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The Role of Complement in Stroke and Traumatic Brain Injury

Christine Couch
Medical University of South Carolina

THE ROLE OF COMPLEMENT IN STROKE AND TRAUMATIC BRAIN INJURY

BY:

Christine Couch, MS

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CHAPTER 1: INTRODUCTION

Brain and neural injury are a non-specific disease category that includes traumatic brain injury (TBI) and stroke. Both TBI and stroke are common, costly, and leading causes of severe disability in adults. Both stroke and TBI are responsible for substantial disability in working age adults, with stroke being the second leading cause of death worldwide [1] and TBI a major cause of disability in people younger than their 40's [2]. The immune response after brain injury is multifactorial and involves both local and systemic events at the cellular and molecular level. The complement system is a component of both the innate and adaptive immune response and can be activated via one of three pathways: the classical, lectin, or alternative pathway. Studies by our lab and by others have established a prominent role for complement in propagating secondary injury after ischemic or traumatic insult to the brain [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13]. The complement system is recognized as an early and significant contributor to secondary insult after TBI by promoting neuronal loss, edema, and inflammatory cell infiltrate [14]. Clinical studies have shown that TBI patients have elevated levels of complement activation products (C3 and sC5b-9) in their cerebrospinal fluid and increased deposition of complement activation products in the perilesional brain [7], [15], [16], [17], [18]. The source of complement deposited after a TBI is a combined contribution of systemic complement, leaking to the brain after trauma-induced blood brain barrier dysfunction, and locally produced complement proteins by the brain parenchyma and infiltrating cells [7], [16], [17], [19]. A similar pattern of complement deposition is seen in experimental models of TBI that also implicate complement in the acute neuronal cell death, neutrophil extravasation, and worsening of outcomes after TBI [9], [10], [11], [12], [13], [20], [21], [22], [23], [24], [25].

Complement is also a major mediator of acute pathology after stroke, and previous work from our lab and others have shown that complement serves as the recognition arm of the immune system to detect and respond to cellular stress in the penumbra leading to a robust neuroinflammatory response [26]. In addition, preclinical and clinical studies have shown significant complement activation in the ischemic penumbra, and elevated serum complement is associated with stroke outcomes [26]. We have also shown that complement increases acute neuronal loss, increases distal thrombosis, and promotes a chronic neuroinflammatory response after stroke leading to worsening of acute and chronic outcomes [14], [26]. A challenge in designing medical treatments for TBI and stroke is the location and multifactorial nature of the pathologies, which are complex and involve dysfunction of multiple homeostatic processes. Studies in animal models have greatly enhanced our understanding of the complex pathophysiology that underlies stroke and TBI and has enabled screening of over 1,000 novel therapeutic agents. A major concern in translational stroke research is that therapeutics that are deemed efficacious at the rodent level fail to show efficacy when moved to clinical trials. Reasons for failure of prior therapies include lack of assessment of chronic outcomes, lack of gender consideration, exclusion of age and other co-morbidities, administering therapeutics at time points not clinically relevant, failure to assess risk profile of novel therapies, and lack of significant motor and cognitive behavioral assessment. While it has long been recognized that neuroinflammation is injurious and represents a therapeutic reparative target, only more recently has it been recognized that neuroinflammation can also contribute to homeostatic and reparative mechanisms after brain injury. Consequently, an emerging paradigm is that systemic and complete

inhibition of neuroinflammation after brain injury is unlikely to be an optimal approach, and that localized and targeted inhibitory strategies, possibly of limited duration, will provide a better therapeutic approach. The goal of treatment has transitioned from symptomatic management to approaches for neuroprotection and regeneration [27]. The complement system is being discussed as a therapeutic target for TBI as well as stroke, due to data supporting a pivotal role for complement in supporting several downstream activities that promote neuroinflammation and degeneration [27]. An extensive understanding of the acute, subacute, and chronic consequences of complement activation is needed in both stroke and TBI and may lead to new therapeutic strategies, including the ability of targeting selective steps in the complement cascade.

CHAPTER 2: LITERATURE REVIEW

2.1 The Complement System:

The complement system is a component of innate and adaptive immune response. It includes more than 30 proteins (both soluble and cell surface bound) that are zymogens. There is noteworthy amplification in the complement pathway and when these zymogens have been activated, the resulting active enzymes can then travel on to repeatedly act on their substrate [28]. The complement system can be activated via any one of three different pathways: the classical, lectin, and alternative pathways. An additional pathway that can lead to complement activation is the extrinsic pathway of the coagulation cascade [29]. Complement activation involves a complex cascade of proteases, providing a first line of defense against pathogens as well as in the elimination of cellular debris. Its function complements that of antibodies and phagocytes by (1) eliminating microbes (through cytolytic membrane attack complex that disrupts the bacterial cell walls), (2) removing apoptotic cells and debris (through opsonization of antigens to promote phagocytosis), (3) augmenting inflammation (through chemoattraction and activation of leukocytes, particularly macrophages and neutrophils), and (4) acting as costimulators of B-cell activation and antibody production and thereby augmenting innate and adaptive immune responses (e.g., via C3d) [30]. Normal host (self) cells express regulatory proteins that protect against complement-mediated attack, whereas foreign pathogens (non-self) and altered host cells are susceptible to such attack, leading to opsonization of pathogens and autoreactive cells.

Soluble proteins of the complement system are synthesized by the liver and circulate initially as inactive pro-enzymes that become functionally active on cleavage by a protease. In the setting of infection or inflammation, the production of certain

complement proteins and other acute phase reactants is augmented by inflammatory cytokines, such as tumor necrosis factor α (TNF- α, interleukin (IL)-1, and IL-6, which are produced by leukocytes (e.g., macrophages and neutrophils) [30]. The initial steps in the complement cascade are triggered by (1) antigen-antibody complexes (classical pathway), (2) lectin binding (lectin pathway), or (3) accelerated C3 tick-over (alternative pathway). After this initial activation, protease-mediated cleavage of complement proteins leads to activation of the aforementioned complement-mediated functions and downstream activation of adaptive immune responses. The other role for complement is immune homeostasis as clearance of apoptotic cells and debris removes self-antigens from the circulation, which reduces the risk of developing autoimmunity.

Regardless of the activation mechanism, three functions result: (1) opsonization, through the deposition of the activation dependent cleavage fragments C3b/iC3b tag pathogens for clearance by phagocytes, (2) leukocyte and microglia engagement to the site of injury by C3a and C5a, and (3) targeted death of pathogens due to the formation of a membranolytic pore in the pathogenic cell membranes instituted by C5b-9 (or the membrane attack complex, MAC). The production of the diffusible chemotactic peptides, C3a and C5a, can also lead to the modification of immune cell functional activities such as production of reactive oxygen species (ROS) and secretion of proinflammatory cytokines [31].

The classical pathway is initiated by C1q. When C1q recognizes certain target molecules (e.g., immune complexes or surface-bound pentraxins), a conformational change occurs, allowing C1q to interact with C1r and C1s and form activated C1qr 2 s 2 [32]. The active enzymatic site is on C1s. Subsequently, activated C1qr 2 s 2 cleaves

C4, then C2, to generate the classical pathway C3 convertase, C4b2a. C4b2a is then able to induce proteolytic activity and activate the common terminal pathway [33]).

The lectin pathway can be triggered by circulating pattern recognition receptors (PRRs) that recognize carbohydrates on microbial surfaces. These include (1) mannose binding lectin (MBL), a "collectin"; and (2) ficolins. Ficolins are PRRs with a fibrinogen-like domain, for example, M-ficolin (ficolin-1, secreted by lung and blood cells), L-ficolin (ficolin-2), and H-ficolin (ficolin-3); the latter two are synthesized in the liver and circulate in blood. MBL-associated serine proteases (MASP-1 and MASP-2) are evolutionarily related to C1r and C1s, and function similarly [34]. MBL complexes with MASPs, which then cleave C4 and C2 to form the lectin pathway C3 convertase, C4b2a.

The alternative pathway is activated by augmentation of C3 tick-over, which is the spontaneous hydrolysis of the thioester bond of C3 that occurs at a low level constitutively but does not proceed further if C3b does not encounter its stabilizing counterpart, Bb [35], [36]. Bb is a proteolytic fragment formed because of factor D-dependent cleavage of factor B. The alternative pathway C3 convertase, C3bBb, is formed when C3b binds to Bb. The activity of C3bBb is stabilized by properdin, which is found on activated surfaces with decreased sialic acid content (e.g., foreign cell membranes), but not on host cell membranes rich in sialic acid. The labile thioester group on C3b is also able to bind covalently and stably exposed amino or hydroxyl groups on the neighboring cell surfaces of pathogens or altered host cell membranes. This process augments C3 tick-over while also promoting subsequent complement activation on the surfaces of targeted pathogens or altered self-structures [37]. The

alternative pathway can also be activated or augmented by C3b generated by the classical or lectin pathways.

After formation of the C3 convertase, C4b2a via the classical and/or lectin pathways, or C3bBb via the alternative pathway, the complement cascade proceeds due to the proteolytic activity of the C3 convertase on C3, which produces an inflammatory mediator or "anaphylatoxin" (C3a) and an enzymatic cleavage component (C3b). This leads to the formation of their respective C5 convertases (C4b2a3b and C3bBbC3b) [32]. Subsequent cleavage of C5 by the C5 convertase also produces an inflammatory mediator or "anaphylatoxin" (C5a) and an enzymatic cleavage component (C5b). The anaphylatoxins C3a, C4a, and C5a activate mast cells and the resulting mediators contribute to the vascular phase of inflammation. C5a is also a chemoattractant that recruits neutrophils and monocytes, contributing to the cellular phase of inflammation. C5b initiates the common terminal pathway and, after complexing with C6, C7, C8, and C9, forms the cytolytic membrane attack complex (MAC).

Complement receptors are G-protein-coupled receptors expressed on various immune cells and rarely on other cells. These receptors are activated by complement protein fragments, principally those derived from C3, which are generated in the plasma during complement activation [38]. C3a is recognized by C3aR, which is expressed on mast cells and basophils and functions as a potent anaphylatoxin. The ligand for complement receptor 1 (CR1) is C3b, which is expressed on red blood cells (RBCs), white blood cells (WBCs), monocytes, dendritic cells (DCs), and podocytes. Its functions include immune complex clearance and phagocytosis. The receptor of C3d is CR2,

which is expressed on B cells and functions in costimulation. The receptor of iC3b is CR3, which is expressed on phagocytic cells and is important in phagocytosis. Finally, C3dg is recognized by CR4, which is expressed on natural killer cells, monocytes, and DCs and may play a role in phagocytosis as well.

2.2 Complement and Stroke:

An ischemic stroke occurs when a blood clot narrows or blocks an artery leading to the brain. Ischemic stroke is frequently followed by reperfusion injury once blood flow is re-established. During ischemia, there is a buildup of xanthine dehydrogenase, which interacts with oxygen when blood flow returns that subsequently leads to production of superoxide, hydrogen peroxide, and hydroxyl radical OH, all leading to endothelial activation and release of proinflammatory cytokines [39]. This can progress to a secondary injury caused by oxidative stress, inflammation and/or blood brain barrier (BBB) breakdown [40], which can exacerbate neurological deficits, tissue damage and cognitive impairment. Therefore, stroke injury can present itself with an acute phase (within 24 h), subacute phase (1–5 days), and chronic phase (weeks to months).

Stroke is one of the leading causes of death worldwide and is a major cause of disability [41], [42], [43]. In the United States it is the fifth most common cause of death and represents a significant disease burden among the veteran population. There is an ongoing need to improve patient management as well as treatments [44], [45]. Current standards of care for ischemic stroke include restoration of blood flow to the ischemic penumbra through pharmaceutical or neuroendovascular approaches [46], [47], [48], [49], [50]. Because of the short treatment window for stroke therapy, together with the requirement for neuroimaging to rule out intracerebral hemorrhage, tissue plasminogen

activator (t-PA) is used for about 7% of acute ischemic stroke patients in the United States [51]. t-PA therapy carries a risk of fatal intracranial hemorrhage and is contraindicated in hemorrhagic stroke [52], [53], [54]. Thus, a current need is an agent that works by a mechanism other than reperfusion, that increases the efficacy of tissue reperfusion, or that decreases the risk profile of current therapies leading to an extended treatment window. A key feature of post stroke pathology that is not addressed during standard patient management is neuroinflammation [55]. Clot dissolution does not prevent the evolution of inflammation in the penumbra, a response that can be self-limiting with early interventions, but can be debilitating when treatment is delayed. Recent data from stroke trials have indicated that vessel recanalization does not mean reperfusion of the capillary bed, and does not necessarily salvage penumbra neurons, since inflammatory responses in the penumbra are lethal to recovering neurons [50]. Ischemia and reperfusion induced neuroinflammation is associated with severity of stroke, acute and chronic outcome, and cognitive recovery [26]. Therefore, a major frontier in neurotherapeutics for stroke is the development of a pharmacological agent that can limit the acute insult and reduce the loss of neurons, while simultaneously blocking acute and chronic neuroinflammatory responses cooperatively with the standards of care.

Complement is a major mediator of the acute pathology after ischemic stroke. Clinical studies have shown that complement components C3a, C5a, C4d, and C5b-9 are raised in the serum of patients during the subacute phase (24–48 hours post stroke) [56], [57]. It has also been shown that the terminal complement pathway remains upregulated in the serum of patients 7 days after stroke [57] accordant to chronic

complement activation. C5b-9 protein levels were significantly associated with volume of ischemic infarction and measures of neurological deficits in stroke patients [56], [57]. Following ischemic insult, ischemic, stressed, and dead cells express neo-epitopes or danger associated molecular patterns (DAMPs) that are recognized by specific natural IgM antibodies that lead to complement activation through the lectin and classical pathways [58]. Our lab has demonstrated that Rag1-/- mice, which lack B and T cells and are therefore also devoid of antibodies are significantly protected from cerebral ischemic reperfusion injury. Reconstitution with one or two natural IgM monoclonal antibodies isolated in our lab (B4-IgM or C2-IgM) and that recognize post ischemic neo-epitopes, restored pathological complement activity and cerebral injury in Rag1-/- mice [58]. In addition, preclinical and clinical studies have shown significant complement activation in the ischemic penumbra, and elevated serum complement is associated with stroke outcomes [26]. Stroke is induced in experimental animal models often by occlusion of the middle cerebral artery (MCAO), and stroke-like conditions can be created in vitro with oxygen and glucose deprivation. Stroke experimental models result in elevated levels of complement components (C1q, C3a, C5a) in the brain [59], [60], [61], [62], [63] and upregulation of C1q mRNA in microglia [59] and C5 mRNA in neurons [60].

Immunohistological evidence supports both a protective and deleterious effect of complement after stroke [62], [63]. We have also shown that complement increases acute neuronal loss and distal thrombosis, and promotes a chronic neuroinflammatory response after stroke leading to a worsening of acute and chronic outcomes [14], [26]. Since complement is an upstream component of inflammatory cascades, it is a

promising target for stroke therapy. However, the use of complement deficient animals is not an optimal strategy to investigate outcome after stroke due to the role of complement in subsequent recovery and regenerative mechanisms. Our lab has pioneered the development of novel injury site targeted complement inhibitors that provide efficacious and local inhibition of complement, specifically at the site of injury. Our lab's studies have demonstrated that of all inhibitors and different targeting strategies (CR2- targeted inhibitors) [64], the inhibitor B4Crry was optimal for providing protection against ischemic stroke. B4Crry is a fusion protein consisting of a single chain antibody (scFv) targeting domain linked to Crry, a complement inhibitor that inhibits all pathways at the central C3 activation step. B4scFv targeting moiety recognizes neoepitopes specifically expressed at sites of ischemic injury [65]. B4Crry specifically targeted the ischemic penumbra, provided acute and chronic protection in terms of infarct, motor and cognitive outcomes in male and female aged mice. It also had a therapeutic window of at least 24 hours and did not increase the risk of infection in pneumonia models [65].

2.3 Complement and Stroke Comorbidities:

Comorbidities in patients present as a cluster of risk factors that increase stroke incidence. What is much less appreciated is that comorbidities also alter stroke pathophysiology, lesion development and recovery in profound ways. Owing to the use of genetic animal models and/or pharmacological interventions, it is possible to capture certain features of comorbidities. Comprehensive reviews on animal models with comorbidities are available elsewhere [66], [67].

