li and Han, 2018
										Xu et al., 2021
										Zhang et al., 2016
										Peng et al., 2018
										Peng et al., 2018
										Hao et al., 2016
										Gu et al., 2017
										Xu et al., 2021
										Wang et al., 2021
										Chen et al., 2019
										Lu et al., 2018
										Shi et al., 2015
										Zhang et al., 2019
	X								X	Shi et al., 2019

Table A-1. (Continued).

Cytokine of Interest	Tissue Type					Species				Study(s)
	Brain	CSF	Plasma	Serum	ISF	Human	Mouse	Rat	Rabbit	
Tumor necrosis factor alpha (Continued)		X				X				Al-Tamimi et al., 2019 Zhou et al., 2015 Wu et al., 2016
			X			X				Lv et al., 2018 Al-Tamimi et al., 2019
				X		X				Wenneberg et al., 2021 Chen et al., 2017
Transforming growth factor beta 1	X							X		Chen et al., 2020 Xu et al., 2021
				X			X			Wang et al., 2020

Notes: CSF = Cerebrospinal fluid, ISF = Interstitial fluid

VITA

Patrick Devlin was born in 1993 and grew up in Mooresville, North Carolina, where he lived with his parents, Gwen and Jeff, and brother, Ryan. After graduating high school, he attended Davidson college. While at Davidson, he was a Division 1 student athlete in Wrestling. He was always interested in science while attending Davidson and pursued a major in Biology. During the summer, he regularly worked as a research intern in labs spanning several areas of science from herpetology to ornithology. At the beginning of his senior year of college, he suffered a severe concussion that ended his athletic career. After that incident, he became interested in brain injury and how the body responds to various types of injury. In his last year of college, he was able to complete a minor in Neuroscience, allowing him to graduate with a major in Biology and minor in Neuroscience. After graduating, he accepted a position as Research Lab Technician in Dr. Soderling's lab at Duke University. While working there for two years, he was able to participate in several projects, one of which investigated the essential role for InSyn1 in dystroglycan complex integrity and cognitive behaviors in mice. He was able to become an author on a manuscript based on this work. He then applied to grad school and was accepted to UTHSC. When deciding a lab to select, he became interested in the work being conducted in Dr. Stanfill's lab, particularly the work involved with brain injury and stroke. While in Dr. Stanfill's lab, Patrick has been an author on three research papers, being first author on some. Patrick's dissertation work specifically focused on the inflammatory response that followed hemorrhagic stroke. He was able to utilize human samples from Dr. Stanfill's lab as well as animal models from Dr. Ishrat's lab to investigate several questions pertaining to this inflammatory response. The two main projects he worked on while in Dr. Stanfill's lab investigated the cytokine landscape following aSAH and the effect of the drug verapamil on the TXNIP-NLRP3 inflammasome following ICH. While in Dr. Stanfill's lab, Patrick was able to present the results of these experiments at several scientific conferences that included the 26th Scientific Conference for the Society on NeuroImmune Pharmacology. At the end of his student tenure, he expects to receive his PhD in Neuroscience in September 2022 from the University of Tennessee Health Science Center.