Stroke comorbidities, including smoking and aging, are major contributors to outcomes after ischemic stroke, and are associated with a poor recovery trajectory in the setting of rehabilitation therapy. Cigarette smoke is the only patient modifiable risk factor of ischemic stroke, and is correlated with an increased risk of mortality, more severe disability, longer hospital stays and worse overall functional recovery [68], [69]. Cigarette smoking remains a common risk factor for stroke, nearly doubling the risk with a dose response relationship between pack-years and stroke risk [70], [71]. It is approximated that smoking contributes to nearly 15% of all stroke deaths per year [72]. Smoking cessation rapidly diminishes the risk of stroke, with excess risk nearly disappearing 2-4 years after smoking cessation [73], [74], [75], [76]. Secondhand smoke has been recognized as an independent risk factor for stroke in the REGARDS cohort, with the risk of stroke increasing 30% after accounting for other stroke risk factors, for those who have been exposed to secondhand smoke versus those who have not been exposed [77]. Similarly, age has been recognized as a significant predictor of outcome, speed and extent of recovery, mortality, and response to thrombolytic therapy [78]. However, the mechanisms underlying how these comorbidities contribute to worse outcomes are poorly investigated, with very few reports on neuroprotective therapies in the context of these comorbidities, despite continuous recommendations from the STAIR committee and funding bodies. There has only been one report that investigated the effects of cigarette smoke on inflammation and oxidative stress in the brain [79]. This study demonstrated that cigarette smoke exposure induced activation of inflammatory cascades and increased oxidative stress. In other models, our lab and others have shown that cigarette smoke is associated with altering systemic

inflammatory profiles towards increased oxidative stress, complement activation, and inflammatory cells activation [80], [81], [82]. In addition, smoking contributes to decreased vessel wall integrity [80] and may be associated with increased risk of hemorrhagic transformation or intracranial hemorrhage after thrombolytic therapy. Our data indicates cigarette smoke induces a more robust complement activation after stroke and synergistically increases complement-mediated edema and hemorrhage, leading to poor outcomes. As with cigarette smoke exposure, there are only a few reports on the consequences of aging on post stroke neuroinflammation. However, the aging brain, in the absence of ischemic pathology, shows a gradual increase in inflammatory signaling [83], reactive oxygen species basally and in response to injury in the central nervous system [84], [85], as well as increased microglial activation with abnormalities in microglial activity [86], [87], and increased expression of innate immune components, including complement proteins in the normal aging brain [88]. The role of aging in the context of neuroinflammation and neuroprotection after stroke remains poorly investigated.

2.4 Inflammation and Stroke Recovery:

Inflammation plays an essential role in the progression and secondary injury mechanisms after stroke [89]. Early and strenuous intervention with targeted rehabilitation efforts post stroke is associated with improved functional recovery [90], but many clinicians are concerned that strenuous rehabilitation could increase inflammation, even though it reduced inflammation in a preclinical model [4]. Understanding the associations during stroke recovery will help in the development and/or refinement of

neuroprotective approaches. Recent consensus-based recommendations encourage the incorporation of stroke recovery biomarkers into rehabilitation research to explore the potential of using inflammatory markers to help clinicians diagnose and determine stroke outcomes [91], [92]. In general, the diagnosis and prediction of functional recovery by biomarkers has become a hot research topic. Based on the existing research, it can be roughly divided into inflammatory biomarkers, growth factors, oxidative damage biomarkers, genetic biomarkers and metabolic biomarkers. Variations in the levels of these molecular biomarkers in the blood and urine correlate with a decline in cognitive function. Thus, the identification of suitable biomarkers can improve the accuracy of diagnosis and prognosis and is helpful for targeted therapy. However, there is still a lack of large-scale clinical trials to further examine and support the existing results. Individual biomarkers may not be sufficient, and multiple biomarkers should be combined for diagnosis and prognosis of post stroke functional recovery.

2.5 Complement and TBI:

Traumatic brain injury is a heterogenous condition characterized by different types of injury and a diverse range of neuropathology and psychopathology. The injury is divided into two main phases: primary and secondary, which lead to temporary or permanent neurological changes [93]. The primary injury includes the direct mechanical damage to the brain. The secondary injury is a multifactorial physiological response to the primary injury, such as cerebral edema, excitotoxicity, hypoxia, and an ongoing inflammatory process creating biochemical changes that include immune system access to the mechanically injured tissue [94], [95]. Several processes, such as free

radical generation, neurotransmitter release, gene activation, calcium-mediated damage, mitochondrial dysfunction, and inflammatory responses, can participate in the secondary injuries associated with TBI [96], [97], [98]. These mechanisms may occur endlessly over patients' lifetimes. It has also been reported that a history of TBI is known to increase the incidence of Alzheimer’s disease (AD) [99] and other neurodegenerative conditions, including Parkinson's disease (PD) [100], amyotrophic lateral sclerosis (ALS) [101], and frontotemporal dementia (FTD) [98].

The complement system is recognized as an early and significant contributor to secondary insult after TBI by promoting neuronal loss, edema, and inflammatory cellular infiltrate [14]. The source of complement deposited in TBI is a combined contribution of systemic complement, leaking to the brain after trauma-induced BBB dysfunction, and locally produced complement proteins by the brain parenchyma and infiltrating cells [7], [16], [17]). A similar pattern of complement depiction is seen in experimental models of TBI that also implicate complement in the acute neuronal cell death, neutrophil extravasation, and worsening of outcome after TBI [9], [10], [11], [12], [13], [20], [21], [22], [23], [24], [25]. However, the mechanism of how complement activation contributes to both acute and chronic neuropathology after TBI is not well understood. Complement inhibition has resulted in acute protection from TBI and reduced neuronal loss [9], [10], [11], [12], [13], [20], [21], [22], [23], [24], [25]. There is strong evidence associating TBI to cognitive decline and early onset dementia [3], [102].

Mechanisms underlying the association of TBI, and the incidence of neurodegeneration are extremely complicated. In TBI, multiple pathologies occur simultaneously. However, systematic analyses of multiple pathologies in individual

cases in a large TBI and control population have not been conducted [103], [104], [105], [106], [107], [108]. These studies could not distinguish a significant association of AD, PD, and APOE-associated dementia and found no evidence that head trauma was a risk factor for patients with these disorders. Several studies have shown that TBI does not affect the development of AD. The single occurrence of moderate-to-severe TBI triggering the development of neurodegeneration remains somewhat controversial [103], [104], [105], [106], [107]. Additionally, TBI can lead to the development of non-AD dementias. However, proper clinical diagnostic criteria for neurodegeneration that results from TBI do not exist. Thus, the exact nature of neurodegeneration after TBI is difficult to characterize.

Examinations of patients with Chronic Traumatic Encephalopathy (CTE) may help clarify this issue. CTE symptoms, which include behavioral disturbances, cognitive dysfunction, and/or motor-related symptoms, generally begin 8–10 years after repetitive mild TBIs [109], [110], [111]. However, the clinical diagnostic criteria for CTE syndrome have only recently been described. CTE can be observed after a single moderate or severe TBI while traumatic encephalopathy syndrome demonstrates progressive deterioration over time. Therefore, clinical judgment must be used to ascertain whether the amount of progression is greater than what is expected for the comorbidities and age of the patient [108], [109], [110], [111]. Several neurodegenerative mechanisms have been used to explain the cognitive dysfunction and behavioral disturbances in patients post TBI owing to the lack of suitable diagnoses. This diagnosis can now be used to determine those symptoms.

The role of complement in chronic phases of TBI and potential contribution to ongoing cognitive decline and neurodegeneration remains poorly investigated, with the exception of recent studies from our lab [10], [112]. Our lab has demonstrated that complement activation signals persist chronically after TBI and can reactivate a neuroinflammatory response after transient inhibition leading to continuous cognitive decline [113]. Inhibition of complement activation has been a major therapeutic target in preclinical models of TBI. The current therapeutics available for TBI patients include anti-coagulant, anti-depressant, or anti-anxiety, but there is no approved neuroprotective agent for chronic protection against a secondary phase of inflammation and injury that can linger for those who have experienced mild or a more severe TBI [27]. The study TBI requires a model system that allows a close representation of the actual event. There is a diverse amount of in vitro and in vivo models [114]. Nonetheless, in vitro models enable better manipulation and provide easier handling for experimental purposes. Advantages to having an invitro model to study include ethics, cost, real time observation, and repeatability. Several experiments can be performed from just one cell vial and can allow investigators to repeat the experiment multiple times using the same injury mechanism and can allow the experiment to resemble real world applications more closely. Hence, they are the preferred model for investigation of mechanisms involved in the sequelae of events following injury. It is believed that in vitro models of mechanical injury are a valuable tool for the study of the cellular consequences of TBI. In addition, they are useful for evaluation of potential therapeutic strategies of TBI [115].

2.6 Article Overview and How They Integrate into Existing Literature:

Inflammation plays a crucial role in the progression and secondary injury mechanisms after stroke, and early and strenuous intervention with targeted rehabilitation efforts post stroke is associated with improved functional recovery. The literature reveals emerging evidence for evaluating inflammatory biomarkers post-stroke, the role of inflammation in functional recovery, and the influence of rehabilitation on inflammation. This is the first systematic review to provide a synopsis of inflammatory biomarkers related to stroke and the recovery process. We incorporated clinical data from studies of ischemic stroke that analyzed the role and presence of inflammation contributing to functional recovery. Additionally, we analyzed which inflammatory biomarkers were related to patient rehabilitation outcomes. The goal was to provide a state of the science update for researchers and clinicians to use as a resource to assist in investigating and developing new stroke therapeutics.

In the research design for this systematic review, we inspected the literature in SCOPUS, CINAHL, and PubMed databases to obtain information on inflammatory biomarkers for stroke and their association with rehabilitation outcomes, according to the PRISMA guidelines. Eleven articles were selected. Immune markers (interleukin 6 [IL-6], C-reactive protein, IL-1α, tumor necrosis factor a, soluble intercellular adhesion molecule 1) and functional status assessments (National Institutes of Health Stroke Scale, Functional Independence Measure, Modified Rankin Score, etc.) were the primary measures used in the reviewed studies.

Stroke comorbidities can promote a pro-inflammatory environment systemically and can exacerbate the inflammatory responses in the brain after injury. While multiple neuroprotective agents have shown beneficial effects in rodent models of stroke, they have failed to be translated in the clinic. Possible explanations for these contrasting results are the inadequate assessment of functional outcomes in stroke models, and the use of young healthy animals that are not representative of clinical cohorts. As such, preclinical stroke models would benefit from incorporating comorbidities that are often seen in patients. For example, aging (65+ years) and cigarette smoking occurs in more than 80% and 35% of patients, respectively, and often correlates with worse functional recovery and treatment outcomes [116], [117], [118]. Factors contributing to worse perfusion at stroke symptom onset are still under investigation, but both advanced age and smoking are predictors of poor cerebrovascular reserve [119]. In this article, we investigated the impact of age and smoking on acute outcomes after stroke and assessed whether increased complement activation contributes to a worsened outcome in the presence of these stroke comorbidities. The research design in this study, investigated how both cigarette smoking and aging contribute to the inflammatory phenotype and acute outcomes after murine stroke as well as in brain endothelial cells (b.End3). We also investigated whether targeted complement inhibition using B4Crry reduces the neuroinflammatory response associated with these stroke comorbidities. Our results were validated by immunohistochemical and immunofluorescence staining, by imaging, and by assessment of infarct volume and edema) as well as by functional outcomes such as neurological deficit scoring and survival.

Clinical data from TBI patients suggest that chronic TBI is associated with a similar neurodegenerative mechanism that mimics neurodegenerative disorders, such as AD. It is also known that TBI is considered a risk factor for AD and other cognitive disorders. Studies from AD have demonstrated that cognitive deficits occur early in the disease and are dependent on the extent of synaptic and neuronal loss, which correlates with overall cognitive performance. These experiments will use defined conditions to investigate complement-mediated phagocytosis of neurons and/or synapses after excitotoxic insult to the brain. This article will give us a mechanistic understanding of how C3 activation leads to deposition of C3d opsonins on the surface of neurons and/or synapses and how this leads to a C3d-CR3 mediated engagement of brain microglia inducing an upregulation and activation of the phagocytic machinery and activation of pro-inflammatory signaling. The research design of the last study will utilize in vitro cultures of neurons, microglia, and co-cultures of neurons and microglia to assess complement-mediated synaptic-microglial interaction during the neurodegenerative loss of neurons and synapses after TBI.

CHAPTER 3: METHODOLOGY

AIM I:

3.1a: Research Question:

Will rehabilitation alleviate inflammation due to stroke and aide in the functional recovery process?

3.1b: Hypothesis:

It is hypothesized that inflammatory biomarkers (IL-6, CRP, IL-1α, TNF-α) related to stroke contribute to poor patient functional recovery and rehabilitation outcomes.

3.1c: Methods/Statistical Analysis:

This review was conducted in accordance with PRISMA guidelines [120]. We conducted a systematic review of PubMed, SCOPUS, and CINAHL databases to identify clinical studies addressing the role of inflammatory biomarkers in relation to functional recovery in stroke patients. The initial search involved the terms “stroke”, “inflammation”, and “rehabilitation”. The search was limited to human clinical studies published in English and was supplemented by reviewing additional references from the selected studies. We included studies that reported the role of inflammatory biomarkers in stroke patients, evaluated inflammatory biomarkers in stroke patients, and evaluated functional recovery post-stroke.

We screened a total of 117 studies for initial review, out of which we selected 11 studies that were fully examined. For the 11 studies included in this review, the primary baseline characteristics are described in Table 1, and study outcomes described in Table 2. Baseline characteristics were extracted from these 11 articles and included patient number, age, sex, current stroke diagnosis, and concurrent conditions including

diabetes, hypertension, depression, hyperlipidemia, or atrial fibrillation. All 11 articles reported smoking status, drinking status, time since stroke, inflammatory markers such as interleukin 6 (IL-6), C-reactive protein (CRP), IL-1α, tumor necrosis factor (TNF-α), and body mass index (BMI). A subset of articles also reported the US National Institutes of Health Stroke Scale (NIHSS) score, cholesterol levels, and while blood cell count, weight, obesity, soluble intracellular adhesion molecule 1 (sICAM-1), Modified Rankin Score (mRS), plasma glucose, systolic and diastolic blood pressure. Patient outcome measurements were extracted from all articles and included serum levels of the inflammatory biomarkers IL-6 (pg/ml), CRP (mg/dl), IL-1α (pg/ml), TNF-α (pg/ml), and sICAM-1 (ng/ml). Data for the Mini Mental State Examination (MMSE), NIHSS, mRS, and Barthel Index to characterize functional recovery post stroke were reported only in a subset of articles. The Functional Independence Measure (FIM) to evaluate functional status was available only in 2 articles. Appendix A.1 describes all the studies included in our systematic review. The methodological quality and risk of bias were assessed by Downs and Black valid and reliable checklist [121]. Ten articles were characterized as “good” quality, and one [122] had “fair” quality, described in Appendix A.2.

AIM II:

3.2a: Research Question:

Will comorbidities such as aging and cigarette smoking contribute to worse acute outcomes post-stroke? Will complement inhibition reduce the impact of these comorbidities on the neuroinflammatory response and functional outcomes after stroke?

3.2b: Hypotheses:

It is hypothesized that both cigarette smoking and aging contribute to the complement dependent neuroinflammation post stroke and worsen acute outcomes. I also hypothesize that targeted complement inhibition will suppress the impact of these stroke comorbidities on the neuroinflammatory response and functional outcomes.

3.2c: Methods:

Study Design:

For all studies, before the initial acclimation on behavior tasks, mice were randomly assigned to treatment groups using a random number generator. Lab personnel involved in surgeries, testing, and scoring were blinded to group allocations for the duration of the study. Animals were excluded only if mortality occurred during surgery or before administration of treatment, as well as if there was less than an 80% reduction in ipsilateral cerebral blood flow compared with the presurgical baseline (see below). Both young adult and aged mice were sacrificed 24 hours after transient MCAO, at which time brain infarct volume and immunofluorescence (IF) parameters were measured. Neurological deficit scoring was measured at 24 hours after MCAO, just prior to sacrifice. End points were determined by time after reperfusion and were 24 hours, or for human reasons as defined by institutional guidelines. Well defined humane and experimental endpoints were developed through a collaboration with our veterinarian and a scoring matrix was used to determine when pre-emptive euthanasia was necessary.

Recombinant Proteins:

The recombinant complement inhibitor B4-Crry used in this study was constructed, expressed, purified, and subjected to quality control as described previously [16], [123].

The MCAO model and Treatment Paradigm:

Male adult C57BL/6 mice were obtained from The Jackson Laboratory and allowed 1 week of acclimation before use. Adult animals were 6 months old and aged animals were 16 months old at the time of stroke surgeries. Anesthesia and transient MCAO was performed as described previously [16]. Cerebral blood flow was assessed at baseline (just before MCAO procedure) and during MCA occlusion, as well as blood pressure and heart rate monitored. Laser Doppler flow monitoring (moorVMS-LDF1 device; Moor Instruments) was used to assess uniform induction of ischemia across animals. Animals with less than 80% reduction in ipsilateral cerebral blood flow compared with presurgical baseline were excluded from study. After surgery and during recovery from anesthesia, temperature was maintained at 37°C and animals were housed in a temperature and humidity-controlled chamber until recovery from anesthesia, and then returned to regular housing. B4Crry (16mg/kg) or vehicle (PBS) was administered intravenously via tail vein injection 2 hours post-MCAO. We have previously performed dose-response experiments with B4Crry and have shown optimal benefit plateaus between 8-16 mg/ml and is an effective dose in both adult and aged male mice [65]. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina.

Cigarette Smoke exposure paradigm:

Adult mice (n=16) were either 6-8 week old and aged mice (n=20) were 12 months old when initially exposed to cigarette smoke (CS) (3R4F, Kentucky) for 5hrs/day, 5days/week for either 4 months, using a Teague TE-10 automated exposure system as previously described [124], [125]. Animals were exposed to 89% side stream and 11% mainstream CS. To standardize intra-day/batch smoke exposure levels, total suspended particulate matter (TSP) was measured 3 times/day, to maintain a target TSP of 130ppm/m^3 as we have described [82], [125]. Exposure at these levels is non-lethal and is associated with carboxyhemoglobin levels between 8% and 12% immediately after exposure, as determined in venous blood by dual beam spectrophotometry [82]. The CS exposed mice were subjected to MCAO and treated as described above. Note that in an initial experiment, mice were exposed to 6 months of CS which resulted in 100% mortality by 24 hr after MCAO. Thus the data shown herein was derived from a 4 month CS exposure paradigm.

Neurological Deficit Scoring and Assessment:

To assess functional recovery, animals were scored daily by two blinded investigators using the neurological deficit score described previously and measured 24 hours after MCAO, just prior to sacrifice [58]. Animals received a score of 0 for normal motor function, 1 for torso and contralateral forelimb flexion when lifted by tail, 2 for contralateral circling when held by tail on flat surface, 3 for contralateral leaning when at rest, and 4 for no spontaneous motor activity.

Immunofluorescence Staining and Imaging:

Following euthanasia, cardiac perfusion was performed with cooled PBS followed by 4% Paraformaldehyde (PFA) mixed in PBS . Brains were extracted and placed in 4% PFA solution overnight at 4°C. The brains were moved to a new vial with 30% sucrose mixed with 4% PFA and PBS. For tissue cutting, brains were embedded in optimal cutting temperature (OCT) compound and frozen. At the time of cutting, brains were cut in 40 µm coronal sections using a cryostat. The complete brain was collected in 12-well plates and kept in PBS wells until analysis. Brain slices were taken for each brain and were identified by stereometric measurement using a mouse brain atlas and used for immunofluorescent staining as described [4]. Slices were washed with PBS and permeabilized using 3% H_2O_2 followed by 0.1% Triton-X in PBS. Slices were blocked in donkey serum (5% in PBS), washed, and incubated with the primary antibody overnight followed by washing and incubation with the fluorescently tagged secondary antibody. High-resolution imaging was performed using a Zeiss LSM 880 confocal microscope (Zeiss) at 40x zoom with water-media overlay and using the Z-stacking feature of the microscope. Images were deconvoluted using the ZEN 2.5 software (Zeiss) and reconstructed in 3D plane. The primary antibodies used for staining were anti-NeuN (Abcam, Cat. #: ab104225, 1:200) for neurons, anti-Iba1 (Invitrogen, Cat. #: PA5-21274, 1:80) for microglia/macrophages, and anti-MAP2 (Abcam, Cat. #: ab32454, 1:200) for dendrites. Image analysis was performed using both ZEN (Zeiss) and ImageJ (NIH) software. Imaging quantification was performed using unbiased stereology and using full brain slices at similar brain atlas reference coordinates from each group to avoid bias in field selection. Quantification of MAP2 signal was performed by computing the

area of MAP2 signal was performed by computing the area of MAP2 signal loss in the ipsilateral hemisphere as a percentage of total area of the contralateral hemisphere and reported in percentage. Quantification of Iba1 signal was performed as the percentage of area of increased Iba1 signal density of the area of the contralateral hemisphere and reported in percentage.

Infarct Volume, Edema, and Survival analysis:

Acute infarct volume was estimated using triphenyltetrazolium chloride (TTC) staining on 2 mm thick coronal sections of mice brains as previously described [58]. Images were quantified using ImageJ (NIH), and edema-corrected infarct volume was calculated as infarct area x (edema index = area of contralateral hemisphere / area of ipsilateral hemisphere) as described [126]. A Kaplan-Meier curve using Mantel-Haenszel log-rank test analysis was performed to compare survival curves.

3.2 d: Statistical Analysis:

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad). Parametric testing was used unless otherwise specified, in the event of Brown-Forsythe test for homogeneity of variance or if normality fails. Histologic analysis for stroke was performed using Chi-squared test. Statistical analyses for infarct and IF analyses were performed using one-way ANOVA test with Bonferroni's correction for multiple comparison. P values below 0.05 were considered significant. Student's t test and Mann Whitney test were used to compare two groups and was used as two-tailed. Pearson correlation coefficients were used to compute correlations.

AIM III:

3.3a: Research Question:

What is the role of complement and microglia in synaptic and neuronal degeneration after traumatic brain injury?

3.3b: Hypothesis:

It is hypothesized that after TBI, complement opsonin C3d deposits on injured neuronal cell bodies and/or synaptic terminals and is recognized by activated microglia, leading to phagocytosis, internalization, and lysosomal localization of synaptic markers.

3.3c: Methods:

Animal husbandry and care:

The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all experiments involving vertebrate animals. Wild-type (WT) C57BL/6J mice were obtained from The Jackson Laboratory and housed in *Helicobacter* negative rooms at the Medical University of South Carolina (MUSC) animal facility.

Cell lines description and monitoring:

Mouse hippocampal neuronal cells (HT22, Millipore-Sigma) used in in-vitro experiments were obtained from Millipore-Sigma, passaged and frozen in liquid nitrogen for use until experiments, and were used to validate the efficacy of our excitotoxic injury model. Prior to their use, cells were monitored daily for their morphology by light microscopy, growth rate, and lack of mycoplasma contamination. Cells were passaged no more than ten times. Cells were incubated in a designated incubator not shared with other cell lines during the experiment. Lack

of mycoplasma contamination was verified by absence of filamentous growth. Cells were grown and maintained as per supplier's recommendation in Dulbecco's Modified Eagle's Medium (DMEM; ATCC 30-2002) with 10% fetal bovine serum (Sigma-Aldrich; Product# F0392), and 1x penicillin streptomycin solution (Corning; Catalogue# 30-002-CI).

Serum collection:

Normal mouse serum used in in-vitro experiments was prepared from wild-type C57bL/6J male mice (Jackson Laboratory). Blood was withdrawn by cardiac puncture of unmanipulated mice and maintained on ice for 2 h to clot. Clotted blood was centrifuged at 3000 rpm for 20 minutes, and serum was collected and stored at -80ºC for future use.

Isolation of Primary Neurons:

Primary cultures of neurons were prepared from embryonic day 18-20 (E18-20) from C57BL/6J mouse embryos by standard techniques. A pregnant female WT mouse was euthanized by CO2 overdose and sprayed with ethanol to prevent contamination. The following occurred inside a laminar flow hood. A V shaped incision was made across the pregnant female belly and cut through the skin and fat. Amniotic sacks were removed, and pups were transferred to a clean petri dish. The pup heads were decapitated, and the skin was removed. The skull was cut through with micro scissors and the brains were removed with a spatula and placed in a clean petri dish with ice cold HBSS. The pup brains were dissociated by using a pestle over a wire mesh, and media with the cells was collected and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and 5 mls of lysis buffer was added to dissociate blood cells. The cells were centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and the cell pellet was further dissociated by trypsinization (0.25%) for 15 minutes at 37°C. Afterwards, trypsin was inactivated with 10% heat inactivated FBS in complete 1x-DMEM media. The cells

were triturated and passed through 70um and 40um cell strainers before being centrifuged for 10 minutes at 1000 rpm. The cell pellet was washed with complete 1x-DMEM with a repeated centrifugation step. Then, neurons were enriched by allowing cells to adhere to poly d lysine coated plates or coverslips for 10 minutes in complete 1x-DMEM. The nonadherent cells (microglia, astrocytes, oligodendrocytes) are removed in this step and discarded. The adherent cells (mostly neurons) then received neurobasal media with B27 supplement media was changed on day 5 after initial neuron plating until confluency is reached at day 7-10.

Isolation of Primary Microglia:

Primary cultures of neurons were prepared from P0-P4 post-natal day pups from C57BL/6J mice. The pups were euthanized by decapitation and the heads of the pups were soaked in 70% ethanol to disinfect the tissue. The heads were removed with a pair of scissors and brain was dissected using a small spatula. Brains were placed over a wire mesh in a sterile petri dish consisting of DMEM/F-12, GlutaMax, 10% FBS, and 5% penicillin–streptomycin. Brain tissue was dissociated with a sterile pestle upon the wire mesh. The cells and media were triturated and placed into a 50 ml centrifuge tube and centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and 5 ml of RBC lysis buffer was added. The centrifugation step was repeated. Next, cells were washed with 5 ml of complete DMEM/F-12 media and centrifuged again. In the meanwhile, media was added to T-75 flasks and cells were distributed. Media was changed every 3 days and on the 9^{th} day of cell culture, microglia was dissociated from mixed glial cultures by mild trypsinization. Briefly, 0.08% trypsin (dilute bottle in 1:3.5 ratio) + 0.35 mM EDTA (25200-072, Life Technologies, Somerset, NJ) in Dulbecco's modified Eagle medium (DMEM/F-12 only) is applied to mixed cultures for 35 min to dissociate all cells but microglia. The trypsinization is stopped by diluting the trypsin with DMEM and immediately removing all medium on the plate. The remaining pure microglial population was incubated with 0.25% trypsin + 1 mM EDTA for 10 min, dissociated by vigorous pipetting, resuspended in culture media, and plated at

75,000/cm^2 for 24 h in DMEM/F12 supplemented with 10% FBS and 1% penicillin–streptomycin. (To remove microglia for experiments: 10 mins with full trypsin, centrifuge etc).

Co-Culture Model:

Our hypothesis was tested using an in vitro model of TBI using immortalized neurons (HT22) to validate the injury model or primary neurons (embryonic day ~E18-20) co-cultured with primary microglia prepared from post-natal day P0-4 from WT mice by standard techniques.

Once primary neurons reached confluence at day 7-10 and primary microglia were isolated at day 9, neurons and microglia were plated in a 5:1 ratio of neurons: microglia for phagocytosis experiments compared to 4 h and 8 h timepoints. This occurred following neuron injury, described below.

This co-culture system allows for the in vitro investigation of complement-mediated synaptic-microglial interaction during the neurodegenerative loss of neurons. Additionally, various aspects of the experimental design can be modified to address particular experimental questions. For example, the length of treatment and co-culture can be adjusted to better assess the time-course of an observed effect.

Glutamate Injury:

It is known that following TBI, there is a release of excess glutamate which results in glutamate excitotoxicity, specifically the buildup of extracellular glutamate causing neurotoxic activation of post synaptic NMDA receptors and metabolic energy failure [127], [128]. We have generated data by chemical injury showing that exposure of HT22 neurons to 20mM of the glutamate, at 8 hours results in approximately 80% early apoptotic cells, as well as exposure of primary neurons to 2uM glutamate.

Immunofluorescence staining and imaging:

This was used solely for validation of our glutamate injury. Neurons and microglia were fixed in 4% PFA solution for 20 minutes for immunocytochemistry. The cells were washed with PBS and permeabilized using 3% H_2O_2 followed by 0.1% Triton-X in PBS. Neurons were blocked in donkey serum (5% in PBS), washed, and incubated with the primary antibody overnight followed by washing and incubation of the fluorescently tagged secondary antibody. The primary antibodies used for staining in the first experiment were anti-NeuN (Abcam, Cat. #: ab104225, 1:200) for neurons and anti-caspase 3 (R+D, Cat. # AF835, 1:200) for apoptotic cells. Simultaneous immunostaining of caspase-3 and NeuN were determined whether C3d is strictly deposited on apoptotic neurons. High-resolution imaging was conducted using a Zeiss LSM 880 confocal microscope (Zeiss) at 40x zoom with water-media overlay and using the Z-stacking feature of the microscope. Images were deconvoluted using the ZEN 2.5 software (Zeiss) and reconstructed in 3D plane. Image analysis was performed using both ZEN (Zeiss) and ImageJ (NIH) software.

Multiparameter Flow Cytometry and Sequential Gating Analysis:

This was performed to discriminate between neurons and microglia and other glial cells, as well as for the characterization and quantification of apoptosis. First, neurons were labeled and stained with cell trace violet. A Cell trace stock solution (5mM) was prepared prior to use by adding 20uL DMSO to 1 vial of the cell trace reagent. 1uL of cell trace stock solution in DMSO was added to each mL of cell suspension in PBS for a final working solution of 5uM. Cells were incubated at 37°C for 20 minutes, protected from light. 5x the original staining volume of complete culture medium was added and mixed (this removed any free dye remaining in solution) and incubated for 5 minutes. Cells were pelleted by centrifugation and supernatant was removed. Cell pellet was resuspended in fresh pre-warmed culture medium. The cells were incubated for at least 10 minutes before analysis (this allowed the reagent to undergo acetate

hydrolysis), and then a live/dead staining solution was added to the microglia cultures in the collected co-cultures and incubated for 6 minutes at 4°C. Cells were then washed 2x with PBS. 100-150uL of the mix was added to the cell co-cultures and incubated for 20 minutes at 4°C, followed by 2 PBS washes, and then final storage in FACS buffer. Flow cytometry was conducted within 10 minutes. For flow cytometry, BV-421 (Pacific Blue) channel was used to detect the cell trace violet (CTV), with excitation wavelength of 405 nm. PE-Cy7 channel was used to detect CD45.1. Normal cells without apoptotic induction were used as control for fluorescence compensation to remove spectral overlap and determine the position of cross-over.

Imaging Flow Cytometry and Gating Strategy:

Imaging flow cytometry was performed on an ImageStream X Mark II operated by INSPIRE software (Amnis Corporation). CD45 (PE-Cy7) was recorded and collected with a 740-800 nm filter (channel 6). Cd11b (BV-711) was recorded and collected with a 660-740 nm filter (channel 11) and cell trace violet (BV-421) was recorded and collected with a 425-505 nm filter (channel 7). Brightfield images were collected in channel 1 (camera 1) and channel 9 (camera 2). A total of 1000 events were collected for each sample. Unstained controls and single stained controls were also collected (CTV only, Cd11b only, and CD45 only stained cells) at the same settings, to develop a compensation matrix for removing spectral overlap of the dyes from each of the channels. Data was analyzed using IDEAS Application v 6.2 software (Amnis Corporation). In each experiment, a compensation matrix was created using single color controls and was applied to all files. Image-based gating was performed as follows: Focused cells were obtained by gating on objects with high gradient RMS (root mean square for image sharpness) values, a measure of the amount of change of pixel values above background in the image. Objects with small RMS are generally out of focus, whereas those with large RMS

demonstrate a sharpness in the images. Single cells were identified by gating on objects with high aspect ratio and low area. The internalization wizard was used to create an analysis for measuring the internalization of neurons (CTV) by microglia (CD45), which represented phagocytosis.

3.3d: Statistical Analysis:

We used 3 experiments per study and 3 replicates for each cell culture for robustness. All quantifications were automated via macros in ImageJ and Zeiss Zen software to eliminate bias. Group comparisons were performed using standard t tests when comparing 2 groups or ANOVA with Bonferroni for multiple comparisons. Data was presented as mean ± standard error of two or more independent experiments. Data was analyzed as parametric measure unless tests for normality and homogeneity of variance fails; non-parametric testing was used.

CHAPTER 4: AIM 1

Manuscript 1: State of the Science in Inflammation and Stroke Recovery: A Systematic Review

The following manuscript was accepted for publication in the *Annals of Physical and Rehabilitation Medicin*e on November 2021. The text below is from the final draft accepted prior to publication. A copy of the published version can be found at:

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TITLE PAGE:

Title: State of the Science in Inflammation and Stroke Recovery

Authors: Christine Couch[1],[2] Khalil Mallah[1], Davis M. Borucki1,[3,4], Heather Shaw Bonilha[2],
Stephen Tomlinson[1,5].

Affiliations: 1Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, USA 29425
2Department of Health Sciences and Research, College of Health Professions, Medical University of South Carolina, Charleston, SC, USA 29425
3Medical Scientist Training Program, Medical University of South Carolina, Charleston, SC,
USA 29425
4Department of Neurosciences, Medical University of South Carolina, Charleston, SC, USA,
29425
5Ralph Johnson VA Medical Center, Charleston, SC, USA 29401 United States

Word Count: 4,000, Tables: 3, Figures:1

***Correspondence:** Stephen Tomlinson, PhD, Department of Microbiology and Immunology
173 Ashley Avenue, BSB 204, MSC 504, Charleston, SC, USA 29425

Email: tomlinss@musc.edu

Abstract:

Stroke is a major cause of mortality worldwide, and affected people who survive often suffer from major life changing disabilities. Annually in the United States, it is estimated that 795,000 people suffer from either a new or recurrent stroke. All types of stroke involve an inflammatory reaction that follows the initial phase of incidence. However, investigations into any links between inflammatory markers and recovery processes in the context of post-stroke rehabilitation are lacking. In this systematic review, we performed a literature search in PubMed, SCOPUS, and CINAHL databases to depict the inflammatory biomarkers related to stroke and their relationship with rehabilitation outcomes, according to the PRISMA guidelines. Eleven articles (N=1,773 stroke patients) were selected for review. Immune markers (IL-6, CRP, IL-1α, TNF-α, sICAM-1) and functional status assessments (mRS, NIHSS, FIM, etc.) were the primary measures used in the reviewed studies. The results indicate that there is preliminary evidence for the evaluation of inflammatory biomarkers post-stroke including the role of inflammation in functional recovery, and the influence of rehabilitation on inflammation. This is the first systematic review of the topic. The review identifies several gaps in the literature that are critical to understanding the potential use of inflammatory markers to improve post-stroke outcomes.

Keywords: stroke, inflammation, inflammatory markers, rehabilitation, functional recovery

Introduction:

There are over 4 million survivors of stroke living in the United States, and up to 50% experience chronic disability [1]. Disabilities following stroke are numerous and include cognitive and communication issues, reduced ability to walk, and motor deficits [2]. Stroke survivors are also at high risk for a second stroke [3], pulmonary or urinary tract infections [4], and depression [5]. Stroke is a global healthcare and financial burden with associated costs of $34 billion annually in the United States alone [6], and yet the only FDA-approved treatments are intravenous thrombolysis (IVT) and thrombectomy, both of which have lower than 20% eligibility rates and common serious side effects. Due to the short treatment window for stroke therapy, together with the requirement for neuroimaging to rule out intracerebral hemorrhage, tissue plasminogen activator (t-PA) is used in around 7% of acute ischemic stroke patients in the United States [7]. In addition, t-PA therapy carries a risk of fatal intracranial hemorrhage and is contraindicated in hemorrhagic stroke [8-10]. This has motivated researchers to investigate therapeutic targets beyond restoring blood flow, such as reducing inflammation. Unfortunately, nearly all investigational anti-inflammatory and neuroprotective drugs have failed in clinical trials, despite showing promise in preclinical studies, and very few candidates reached phase III trials [11]. In many cases, this failure has been attributed to inadequate assessment of functional and/or long-term outcomes, and the use of young healthy animals in preclinical studies that is not representative of clinical cohorts.

Inflammation is known to play a key role in the progression and secondary injury mechanisms after stroke [12]. Early and strenuous intervention with targeted rehabilitation

efforts post-stroke is associated with improved functional recovery [13], but many clinicians are concerned that strenuous rehabilitation could increase inflammation, despite it reducing inflammation in a preclinical model [14]. Understanding this relationship during stroke recovery will assist in the development and/or refinement of neuroprotective approaches. Recent consensus-based recommendations encourage the incorporation of stroke recovery biomarkers into rehabilitation research in order to explore the potential of using inflammatory markers to assist clinicians in diagnosing and determining stroke outcomes [15-16].

This is the first systematic review that provides a synopsis of inflammatory biomarkers related to stroke and the recovery process. We incorporated clinical data from acute ischemic stroke patient studies that analyzed the role and presence of inflammation contributing to functional recovery. We selected specific inflammatory biomarkers that were reported at different timepoints during patient recovery. Furthermore, we analyzed which inflammatory biomarkers were related to patient rehabilitation outcomes. Our goal is to provide a state-of-the-science update for researchers and clinicians to use and provide a resource to assist in the investigation and development of new stroke therapeutics.

Methods:

Literature Search and Selection Criteria

This review was conducted in accordance with the PRISMA guidelines [17]. Articles were identified by searching PubMed, SCOPUS, and CINAHL databases. Systematic literature

search of PubMed databases was used to indicate clinical studies addressing the role of inflammatory biomarkers in relation to functional recovery in stroke patients. The initial search encompassed the terms *stroke*, *inflammation*, and *rehabilitation.* The search was limited to human clinical studies with the English language restriction applied. The search was supplemented by reviewing additional references from included studies. The references of selected articles were analyzed for relevance. The search of databases was conducted on October 6, 2020. We included studies that met the following criteria: 1) reported the role of inflammatory biomarkers in stroke patients, 2) published in English, 3) evaluated inflammatory biomarkers in stroke patients, 4) evaluated functional recovery post-stroke.

Results:

Data extraction

One hundred and seventeen articles were selected for initial review (Figure 1). The process of article selection for final review resulted in 11 articles and is summarized in Figure 1. For the 11 studies included in this review, the primary baseline characteristics are described in Table 1, and study outcomes measured reported in Table 2. Baseline characteristics were extracted from eleven articles including patient number, age, gender, current stroke diagnosis, and concurrent conditions, which included: diabetes, hypertension, depression, hyperlipidemia, or atrial fibrillation. Smoking status, drinking status, time since stroke, inflammatory markers interleukin-6 (IL-6), C-reactive protein (CRP), interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α), Body Mass Index (BMI), The National Institutes of Health Stroke Scale score (NIHSS), cholesterol levels, white

blood cell count were also reported in all 11 articles. Weight, obesity, soluble intercellular adhesion molecule-1 (sICAM-1), Modified Rankin Score (mRS), plasma glucose, systolic blood pressure, and diastolic blood pressure were also reported in a subset of articles. Patient outcome measurements were extracted from all articles including serum levels of the inflammatory biomarkers IL-6 (pg/ml), CRP (mg/dl), IL-1α (pg/ml) TNF-α (pg/ml), and sICAM-1 (ng/ml). MMSE, NIHSS, mRS, and BI were available in a subset of articles to characterize functional recovery post stroke, and Functional Independence Measure (FIM) was available in two articles to evaluate participant functional status.

Quality Assessment:

Table A.1 (see supplemental) describes all of the studies included in this systematic review. The methodological quality and risk of bias was assessed through Downs and Black's valid and reliable checklist [18]. Ten of the articles were characterized as "good" quality, and one [19] as "fair" quality, as Table A.2, described in the supplemental material.

Patient Demographics

A total of 1,773 patients (age [mean (SD)] 67.5(8.7) years, 46% male) with a diagnosis of acute stroke were included in this review. The most common concurrent conditions (n[% patients]) mentioned across all studies included: diabetes (407[29] patients, 3 articles did not report), hypertension (918[64] patients, 3 articles did not report), depression (172[18] patients, 9 articles did not report), hyperlipidemia (55[32]patients, 9 articles did not report), and atrial fibrillation (298[29]patients, 8 articles did not report). In order to treat these concurrent conditions, several patients were currently taking medications including

aspirin (134[64] patients), statins (68[49] patients), beta blockers (8[33]), and ACE inhibitors (65[40]patients). The mean weight was 76.8(7.4) kilograms, with 20.8(9.6) patients with a BMI $\geq$ 30. The average time since a patient's first stroke was 20.9 days, with a range from 1 to 74.9 days. NIHSS (3.9(3.0)) and mRS (3.3(1.7)) indicated stroke severity and functional impairment in the sample.

Inflammatory Biomarkers, Functional Recovery, & Relationship Between Inflammatory Markers and Functional Recovery

C-reactive protein (mg/dl) was the most common inflammatory marker discussed in 8 of the studies, with Interleukin-6 (pg/ml) being the second most discussed in 5 of the studies. The average baseline inflammatory biomarker values across the 11 studies were: IL-6 of 8.8(4.7) pg/ml, CRP of 1.4(1.0) mg/dl, IL-1α of 2.8(0.0) pg/ml, TNF-α of 62.3(89.0) pg/ml, and sICAM-1 of 387.7(167.3) ng/ml.

Only four studies provided repeated measures of inflammatory markers:

Manolescu et al.[8] stated an IL-6 of 19.2(1.7) pg/ml upon patient admission to inpatient rehabilitation, which had a significant increase to 28.9(10.1) pg/ml at patient discharge with an average hospitalization period of 13(1.0) days, at $p<0.05$.

Aquilani et al.[20] indicated an IL-6 inflammatory marker mean value at patient admission to inpatient rehabilitation of 19.24(23.01) pg/ml compared to a mean at patient discharge of 10.4(7.8) pg/ml, with an average hospitalization period of 45(7) days.

Ryan et al. [21] specified an IL-6 mean value of 10.9(11.1) pg/ml in stroke patients pre-resistive training compared to a mean value of 12.0(12.6) pg/ml after 12 weeks of resistive training.

Yeh et al.[19] noted a sICAM-1 inflammatory marker mean value pre-robot assisted therapy of 275.3(80.4) ng/mL compared to a mean value of 258.5(75.0) ng/mL post-robot assisted therapy.

The most common functional recovery measure, reported in 4 of the 11 studies, was NIHSS. mRS was only used in 4 studies. Other recovery measures included functional independence measure (FIM) or the Barthel Index (BI). Four studies used the Mini Mental State Examination (MMSE) to assess patient mental state upon hospital admission and to define cognitive recovery, and for symptoms of post-stroke depression, the Hamilton Depression Scale (HAMD) was used in 1 study. Stroke patient outcomes were assessed at various timepoints, most commonly at patient admission or discharge.

Inflammatory marker and Functional Recovery findings:

Rothenburg et al.[22] studied 48 ischemic stroke patients within their first month post-stroke (NIHSS score of 6.8(4.0) and a MMSE of 26.4(3.8)) and found a relationship between inflammation and the development of cognitive impairment post-stroke. Specifically, IL-6 ($r=-0.33$, $p=0.025$) and CRP ($r=-0.46$, $p=0.002$) were significantly correlated with MMSE scores and were significantly intercorrelated ($r=0.48$, $p=0.001$). When patient risk factors (diabetes, smoking, obesity, hypertension, and hyperlipidemia) were included in the analyses, serum CRP values remained significantly associated to

MMSE scores (β_{CRP}=-.46, p=0.002). Similarly, CRP values were the only significant predictor of MMSE ($F_{1,44}$= 11.3, p=0.002) when other variables of age, infarct side, and level of education were accounted for. MMSE scores ranged from 13 to 30, with 21% of the sample having evidence of cognitive impairment (MMSE $\leq$ 24), thus the sample size for this exploratory study was limited and may have influenced the detection of relationships between cognitive impairment, stroke severity, and inflammatory markers, specifically IL-6 and IFN-δ.

Meng et al.[9] assessed levels of inflammatory biomarkers the morning after admission in patients with and without post-stroke depression (PSD). The results demonstrated that levels of IL-6 and TNF-α were significantly higher in the PSD group (IL-6 4.6(1.0), TNF-α 224.4(44.1)) than in the non-PSD group (IL-6 3.4(0.9), TNF-α 164.9(43.7)). Patients with PSD were also more likely to have lower nerve growth factor levels (average 6.5 vs 8.1 ng/L). In general, patients with post stroke depression were more likely to present higher NIHSS scores (median, 3 vs 1), as well as lower MMSE scores (median, 25 vs 29) compared to patients without post-stroke depression. It is important to note that the NIHSS (median, IQR) of 1.0(0;2) in the non-PSD, and of 3(1;6) in the PSD group is not inclusive of more severe strokes and limits the generalizability of the results.

Regarding stroke patient recovery, *Manolescu et al*.[8] found a decreasing trend for Lp(a) concentrations, but an increasing trend for IL-6, IL-8, and sICAM-1 concentrations at discharge from inpatient rehabilitation. There were statistically significantly increased values of inflammatory markers in stroke patients compared to healthy control subjects at inpatient rehabilitation hospital admission for IL-6, TNF-α, and

Lp(a). Improvement in functional recovery, as measured by the Barthel index, was correlated with decreases in IL-1α TNF-α, and LP(a). It is important to note that only 18 patients with stroke were included in this study and that inflammatory markers may be confounded by age and comorbidities of the study participants.

Aquilani et al.[20] studied inflammatory markers at admission and discharge of inpatient rehabilitation in patients admitted within 90 days post-stroke. Upon admission, the study sample had elevated IL-6 and CRP values. An inflammatory status was found in 55% of the 94 stroke patients and related to significantly lower FIM scores. At admission, there was no significant correlation between functional disability and IL-6, although a significant relationship was found between functional disability, measured by FIM, and the liver shift of protein synthesis. Serum albumin was an independent predictor of functional independence at admission and had an inverse relationship with IL-6. During the rehabilitation stage of stroke, IL-6 and albumin maintained their inverse relationship. The gain in functional independence (FIM) averaged 17.8 points for stroke patients with inflammation and 15.6 points in stroke patients without inflammation. At discharge, the inflammation rate, although reduced, was still present in 60% of the inflamed subjects and related to poorer functional status ($p<0.03$). FIM positively correlated with blood hemoglobin levels, serum albumin concentrations, plasma histidine, tryptophan, and tyrosine concentrations. The study separately analyzed inflammatory markers for the subset (32%) of patients diagnosed with dysphagia and found that the prevalence of inflammation was higher in persons with dysphagia (73% vs. 43%) and related to lower FIM scores for patients with high inflammation and dysphagia (35.3 points lower). Patients with and without dysphagia improved FIM scores during rehabilitation by 14 and 17

points, respectively. Patients with dysphagia were more likely to develop infection during rehabilitation (66% vs. 37%).

Smith et al.[23] reported on an association of IL-6 peak plasma measures with CT infarct volume (0.26, p=0.19) and stroke severity (0.37, p=0.03) at 5 to 7 days post-stroke, and functional recovery outcomes, mRS and BI. Specifically, peak plasma IL-6 concentration correlated significantly with BI at 3 months (r=-0.074), mRS at 12 months (r=0.77), and BI at 12 months (r=-0.77). Strong correlations were found for CRP and WBC and functional recovery outcomes, NIHSS, mRS, and BI, at all timepoints, 1 week, 3 months and 12 months. This was the only article in the review to compare CRP values with functional recovery (mRS) from 5 to 7 days post stroke out to 1 year. Here*, Smith et al.[23]* noted a mean of 0.54 CRP (mg/dl) was analyzed in the NIHSS cohort at 5 to 7 days post stroke, 0.63 CRP (mg/dl) at 3 months poststroke, and 0.65 CRP (mg/dl) at 12 months post stroke. It is important to note the limited sample size of this study with 34 participants available for week 1 data and 19 participants available for 12-month data.

Winbeck et al.[24] also demonstrated a relationship between serial CRP measurement during the acute phase of stroke with functional outcomes. *Winbeck* et al.[24] analyzed three CRP concentrations at three different timepoints. The first CRP 1 (.86(.33)) mg/dl concentration determined immediately after patient admission within 12 hours of symptom onset, CRP 2 (1.22(.67)) mg/dl was taken 24 hours after stroke symptom onset, and CRP 3 (1.8(1.0)) mg/dl was evaluated 24 hours after the second measurement. Results indicate that the initial CRP value determined within 12 hours of symptom onset failed to predict new fatal or nonfatal cerebrovascular events as well as functional outcome after follow up at 1 year. CRP assessed at 12-24 hours after ischemic

stroke predicted end point events and an unfavorable outcome after stroke (odds ratio, 3.5). Furthermore, patients with lower CRP values at 24 hours after symptom onset had poorer functional recovery as measured by the BI at one year. The authors concluded that the combination of initial diffusion weighted imaging (DWI) lesion volume and CRP measurement at 24 hours after symptom onset may aid in identifying patients who are predisposed to activation of the inflammatory process due to even small infarcts.

Geng et al.[7] found that CRP values within the first 24 hours of admission (OR 4.89) and direct bilirubin (OR 1.79) predicted poor functional status (mRS between 3 to 6) at discharge, on average two weeks after admission. Age and residence in rural regions were also associated with poorer functional status which may also relate to other causes of inflammation.

Rabadi et al.[25] investigated the relationship between CRP at inpatient rehabilitation admission and change in functional status at discharge. This study found that functional measures at admission and not CRP predicted improvement in function at discharge. Elevated CRP at admission was related to discharge to an institution (vs. home) (66% to 44%, $p<0.03$), as well as lower functional status, motricity index and FIM, and higher likelihood of infection at admission (57% vs. 29%, $p<0.005$).

Winovich et al.[26] found that CRP (p value =0.01 adjusted for interaction) and IL-6 (p value =0.04 adjusted for interaction) levels were associated with cognitive decline in men post-stroke. These authors also found that IL-6 was associated with post-stroke survival for persons under 80 years old. One limitation of the study was the amount of missing data for ADLs. When ADL data was imputed and characterized as

ADL decline or no ADL decline, higher CRP and IL-6 values were statistically significant risk factors for poor functional outcomes.

Yeh et al.[19] demonstrated that 4 weeks of rehabilitation reduced oxidative stress biomarker level (malondialdehyde, MDA, $z=2.54$, $p<0.05$), indicating that rehabilitation alleviated oxidative stress in these patients. There was a significant correlation between pre-MDA and post-Wolf Motor Function Test (WMFT) time ($\rho=0.462$, $p<0.05$). After rehabilitation, patients significantly improved on functional outcomes as indicated by the mRS ($z=3.45$, $p=0.001$) and the Stroke Impact Scale (SIS) ($z=2.98$, $p=0.003$).

Ryan et al.[21] found that inflammatory markers did not increase after 12 weeks of resistive training in a pilot study of 20 subacute patients' post-stroke. CRP did decrease 12%; however, this was not a statistically significant change. Of note, baseline inflammatory values for this patient population were within or near normal limits for the inflammatory markers, IL-6 10.9(11.1)ρg/mL, IL-8 13.6(15.3) ρg/mL, CRP 6.5(8.5) mg/mL, and TNF-α 16.3(5.9)ρg/mL, which may limit generalizability to patients with higher baseline inflammation.

There was not sufficient longitudinal data across any of the 12 studies to perform outcome analyses at multiple timepoints.

Discussion:

In this review, we sought to understand changes in inflammatory biomarkers post-stroke, the relationship between these changes and functional outcomes, and the impact of rehabilitation on inflammation post-stroke. Eleven studies met inclusion criteria for this review, and in summary they indicate that there is preliminary evidence for the evaluation

of inflammatory biomarkers post-stroke including the role of inflammation in functional recovery, and the influence of rehabilitation on inflammation. There is more substantial evidence to support the use of inflammatory biomarkers to predict post-stroke mortality.

Inflammatory Biomarkers Post-Stroke

There is limited and conflicting evidence on the time course of inflammation post-stroke. Following stroke, IL-6 is released by activated microglia within the CNS [27], and this release is mirrored by increased peripheral IL-6 concentrations [28]. *Rothenburg et al.[22]* found no association with time post stoke and levels of CRP and IL-6 in patients ranging from 5-days to one-month post-stroke. *Manolescu et al.[8]* noted an increase in IL-6 from admission to discharge from inpatient rehabilitation, while *Aquilani et al.[20]* found a reduction in IL-6 from admission to discharge from inpatient rehabilitation. *Ryan et al.[21]* found no change in IL-6 from pre- to post-resistive training. *Winbeck et al.[24]* found that CRP level between 12 to 24 hours post-stroke, but not prior to 12 hours or between 24 and 48 hours, was associated with mortality and functional recovery. In *Rabadi et al.[25]*, patients with greater time since stroke also had higher levels of CRP; however, they were also likely to have longer acute hospitalizations and, presumably, more severe strokes. If inflammatory markers are to be used as a prognostic factor or an indicator for a specific intervention, it is vital that there is first a good understanding of the natural progression of inflammation post-stroke. Furthermore, an understanding of the degree to which inflammation is related to the stroke event versus indicative of comorbidities, age, and medication will be important in determining the role of inflammatory markers in patient care post-stroke.

Relationship Between Inflammatory Markers and Functional Status

There is emerging evidence that inflammation is related to functional status. *Meng et al.[9], Manolescu et al.[8], Aquilani et al.[20], Smith et al.[23], Winbeck et al.[24], Geng et al.[7], Rabadi et al.[25],* and *Winovich et al.[26]* all reported associations between inflammation, and functional status (mRS, BI, FIM, dysphagia, etc.). *Rothenburg et al.[22]* and *Winovich et al.[26]* found relationships between inflammation (IL-6 and CRP) and cognitive impairment post-stroke. *Winovich et al.[26]* and *Winbeck et al.[24]* found an association with inflammation and stroke survival. *Meng et al.[9]* found that IL-6 and TNF-α were associated with PSD.

Impact of Rehabilitation on Inflammation

Four of the 11 studies included in the review measured inflammation pre- and post-rehabilitation. These studies generally found that inflammatory markers were decreased after rehabilitation. *Manolescu et al.[8]* found decreases in IL-1α TNF-α, and LP(a) related to functional improvement (BI); however, this study had a small sample size (n=18), and no description of the rehabilitation was provided. *Aquilani et al.[20]* found that both patients with and without inflammation improved their functional status (FIM) with rehabilitation (specific rehabilitation unspecified). While inflammation levels were generally decreased, 60% of patients with inflammation on admission had inflammation at discharge which was associated with poorer functional status (FIM). *Yeh et al.[19]* found that after four weeks of upper limb intervention oxidative stress (MDA) was

decreased. *Ryan et al.[21]* found no change in inflammation after 12 weeks of lower limb resistive training. It is important to note that stroke patients represent a heterogenous population, and therefore inclusion criteria and participant demographics should be carefully considered when evaluating changes in inflammatory markers. Additionally, the time post stroke that a study is conducted is critical as we expect functional differences and rehabilitation strategies to vary in the acute, subacute, and chronic stages.

Given the small number of available clinical studies, there is not sufficient information on the relationship of inflammation and recovery outcomes in stroke patients. Based on the results of this review, it is evident that inflammatory biomarkers CRP and IL-6 are indicators of this process. Patient comorbidities, stroke severity, and age also contribute to acute stroke patent recovery trajectory and overall worse functional outcomes and are associated with a more pronounced elevation in these biomarkers.

Clinical Implications:

This review indicates an emerging understanding of the role of inflammation in the natural progression of post-stroke recovery as well as in rehabilitation-aided recovery. Ultimately, such knowledge may help clinicians know when and how to optimize post-stroke rehabilitation. For example, there is controversy amongst rehabilitation professionals about when to start rehabilitation [29]. Data on the progression of inflammation and inflammatory changes during rehabilitation would help clarify whether early rehabilitation is helpful or harmful. In addition to the timing of rehabilitation, the intensity and type of rehabilitation is often debated by clinicians. It would be particularly helpful to understand

if intense rehabilitation, e.g. rehabilitation that stresses the patient such as strenuous exercise or long duration exercise, has a meaningful negative impact on inflammation as clinicians largely avoid these due to health safety concerns.

Limitations:

This review has several limitations. Most of the studies reviewed present pilot data with small sample sizes. Several of these studies were performed at a single center, and future studies should test whether observations in these studies are generalizable to larger patient populations. The studies also varied greatly regarding stroke patient selection and recruitment, stroke baseline severity, time to intervention, and the measures used to assess functional recovery and inflammation. Although stroke severity was assessed and included as a covariate, the timing of administration of the NIHSS relative to stroke and other assessments varied between participants. Most of the studies were not designed or powered to assess cerebrovascular risk factors. Additionally, in most of the studies, inflammatory biomarkers were measured only once at admission. Future studies are needed to determine whether other inflammatory biomarkers would be more useful than CRP. Further research including longitudinal data is crucial to assess inflammatory biomarkers at several timepoints during stroke recovery and rehabilitation. Based on results of this review, there is primarily evidence that inflammation biomarkers have a relationship with functional status post-stroke; however further research is needed to define the nature and time course of the relationship during specific rehabilitation treatments, that vary in intensity. Additionally, studies need to standardize the functional

status measures and inflammatory biomarkers that are reported to improve comparisons and generalizability.

Conclusion:

The intention of this review was to determine the state of science for specific inflammatory biomarkers in post-stroke patients at various timepoints after stroke symptom onset, and to determine if there is a relationship between inflammation and functional recovery. The literature reveals emerging evidence for evaluating inflammatory biomarkers post-stroke, the role of inflammation in functional recovery, and the influence of rehabilitation on inflammation. There is more substantial evidence to support the use of inflammatory biomarkers to predict post-stroke mortality. This review supports the need for further investigation, using longitudinal data, into the relationship between inflammation and rehabilitation-related recovery to determine and, optimize, patient outcomes after stroke.

Author Contributions:

CC and HSB were responsible for conceptualization of the review topic. CC, KM, DMB, HSB, and ST contributed to conducting the review and writing the manuscript. All authors contributed to reviewing the manuscript.

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Conflict of Interest: ST is cofounder and consultant for Q32Bio, a company developing complement inhibitors. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

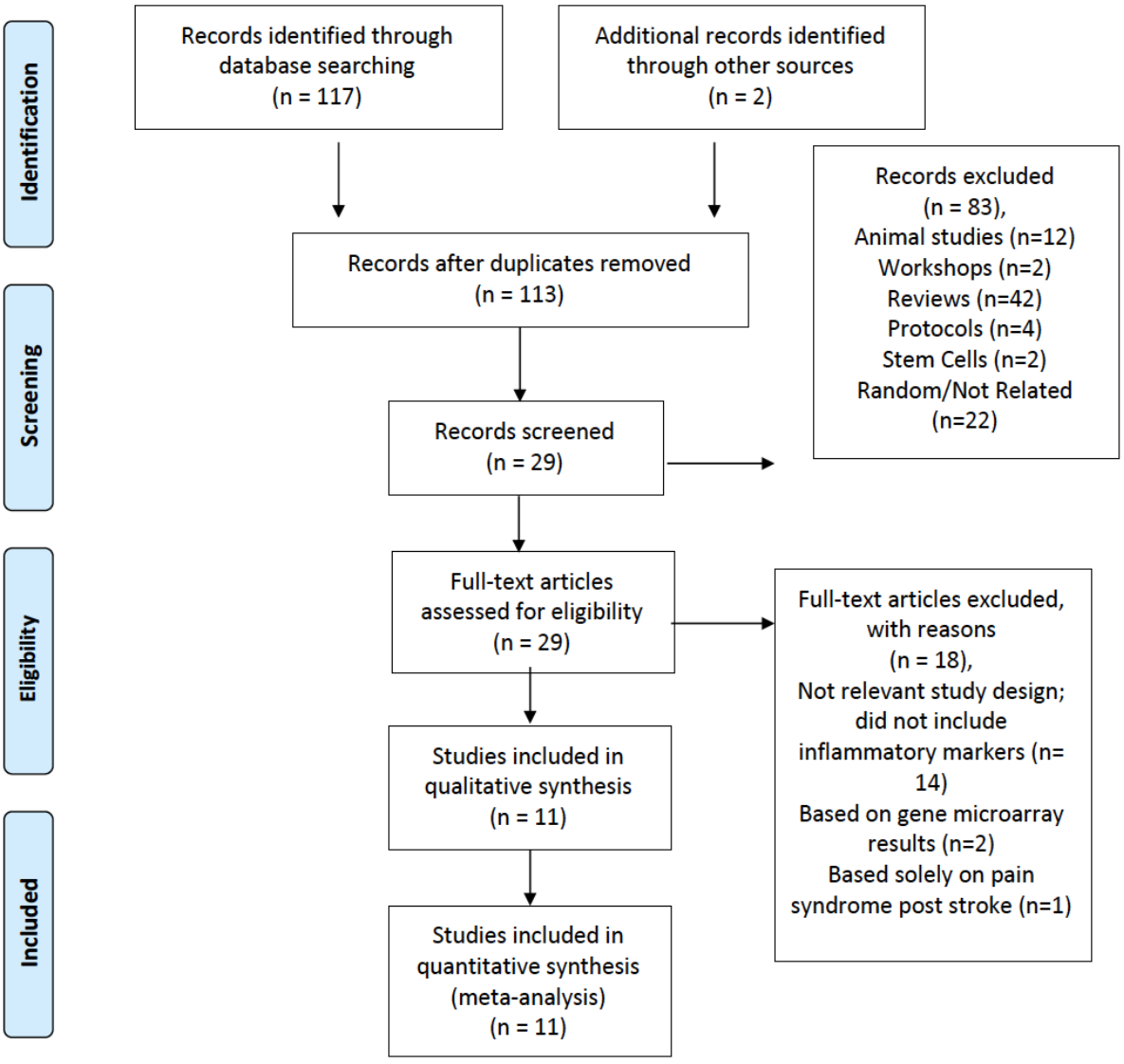

FIGURE 1: PRISMA GUIDELINES

Table 1: Baseline Characteristics of Stroke Patients	
	Totals
	Acute Stroke Patients without Intervention
Age (years)	67.5
Female (n=1,581)	829(52)
Male (n=1,581)	723(46)
Primary Diagnosis	Acute stroke
Concurrent Condition:	
Diabetes (n=1,419)	407(29)
Hypertension (n=1,443)	918(64)
Depression (n=941)	172(18)
Hyperlipidemia (n=174)	55(32)
Atrial Fibrillation (n=1,032)	298(29)
Medications:	
Aspirin (n=208)	134(64)
Statins (n=139)	68(49)
Beta Blockers (n=24)	8(33)
ACE Inhibitors (n=163)	65(40)
Smoker (n=147)	110(75)
Drinker (n=448)	107(24)
Weight (kg)	76.8
Obesity (BMI≥30 kg/m^2) (n=42)	14(33)
BMI (kg/m^2)	26.1
Time Since Stroke (days)	20.9
IL-6 (pg/ml)	8.8
CRP (mg/dl)	1.5
IL-1α (pg/ml)	2.8
TNF-α (pg/ml)	62.3
sICAM-1 (ng/ml)	387.7
Modified Rankin Score	3.3
NIHSS	3.9
White Blood Cell (x10^3 cell/uL)	7.0
Plasma Glucose (mg/dl)	124.0
Systolic Blood Pressure (mmHg)	148.0
Diastolic Blood Pressure (mmHg)	83.0
Cholesterol (mg/dl)	137.8
HDL (mg/dl)	30.4
LDL (mg/dl)	81.2

Data is represented as the overall mean value across all 11 studies; sex, concurrent conditions, & medications are represented as n(%).

Values reflect an average of data presented in the 11 papers; however, not all papers included all variables reflected in the table.
ACE inhibitor- Angiotensin Converting Enzyme inhibitor; BMI- Body Mass Index; CRP- C Reactive Protein; HDL- High Density Lipoprotein; IL-1α- Interleukin-1α; IL-6- Interleukin-6; LDL- Low Density Lipoprotein; TNF-α- Tumor Necrosis Factor-α

Table 2: Patient Inflammation and Functional Measures							
		IL-6 (pg/ml)	*CRP (mg/dl)*	*IL-1α (pg/ml)*	*TNF-α (pg/ml)*	*sICAM-1 (ng/ml)*	*Functional Measures*
Lana Rothenburg, 2010, [22]	**Individuals with cognitive symptoms post stroke**	8.5 (11.3)	0.7 (0.5)				NIHSS: 6.8 (4.0); MMSE: 26.4(3.8)
Guilin Meng, 2017, [9]	**Ischemic Stroke Patients without PSD**	3.4(0.9)			164.9(43.7)		NIHSS: 1(0-2); MMSE: 29(26;30): HAMD: 0(0;1)
	Ischemic stroke patients with PSD	4.6(1.0)			224.4 (44.1)		NIHSS: 3(1-6); MMSE: 25(22;27); HAMD: 9.4(8.5;10.8)
Bogdan Manolescu, 2011, [8]	**Admission**	19.2 (1.7)		3.2(0.9)	7.6(0.6)	315.3(17.1)	BI: 56.5(3.3)
	Discharge	28.9 (10.1)		3.0 (1.0)	7.5 (0.6)	323.6 (20.2)	BI: 60.5(3.4)
Roberto Aquilani, 2014, [20]	**Admission (Inflam)**	19.2(23.0)	1.5 (2.2)				FIM: 56.1(26.0)
	Discharge (Inflam)	10.3(7.8)	0.85(1.6)				FIM: 73.9(28.5)
	Admission (No Inflam)	4.1(1.6)	0.4(0.5)				FIM: 75.3(23.6)
	Discharge (No Inflam)	5.5(3.4)	0.6(1.2)				FIM: 90.0(24.1)
Craig Smith, 2004, [23]	**NIHSS 5-7 days**		0.5				NIHSS: 11(1,39)
	mRS 3 months		0.6				mRS: 3(0,5)
	mRS 12 months		0.7				mRS: 3(0,5)
Kerstin Winbeck, 2002, [24]	**Admission (CRP 1)**		0.8 (.6-1.0)				
	Admission (CRP 2)		1.2 (0.8-1.5)				BI:50(10-83.8); BI: 62.5(25-95)
	Admission (CRP 3)		1.7 (1.2-2.2)				
He-Hong Geng, 2016, [7]	**Good Outcome (mRS≤2)**		0.5(0.6)				NIHSS≤15: 138(61.9); NIHSS>15: 85(38.1)

	Poor Outcome (mRS>2)		1.8(0.2)				NIHSS≤15: 32(41.0); NIHSS>15: 46(59.0)
Meheroz Rabadi, 2008, [25]	**Normal CRP**		4.2(3.0)				FIM: 53.0(20.4); MMSE: 16.2(9.7); UE MI:54.5(40.1); LE MI: 65.6(31.7)
	Abnormal CRP		41.6(41.8)				FIM: 42.4(15.7); MMSE:14.1(10.7); UE MI:36.6(38.2); LE MI:40.2(35.7)
Divya Winovich, 2017, [26]	**Survival analysis population**	2.9(1.9-4.3)	3.0(1.5-6.5)				
	Eligible recovery population	2.7(1.7-4.2)	2.9(1.5-6.7)				
Ting-ting Yeh, 2017, [19]	**Pre-treatment**					275.3(80.3)	mRS: 3.3(0.9); SIS: 59.4(13.2); WMFT-FAS: 2.2(0.9)
	Post-treatment					258.4(74.9)	mRS: 2.4(1.0); SIS:65.3(13.8); WMFT-FAS: 2.7(1.0)
Alice Ryan, 2017, [21]	**Before resistive training in stroke survivors**	10.9(11.1)	6.5(8.5)		16.3(5.9)	580(215)	
	After resistive training in stroke survivors	12.0(12.6)	5.7(7.3)		16.5(8.2)	536 (140)	

Data is represented as mean (standard deviation).
Meng, 2017 listed MMSE and HAMD as median (IQR); Smith, 2004 listed NIHSS and mRS as median (min, max); Winbeck, 2002 listed BI as median (IQR) of CRP2 ≥0.86 mg/dL and CRP 2 <0.86 mg/dL; Geng, 2016 listed NIHSS as n(%).

BI- Barthel Index; CRP- C Reactive Protein; FIM- Functional Independence Measure; HAMD- Hamilton Depression Rating Scale; IL-1α- Interleukin-1α; IL-6- Interleukin-6; LE MI- Lower Extremity Motricity Index; MMSE- Mini-Mental State Exam; mRS- Modified Rankin Score; NIHSS- NIH Stroke Scale; sICAM-1- Soluble Intercellular Adhesion Molecule-1; SIS- Stroke Impact Scale; TNF-α- Tumor Necrosis Factor-α; UE MI- Upper Extremity Motricity Index; WMFT-FAS- Wolf Motor Function Test-Functional Ability Scale

A.1: Summary of Studies Conducted in Systematic Review

Authors	Population	Outcomes	Intervention	Main Results
Rothenburg (2010), [22]	Acute Ischemic Stroke Patients with post-stroke cognitive symptoms	NIHSS, MMSE	-	Relationship between inflammation & development of cognitive impairment post stroke; CRP significantly correlated with MMSE; IL-6 & CRP significantly correlated; NIHSS score of 6.8(4.0) & MMSE 26.4 (3.8) within 1st month post stroke
Meng (2017), [9]	Acute ischemic stroke patients with post stroke depression	NIHSS, MMSE	-	IL-6 & TNF-α were significantly higher in PSD group; PSD patients were more likely to have lower nerve growth factor levels; patients with PSD were more like to represent higher NIHSS scores & lower MMSE scores compared to patients without post stroke depression
Manolescu (2011), [8]	Ischemic or Hemorrhagic stroke patients	BI at admission & discharge	Rehabilitation	Regarding stroke patient recovery, decreasing trend of Lp(a) concentrations; increasing trend of IL-6, IL-8, & sICAM-1 concentrations
Aquilani (2014), [20]	Ischemic or Hemorrhagic stroke patients	1st week of admission & before discharge, FIM	Rehabilitation	Upon admission to rehabilitation, inflammatory status found in 55% of stroke patients; significant relationship between FIM & the liver shift of protein synthesis. At rehabilitation stage of stroke, gain in FIM averaged 17.8 points for stroke patients with inflammation and 15.6 in stroke patients without inflammation; FIM positively correlated with blood hemoglobin levels & serum albumin levels
Smith (2004), [23]	Acute ischemic stroke patients	NIHSS, mRS, BI, Mortality	-	Peak plasma IL-6 correlated significantly with stroke severity at 5-7 days & mRS at 3 months; CRP correlated with functional recovery at all timepoints
Winbeck (2002), [24]	Ischemic stroke patients	BI & mRS at admission & after follow-up, mortality	-	CRP assessed at 12-24 hours after ischemic stroke predicts end point events & an unfavorable outcome after stroke
Geng (2016), [7]	Acute ischemic stroke patients	mRS at discharge	-	Poor outcome at discharge was associated with recurrent acute ischemic stroke, direct bilirubin (DBIL), & CRP
Rabadi (2008), [25]	Ischemic or hemorrhagic stroke patients	TFIM, FIM motor & cognitive sub scores, length of stay	Rehabilitation	Those with abnormal admission CRP levels had a longer interval between stroke onset & admission to the rehabilitation hospital, lower TFIM & motor FIM sub score; admission CRP

				level correlated with stroke severity & was a predictor of home discharge. However, CRP did not predict change in functional status measures at discharge.
Winovich (2017), [26]	Ischemic stroke patients	Survival time, ADL, MMSE	-	CRP & IL-6 levels were associated with cognitive decline in men; IL-6 was associated with post stroke survival in patients under 80; CRP & IL-6 were associated with ADL decline at one year in imputed data
Yeh (2017), [19]	Subacute stroke patients	mRS, SIS, WMFT	Rehabilitation (robot assisted therapy)	4 weeks of rehabilitation reduced the oxidative stress biomarker level MDA; no change was noted in the inflammatory biomarker, sICAM-1
Ryan (2017), [21]	Ischemic stroke patients	-	Rehabilitation (resistive training)	Inflammatory markers (TNF-α, IL-6, CRP, serum amyloid A, sICAM-1, sVCAM-1) did not change with 12 weeks of resistive training

ADL- Activities of Daily Living; BI- Barthel Index; CRP- C Reactive Protein; DBIL- Direct Bilirubin; FIM- Functional Independence Measure; IL-6- Interleukin-6; IL-8- Interleukin-8; Lp(a)- Lipoprotein a; MMSE- Mini-Mental State Examination; mRS- Modified Rankin Score; NIHSS- NIH Stroke Scale; PSD- Post-Stroke Depression; sICAM-1- Soluble Intercellular Adhesion Molecule-1; SIS- Stroke Impact Scale; sVCAM-1- soluble form of Vascular Cell Adhesion Molecule-1; TFIM- Total Functional Independence Measure; TNF-α- Tumor Necrosis Factor-α; WMFT- Wolf Motor Function Test

A.2: Methodological Quality of Each Study Assessed by the Downs and Black Checklist

Author (Year Published)	Q1	Q2	Q3	Q5	Q6	Q7	Q10	Q11	Q12	Q16	Q18	Q20	Q21	Q22	Q25	Score	%
Rothenburg (2010), [22]	1	1	1	1	1	NA	1	1	NA	1	1	1	0	1	NA	10 out of 11	91%
Meng (2017), [9]	1	1	1	NA	1	1	1	1	0	1	1	1	1	1	1	14 out of 15	93%
Manolescu (2011), [8]	1	1	1	0	1	0	1	1	0	1	1	1	1	1	0	11 out of 15	73%
Aquilani (2014), [20]	1	1	1	NA	1	NA	1	NA	NA	1	1	1	NA	NA	1	9 out of 9	100%
Smith (2004), [23]	1	1	1	0	1	0	1	1	NA	1	1	1	1	1	1	12 out of 14	86%
Winbeck (2002), [24]	1	1	1	0	1	1	1	1	NA	1	1	1	1	1	NA	12 out of 13	92%
Geng (2016), [7]	1	1	1	1	1	1	1	1	1	1	1	1	NA	1	1	14 out of 14	100%
Rabadi (2008), [25]	1	1	1	1	1	1	1	1	NA	1	1	1	1	1	NA	13 out of 14	93%
Winovich (2017), [26]	1	1	1	1	1	1	1	1	0	1	NA	1	0	1	NA	11 out of 13	85%
Yeh (2017), [19]	1	1	1	0	1	0	1	0	NA	1	1	1	1	1	NA	9 out of 13	69%
Ryan (2017), [21]	1	1	1	NA	1	0	1	1	NA	1	NA	1	1	NA	NA	9 out of 10	90%

Yes (1 point), No (0 point), NA not applicable (question excluded from quality analysis). Reporting: Q1: Is the hypothesis/aim clearly described? Q2: Are the main outcomes of the study clearly described? Q3: Are the characteristics of the patients included in the study clearly described? Q5: Are distributions of principle confounders clearly described? Q6: Are the main findings of the study clearly described? Q7: Does the study provide estimates of the random variability in the data for the main outcomes? External Validity: Q10: Have actual probability values been reported? Q11: Were the subjects asked to participate in the study rep of the entire population from which they were recruited? Q12: Were those subjects who were prepared to participate rep of entire population? Bias: Q16: Were there any of the results based on data dredging? Q18: Were the statistical tests used to assess main outcomes appropriate? Confounding: Q20: Were the main outcome measures used accurate? Q21: Were cases and controls from the same population? Q22: Were the cases and controls recruited over the same period of time? Q25: Was there adequate adjustment for confounding in the analysis?

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CHAPTER 5: AIM 2

Manuscript 2: Evaluating the Comorbidities of Age and Cigarette Smoking on Stroke Outcomes in the Context of Anti-Complement Mitigation Strategies

The following manuscript was submitted to *Frontiers in Immunology*: *Molecular Innate Immunity* on February 7, 2023.

TITLE PAGE:

Evaluating the comorbidities of age and cigarette smoking on stroke outcomes in the context of anti-complement mitigation strategies

Christine Couch[1†], Ali M. Alawieh[2†*], Amer Toutonji[1], Carl Atkinson[3], Stephen Tomlinson[1,4*]

† Contributed equally to the work

[1]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425, USA.

[2]Department of Neurosurgery, Emory University School of Medicine, Atlanta, GA, United States.

[3]Division of Pulmonary, Critical Care and Sleep Medicine, University of Florida, Gainesville, FL, United States.

[4]Ralph H. Johnson VA Medical Center, Charleston, SC 29425, USA.

*** Correspondence:**
Stephen Tomlinson: tomlinss@musc.edu and Ali Alawieh: ali.mostafa.alawieh@emory.edu

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Abstract

Multiple neuroprotective agents have shown beneficial effects in rodent models of stroke, but they have failed to translate in the clinic. In this perspective, we consider that a likely explanation for this failure is that there has been inadequate assessment of functional outcomes in preclinical stroke models, as well the use of young healthy animals that are not representative of clinical cohorts. Although the impact of older age and cigarette smoking comorbidities on stroke outcomes is well documented clinically, the impact of these (and other) stroke comorbidities on the neuroinflammatory response after stroke, as well as the response to neuroprotective agents, remains largely unexplored. We have shown that a complement inhibitor (B4Crry), that targets specifically to the ischemic penumbra and inhibits complement activation, reduces neuroinflammation and improves outcomes following murine ischemic stroke. For this perspective, we investigated the impact of age and smoking comorbidities on acute outcomes after stroke, and assessed whether increased complement activation contributes to a worsened outcome with these comorbidities. We found that the pro-inflammatory effects of aging and smoking contribute to worse stroke outcomes, and these effects are mitigated by complement inhibition.

Introduction

Acute ischemic stroke occurs secondary to thrombosis or embolization within the cerebral vasculature, which leads to an infarct within the brain and clinical deficits. The standard of care for stroke therapy is rapid recanalization of the target vessel, either pharmacologically or endovascularly. Over the past decade, outcomes following acute stroke have improved significantly due to both stroke prevention efforts and the introduction of endovascular thrombectomy as the routine standard of care. Nevertheless, stroke remains a major cause of disability and mortality in the United States and Worldwide. The current standard of care for stroke patients remains rapid reperfusion using thrombolysis and/or thrombectomy for eligible patients that present within about 24 hours of onset (1-3). However, despite a successful recanalization rate of over 85%, the rate of functional independence at 90 days remained at less than 50% in successfully recanalized patients (3-4). Reasons for this mismatch between recanalization and recovery are multiple and include the rapid progression of infarct secondary to inflammation, microthrombosis in the microvasculature, hemorrhagic complications, and limited rehabilitation support. Several clinical studies have focused on identifying a subset of stroke patients that are termed “fast progressors”, that is patients whose infarct progresses rapidly despite recanalization and who tend to have worse functional outcomes. Poor cerebrovascular reserve and collateral circulation are considered major culprits within this patient group, which is attributed to advanced age and other comorbidities, as well as an enhanced local neuroinflammatory response. Data from thrombectomy trials lend strong support to the concept that neuroprotective adjuvant therapies will leverage the benefit of reperfusion and limit the progression of cerebral tissue loss after stroke. However, there are currently no neuroprotective agents approved for ischemic stroke, with multiple agents having failed in clinical trials. This failure of neuroprotective agents is multifactorial, but one perspective is that this is due in large part to the poor design of preclinical studies that often lacked consideration of long-term outcomes, cognitive recovery, rehabilitative interventions, and relevant to this

perspective article, stroke comorbidities(5-6). The translational importance of incorporating stroke comorbidities in evaluating preclinical efficacy of neuroprotective and anti-inflammatory therapies for stroke is being increasingly recognized. Within this context, previous studies from our lab and others have studied the role of the complement system in initiating and propagating a neuroinflammatory response after stroke , (7- 10), and inhibition of complement has been shown to provide long-lasting neuroprotection in murine stroke models. However, these previous investigations were almost exclusively performed using healthy young adult mice. Complement inhibitors are recognized as potential therapeutic agents for treating stroke, and here we provide an assessment on the effects of two major stroke comorbidities, namely advanced age and cigarette smoking (CS), within the framework of complement-dependent neuroinflammation and recovery.

Impact of Cigarette Smoking and Aging in Ischemic Stroke

Comorbidities in patients present as a cluster of risk factors that increase stroke incidence. What is much less appreciated is that comorbidities also alter stroke pathophysiology, lesion development and recovery in profound ways. With the use of genetic animal models and/or pharmacological interventions, it is possible to capture certain features of comorbidities. Comprehensive reviews on animal models with comorbidities are available elsewhere (11-12).

Cigarette smoke is the only patient modifiable risk factor for ischemic stroke, and is correlated with an increased risk of mortality, more severe disability, longer hospital stays and worse overall functional recovery (13-14). Cigarette smoking nearly doubles the risk for stroke, with a dose response relationship between pack-years and stroke risk (15-16). Age is also recognized as a significant predictor of stroke outcome, affecting speed and extent of recovery, mortality, and response to thrombolytic therapy [78]. However, the mechanisms underlying how either comorbidity contributes to worse outcomes are not well understood, and there are very few reports on neuroprotective therapies in the context of these comorbidities, despite continuous recommendations from the STAIR committee and funding bodies. There has only been a single report investigating the effects of cigarette smoke on in the brain, in which it was shown that cigarette smoke exposure induced activation of inflammatory cascades and increased oxidative stress [79]. In other (non-stroke) models, our lab and others have shown that cigarette smoke is associated with altered systemic inflammatory profiles, including complement activation (19-22). Smoking also contributes to decreased vessel wall integrity [80] and may be associated with increased risk of hemorrhagic transformation or intracranial hemorrhage after thrombolytic therapy. There are also only very few reports on the consequences of aging on post stroke neuroinflammation, even though with increasing life expectancy, aging has become a principal risk factor for stroke. It is known that in the absence of ischemic pathology, the aging brain shows a gradual increase in inflammatory signaling [83]

and an increase in reactive oxygen species both basally and in response to injury (23-24). In the normal ageing brain, there is also increased expression of innate immune molecules, including complement proteins [88]. However, as with CS exposure, the role of aging in the context of neuroinflammation and neuroprotection after stroke remains poorly investigated.

Age and CS-exposure lead to worse acute outcomes after murine stroke, an effect that is reversed with complement inhibition

To investigate how age and CS affect a complement-dependent neuroinflammatory response and behavioral outcomes after stroke, we utilized a murine model of 60 minute transient middle cerebral artery occlusion (MCAO) and the site-targeted complement inhibitor, B4Crry, as previously described [65]. The B4Crry inhibitor targets specifically to the ischemic and perilesional region of the post-ischemic brain after MCAO and locally inhibits all complement pathways at the C3 activation step (9).

In an initial study, we exposed mice to 6 months of CS (see supplement) starting at 6-8 weeks of age. Following CS exposure, mice were randomized into vehicle (PBS) or B4Crry treatment groups, and treatment administered 2 hours post MCAO (1 h post-reperfusion). No animals in the CS exposed PBS treated group survived beyond 24 h post-MCAO, whereas 40% of B4Crry treated mice survived to 6 days post-MCAO (n = 10). For this reason, we switched to a 4 month CS exposure paradigm which resulted in improved 24 hour mortality rates (see below).

We assessed the impact of age and CS-exposure on acute outcomes following transient MCAO in terms of neurological deficit, mortality and infarct volume. As above, mice were randomized into either vehicle or B4Crry treatment groups. B4Crry or vehicle was administered 2 hours post MCAO to aged + CS-exposed mice, young + CS-exposed or aged room air mice. As noted above, CS exposure was for 4 months. Our previously published data using young adult mice and the same MCAO model showed a mortality rate of less than 10% at 24 hour post-MCAO

[124]. Here we show that aged + CS exposed mice had a mortality rate of 50%, and aged mice a mortality rate of 35% at 24 hours after stroke (Figure 1A,B). Thus, the impact of CS on mortality in aged mice was higher than that of aging alone. Complement inhibition with B4Crry resulted in a significant reduction in mortality rates at 24 hours in both aged and aged + CS-exposed mice. B4Crry treatment also resulted in a significant reduction in neurological deficit scores and in lesion volume in both groups compared to vehicle (Figure 1A,B). There was no significant difference in 24 hour survival between vehicle and B4Crry treated mice that were exposed to CS. However, B4Crry treatment did significantly improve neurological deficit scores and reduced lesion volume (Figure 1C). When the effect size of B4Crry on neurological outcome was computed, the protective effect of B4Crry was highest in aged + CS-exposed mice (Cohen's d index 1.50), followed by CS-exposed young mice (Cohen's d index 1.36), followed by aged no CS mice (Cohen's d index 1.21). These findings indicate that the effects of age and CS exposure on neurological outcome after MCAO is at least in part mediated by complement.

Complement Inhibition Reduces the Extent of Dendritic Loss and Microglial Activation in Aged Mice and Aged+Cigarette Exposed Mice:

To assess how CS-exposure affects a complement mediated post-stroke neurodegenerative neuroinflammatory response in aged mice, the extent of dendritic loss and microgliosis 24 hours after MCAO in the context of B4Crry treatment was assessed. This was achieved by high resolution immunoflurescence imaging of MAP2 (dendritic marker), microglia/macrophages (Iba1) and neurons (NeuN) (Figure 2A,B). Unbiased stereology was used for quantification (Figure 2C,D). Compared to vehicle treated controls, B4Crry treatment significantly reduced microgliosis and preserved dendritic signal in the ipsilateral hemisphere in both aged and aged-CS exposed mice. There was no significant difference in the extent of microgliosis or dendritic loss between aged and aged + CS exposed mice.

Discussion and Conclusions

The failure of neuroprotective agents in stroke clinical trials is multifactorial, but one contributing factor is thought to be a lack of accounting of stroke comorbidities when evaluating drugs in preclinical studies. A neuroinflammatory response that occurs after stroke is considered to be a major contributor to secondary injury after stroke, and we investigated whether the complement system, a central component of a neuroinflammatory response after stroke, is involved in the negative effects of stroke comorbidities on stroke outcomes. Specifically, we addressed age, a non-modifiable stroke risk factor, and CS exposure, a modifiable risk factor. Both cigarette smoking and aging are factors known to affect mortality and disability after stroke (13-14), (26). However, the mechanisms underlying the effects of age and CS exposure on stroke outcomes are poorly understood, with limited reports on neuroprotective therapies in the context of either cigarette smoking and aging, despite continuous recommendations from the Stroke Treatment Academic Industry Roundtable (STAIR) committee. We investigated acute inflammatory profiles and outcomes in aged mice, and CS exposed aged and young mice in the context of complement inhibition.

Consistent with prior reports describing a more systemic proinflammatory phenotype associated with CS (19), (21), (28), we demonstrate that CS is associated with increased local complement activation after stroke. In the aging brain, increased local complement activity is documented in the presence or absence of an ischemic or traumatic event (22-25, 29-30). Our previous studies using murine models of ischemic stroke have demonstrated that local complement activation in the post-ischemic brain plays an important role in rapid neurological cell loss and worsening outcomes (4), (9-10), (31-32). We have shown that MCAO promotes neuronal stress in perilesional areas and that stressed, but still viable neurons, display danger associated molecular patterns (DAMPs) that activate complement leading to aberrant neuronal uptake by microglia [78]. Here we show that after MCAO, the stroke comorbidities of age and CS exposure exacerbate both infarct growth and a neuroinflammatory response. Local inhibition

of complement with B4Crry interrupted this response and reversed the effects of age and CS exposure on acute progression of a neurodegenerative inflammatory response.

The findings presented support the hypothesis that the impact of stroke comorbidities on worsening stroke outcomes, and specifically the comorbidities of age and CS exposure, is due at least in part to a complement-mediated neuroinflammatory response. Today, patients who present with a high burden of infarcted brain as measured by perfusion imaging are not eligible for endovascular intervention due to risk of hemorrhage and worsening edema (33-36). In this context, a complement inhibitor has the potential for delaying the progression of infarct in patients en route to a comprehensive stroke center for endovascular surgery, as well as for minimizing post-interventional cerebral edema and hemorrhage risk. Both effects would likely increase the subset of stroke patients eligible for intervention and lead to improved functional outcomes. A key message from this work is the implication that complement-mediated neuroinflammation is a major contributor to exacerbation of cerebral injury after stroke, and that this complement-mediated effect is more prominent in subjects with stroke comorbidities. Using pharmacological interventions that can limit the progression of infarct and temporarily preserve the penumbra remains an unmet clinical need, and one that complement inhibition shows potential for fulfilling.

In summary, this perspective supports the narrative that the inclusion of comorbidities in experimental stroke models is important to more accurately represent the stroke patient population, and which is necessary to create neuroprotective therapeutics with a higher level of success in human clinical trials. In this report, we show that complement inhibition is neuroprotective in a model that includes the comorbidities of age and CS exposure, suggesting a improved likelihood for successful translation to a heterogeneous human stroke population.

Author Contributions

AA, CA and ST conceived and planned the reported studies. CC, AA, AT performed the experiments. All authors contributed to analyzing the data and to writing the manuscript. CC, AA and ST formulated the perspective. ST acquired funding for the study

Funding

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Conflict of interest

ST is a consultant for Q32Bio, a company developing complement inhibitors

SUPPLEMENTAL MATERIAL

Materials and Methods

Study Design

For all studies, before the initial acclimation on behavior tasks, male mice were randomly assigned to treatment groups using a random number generator. Lab personnel involved in surgeries, testing, and scoring were blinded to group allocations for the duration of the study. Animals were excluded if mortality occurred during surgery or before administration of treatment, as well as if there was less than an 80% reduction in ipsilateral cerebral blood flow compared with presurgical baseline (see below). Both young adult and aged mice were sacrificed 24 hours after transient MCAO, at which time brain infarct volume and immunofluorescence (IF) parameters were measured. Neurological deficit scoring was measured at 24 hours after MCAO, just prior to sacrifice. End points were determined by time after reperfusion and were 24 hours, or for humane reasons as defined by institutional guidelines. Well defined humane and experimental endpoints were developed through a collaboration with our veterinarian and a scoring matrix was used to determine when pre-emptive euthanasia was necessary.

Recombinant Proteins

The recombinant complement inhibitor B4-Crry used in this study was constructed, expressed, purified, and subjected to quality control as described previously (10), (37).

The MCAO model and Treatment Paradigm

Male adult C57BL/6J mice were obtained from The Jackson Laboratory and allowed 1 week of acclimation before use. Adult animals were 6 months old and aged animals were 16 months old at the time of stroke surgeries. Anesthesia and transient MCAO was performed as described previously [27]. Cerebral blood flow was assessed at baseline (just before MCAO procedure)

and during MCA occlusion, as well as blood pressure and heart rate monitored. Laser Doppler flow monitoring (moorVMS-LDF1 device; Moor Instruments) was used to assess uniform induction of ischemia across animals. Animals with less than 80% reduction in ipsilateral cerebral blood flow compared with presurgical baseline were excluded from study. After surgery and during recovery from anesthesia, temperature was maintained at 37°C and animals were housed in a temperature and humidity-controlled chamber until recovery from anesthesia, and then returned to regular housing. B4Crry (16mg/kg) or vehicle (PBS) was administered intravenously via tail vein injection at 2 hours post-MCAO. We have previously performed dose-response experiments with B4Crry and have shown optimal benefit plateaus between 8-16 mg/ml and is an effective dose in both adult and aged male mice [80]. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina.

Cigarette Smoke exposure paradigm

Adult mice (n=16) were 6-8 weeks old and aged mice (n=20) were 12 months old when initially exposed to cigarette smoke (CS) (34F, Kentucky) for 5 hours/day 5 days/week for 4 months using a Teague TE-10 automated exposure system as described (26, 31). Animals were exposed to 89% sidestream and 11% mainstream CS. To standardize intra-day/batch smoke exposure levels, total suspended particulate matter (TSP) was measured 3 times/day, to maintain a target TSP of 130ppm/m^3 as we have described (21), (31). Exposure at these levels is non-lethal and is associated with carboxyhemoglobin levels between 8% and 12% immediately after exposure, as determined in venous blood by dual beam spectrophotometry [130]. The CS exposed mice were subjected to MCAO and treated as described above. Note that in an initial experiment, mice were exposed to 6 months of CS which resulted in 100% mortality

by 24 hour after MCAO. Thus the data shown herein was derived from a 4 month CS exposure paradigm.

Neurological Deficit Scoring and Assessment

To assess functional recovery, animals were scored daily by two blinded observers using the neurological deficit score described previously and measured 24 hours after MCAO, just prior to sacrifice [131]. Animals received a score of 0 for normal motor function, 1 for torso and contralateral forelimb flexion when lifted by tail, 2 for contralateral circling when held by tail on flat surface, 3 for contralateral leaning when at rest, and 4 for no spontaneous motor activity.

Immunofluorescence Staining and Imaging

Following euthanasia, cardiac perfusion was performed with cooled PBS followed by 4% Paraformaldehyde (PFA) mixed in PBS. Brains were extracted and placed in 4% PFA solution overnight at 4°C. The brains were moved to a new vial with 30% sucrose mixed with 4% PFA and PBS. For tissue cutting, brains were imbedded in optimal cutting temperature (OCT) compound and frozen. At time of cutting, brains were cut in 40 µm coronal sections using a cryostat. The complete brain was collected in 12-well plates and kept in PBS wells until analysis. Brain slices were taken for each brain and were identified by stereometric measurement using a mouse brain atlas and used for immunofluorescent staining as described [132]. Slices were washed with PBS and permeabilized using 3% H_2O_2 followed by 0.1% Triton-X in PBS. Slices were blocked in donkey serum (5% in PBS), washed, and incubated with the primary antibody overnight followed by washing and incubation of the fluorescently tagged secondary antibody. High-resolution imaging was performed using a Zeiss LSM 880 confocal microscope (Zeiss) at 40x zoom with water-media overlay and using the Z-stacking feature of the microscope. Images were deconvoluted using the ZEN 2.5 software (Zeiss) and reconstructed in 3D plane. The primary antibodies used for staining were anti-NeuN (Abcam,

Cat. #: ab104225, 1:200) for neurons, anti-Iba1 (Invitrogen, Cat. #: PA5-21274, 1:80) for microglia/macrophages, and anti-MAP2 (Abcam, Cat. #: ab32454, 1:200) for dendrites. Image analysis was performed using both ZEN (Zeiss) and ImageJ (NIH) software. Imaging quantification was performed using unbiased stereology and using full brain slices at similar brain atlas reference coordinates from each group to avoid bias in field selection. Quantification of MAP2 signal was performed by computing the area of MAP2 signal loss in the ipsilateral hemisphere as a percentage of total area of the contralateral hemisphere and reported in percentage. Quantification of Iba1 signal was performed as the percentage of area of increased Iba1 signal density of the area of the contralateral hemisphere and reported in percentage.

Infarct Volume and Survival analysis

Acute infarct volume was estimated using triphenyltetrazolium chloride (TTC) staining of 2 mm thick coronal sections of mice brains as previously described [131]. Images were quantified using ImageJ (NIH), and edema-corrected infarct volume was calculated as infarct area x (edema index= area of contralateral hemisphere / area of ipsilateral hemisphere) as described [68]. A Kaplan-Meier curve using Mantel-Haenszel log-rank test analysis was performed to compare survival curves.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad). Parametric testing was used unless otherwise specified, in the event of Brown-Forsythe test for homogeneity of variance or if normality fails. Histologic analysis for stroke was performed using Chi-squared test. Statistical analyses for infarct and IF analyses were performed using one-way ANOVA test with Bonferroni's correction for multiple comparison. P values below 0.05 were considered

significant. Student's t test and Mann Whitney test were used to compare two groups and was used as two-tailed. Pearson correlation coefficients were used to compute correlations.

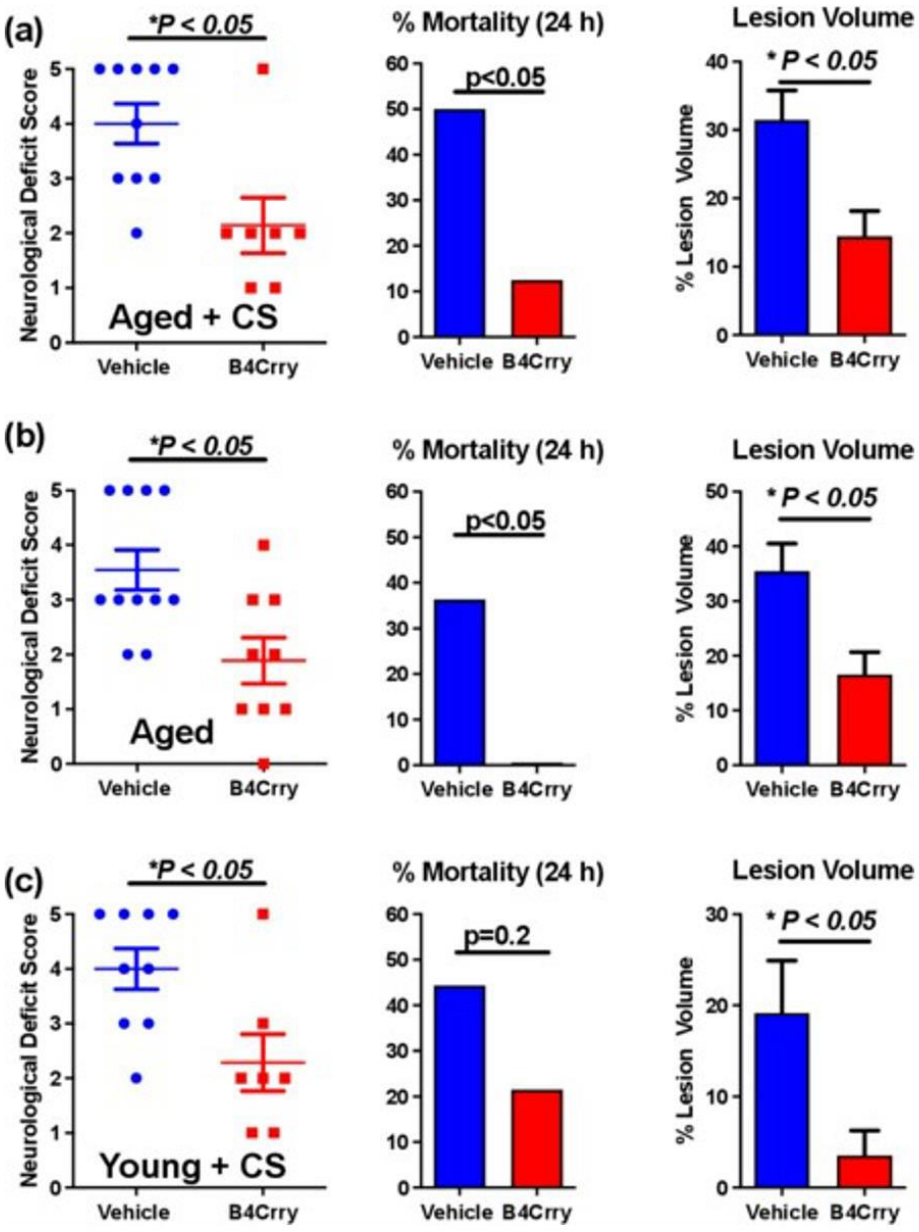
(a)
*P < 0.05
Neurological Deficit Score
Aged + CS
Vehicle
B4Crry
% Mortality (24 h)
p<0.05
Vehicle
B4Crry
Lesion Volume
*P < 0.05
% Lesion Volume
Vehicle
B4Crry
(b)
*P < 0.05
Neurological Deficit Score
Aged
Vehicle
B4Crry
% Mortality (24 h)
p<0.05
Vehicle
B4Crry
Lesion Volume
*P < 0.05
% Lesion Volume
Vehicle
B4Crry
(c)
*P < 0.05
Neurological Deficit Score
Young + CS
Vehicle
B4Crry
% Mortality (24 h)
p=0.2
Vehicle
B4Crry
Lesion Volume
*P < 0.05
% Lesion Volume
Vehicle
B4Crry

Figure 1: Effect of B4-Crry treatment on neurological deficit, mortality and lesion volume 24 hours after MCAO in aged and CS exposed mice. (A) Aged + CS exposed mice. (B) Aged non-CS exposed mice. (C) Young + CS exposed mice. Neurological deficit scores, Mann Whitney test. Mortality, Chi squared test. Lesion Volume, T-test. For all groups, n=7-11. Error bars = SEM.

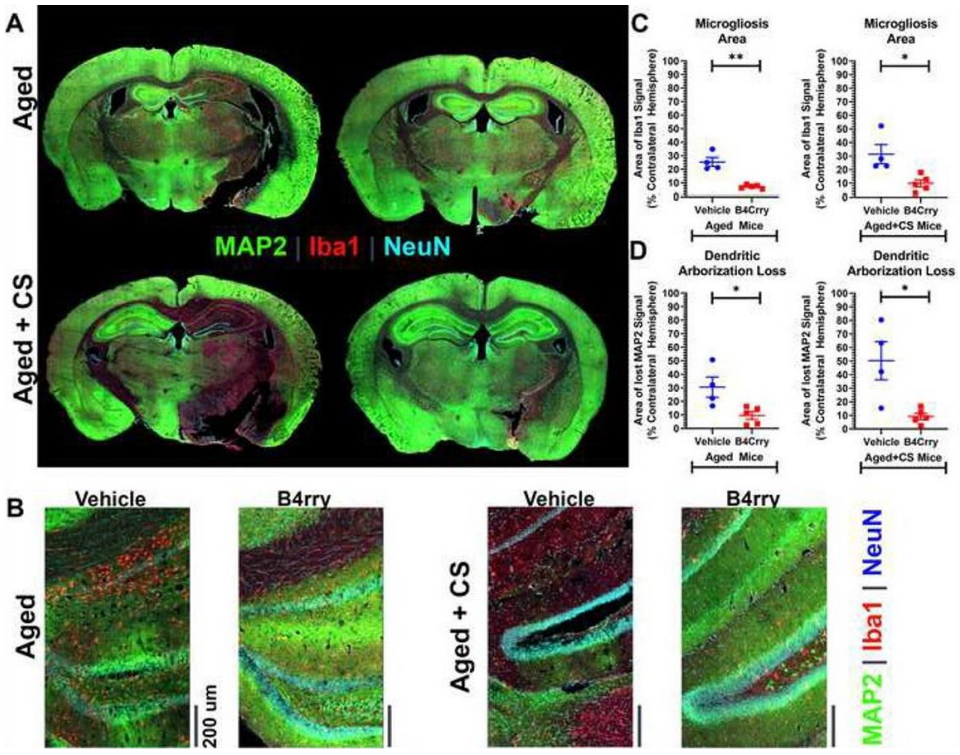
A
Aged
Aged + CS
MAP2 | Iba1 | NeuN
C
Microgliosis Area
**
*
Area of Iba1 Signal (% Contralateral Hemisphere)
Vehicle B4Crry
Aged Mice
Aged+CS Mice
D
Dendritic Arborization Loss
Area of lost MAP2 Signal (% Contralateral Hemisphere)
B
Vehicle
B4rry
200 um
MAP2 | Iba1 | NeuN

Figure 2: Extent of Microgliosis and loss of dendritic arborization at 24 hours after MCAO in the perilesional area of B4-Crry and vehicle treated CS exposed and non-CS exposed aged mice. (A,B) Immunofluorescence staining for MAP2 (dendritic marker, green), microglia (Iba1, red), and neurons (NeuN, cyan) demonstrating wider extent of microgliosis and dendritic loss in vehicle treated compared to B4Crry treated mice. Representative images. (C) Quantification of images in (B) showing percentage area of microgliosis in the ipsilateral hemisphere. Student's T-test. *p<0.05. **p<0.01. Mean +/- SEM. (D) Quantification of images in (B) showing percentage area with loss of MAP2 signal indicating loss of dendritic arborization in the ipsilateral hemisphere in aged mice (left) and aged + CS exposed mice (right), comparing vehicle to B4Crry treatment. Student's t-test. *p<0.05. Mean +/- SEM.

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CHAPTER 6: AIM 3

Manuscript 3: A Co-Culture Protocol Established for A Microglia Phagocytosis Assay

TITLE PAGE:

A Co-Culture Protocol Established for A Microglia Phagocytosis Assay

Christine Couch[1], Moumita Majumder[1], Silvia Guglietta[1], Stephen Tomlinson[1,2*]

[1]Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425, USA.

[2]Ralph H. Johnson VA Medical Center, Charleston, SC 29425, USA.

*** Correspondence:**
Stephen Tomlinson: tomlinss@musc.edu

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Abstract

Clinical data from traumatic brain injury (TBI) patients suggest that chronic TBI is associated with a similar neurodegenerative mechanism that mimics neurodegenerative disorders, such as Alzheimer's Disease (AD). It is also known that TBI is considered a risk factor for AD and other cognitive disorders. Studies from AD have demonstrated that cognitive deficits occur early in the disease and are dependent on the extent of synaptic and neuronal loss, which correlates with overall cognitive performance. These experiments used defined conditions to investigate complement-mediated phagocytosis of neurons and/or synapses after excitotoxic insult to the brain. Our co-culture protocol was established as a microglia phagocytosis assay to better define complement-microglial interactions. This protocol was also developed to give us a better mechanistic understanding of how C3 activation leads to deposition of C3d opsonins on the surface of neurons and/or synapses as well as how this leads to a C3d-CR3 mediated engagement of brain microglia inducing an upregulation and activation of the phagocytic machinery and activation of pro-inflammatory signaling. In vitro cultures of neurons, microglia, and co-cultures of neurons and microglia were utilized to assess complement-mediated synaptic-microglial interaction during the neurodegenerative loss of neurons and synapses after TBI.

Introduction

The complement system is recognized as an early and significant contributor to secondary insult after traumatic brain injury by promoting neuronal loss, edema, and inflammatory cellular infiltrate [14]. Clinical studies have shown that TBI patients have elevated levels of complement activation products (C3 and sC5b-9) in their cerebrospinal fluid and increased deposition of complement activation products in the perilesional brain [7], [15], [18], [16], [17]. The source of complement deposited in TBI is a combined contribution of systemic complement leaking to the brain after trauma induced blood brain barrier dysfunction, and locally produced complement proteins by the brain parenchyma and infiltrating cells [7], [16], [17]. A similar pattern of complement deposition is also seen in experimental models of TBI that also implicate complement in the acute neuronal cell death, neutrophil extravasation, and worsening of outcomes after TBI [9], [10], [11], [12], [13], [133]. Complement proteins can be deposited on dendrites and synapses to promote their removal by microglial cells [134], [135], [136]. The key step in complement activation is proteolytic cleavage of C3 to C3a and C3b, where C3a can recruit and activate microglia, whereas C3b opsonizes synapses and neurons [137].

Neuropathology and clinical data from TBI patients indicate that chronic TBI is associated with a similar neurodegenerative mechanism that mimics neurodegenerative disorders such as Alzheimer's Disease (AD), and TBI is considered a risk factor for AD and other cognitive disorders [138], [139], [140]. Studies from AD have demonstrated that cognitive deficits occur early in the disease and are dependent specifically on the extent of synaptic and neuronal loss may be a significant component of neurodegenerative pathology of TBI, especially since this process continues to occur months after insult. In this context, our lab has shown that complement dependent phagocytosis of live, salvageable neurons in the perilesional area after

stroke [65], and in more recent studies, we identified a complement dependent destruction by microglia phagocytosis of synapses acutely after a severe single hit controlled cortical impact TBI model [10]. However, the role of a complement microglial axis in the degenerative loss of neurons and synapses after TBI is unclear. The following experiments investigate the cooperative role of microglia and complement in synaptic and microglial degeneration after in vitro TBI.

We hypothesize that after TBI, complement opsonin C3d deposits on injured neuronal cell bodies and/or synaptic terminals and is recognized by activated microglia, leading to phagocytosis, internalization, and lysosomal localization of synaptic markers. In vitro cultures and co-cultures permitted the use of well-defined manipulations to better define complement-microglial interactions as well as the mechanism of synapse destruction. We have also demonstrated that stressing cultured neurons with glutamate results in C3d deposition (opsonization) on neurons when incubated in mouse serum. Lastly, we have successfully established a working co-culture protocol for in vitro TBI.

Materials and Methods

Animal husbandry and care:

The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all experiments involving vertebrate animals. Wild-type (WT) C57BL/6J mice were obtained from The Jackson Laboratory and housed in *Helicobacter* negative rooms at the Medical University of South Carolina (MUSC) animal facility.

Cell lines description and monitoring:

Mouse hippocampal neuronal cells (HT22, Millipore-Sigma) used in in-vitro experiments were obtained from Millipore-Sigma, passaged and frozen in liquid nitrogen for use until experiments, and were used to validate the efficacy of our excitotoxic injury model. Prior to their use, cells were monitored daily for their morphology by light microscopy, growth rate, and lack of mycoplasma contamination. Cells were passaged no more than ten times. Cells were incubated in a designated incubator not shared with other cell lines during the experiment. Lack of mycoplasma contamination was verified by absence of filamentous growth. Cells were grown and maintained as per supplier's recommendation in Dulbecco's Modified Eagle's Medium (DMEM; ATCC 30-2002) with 10% fetal bovine serum (Sigma-Aldrich; Product# F0392), and 1x penicillin streptomycin solution (Corning; Catalogue# 30-002-CI).

Serum collection:

Normal mouse serum used in in-vitro experiments was prepared from wild-type C57bL/6J male mice (Jackson Laboratory). Blood was withdrawn by cardiac puncture of unmanipulated mice and maintained on ice for 2 h to clot. Clotted blood was centrifuged at 3000 rpm for 20 minutes, and serum was collected and stored at -80ºC for future use.

Isolation of Primary Neurons:

Primary cultures of neurons were prepared from embryonic day 18-20 (E18-20) from C57BL/6J mouse embryos by standard techniques. A pregnant female WT mouse was euthanized by CO2 overdose and sprayed with ethanol to prevent contamination. The following occurred inside a laminar flow hood. A V shaped incision was made across the pregnant female belly and cut through the skin and fat. Amniotic sacks were removed, and pups were transferred to a clean petri dish. The pup heads were decapitated, and the skin was removed. The skull was cut through with micro scissors and the brains were removed with a spatula and placed in a

clean petri dish with ice cold HBSS. The pup brains were dissociated by using a pestle over a wire mesh, and media with the cells was collected and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and 5 mls of lysis buffer was added to dissociate blood cells. The cells were centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and the cell pellet was further dissociated by trypsinization (0.25%) for 15 minutes at 37°C. Afterwards, trypsin was inactivated with 10% heat inactivated FBS in complete 1x-DMEM media. The cells were triturated and passed through 70um and 40um cell strainers before being centrifuged for 10 minutes at 1000 rpm. The cell pellet was washed with complete 1x-DMEM with a repeated centrifugation step. Then, neurons were enriched by allowing cells to adhere to poly d lysine coated plates or coverslips for 10 minutes in complete 1x-DMEM. The nonadherent cells (microglia, astrocytes, oligodendrocytes) are removed in this step and discarded. The adherent cells (mostly neurons) then received neurobasal media with B27 supplement media was changed on day 5 after initial neuron plating until confluency is reached at day 7-10.

Isolation of Primary Microglia:

Primary cultures of neurons were prepared from P0-P4 post-natal day pups from C57BL/6J mice. The pups were euthanized by decapitation and the heads of the pups were soaked in 70% ethanol to disinfect the tissue. The heads were removed with a pair of scissors and brain was dissected using a small spatula. Brains were placed over a wire mesh in a sterile petri dish consisting of DMEM/F-12, GlutaMax, 10% FBS, and 5% penicillin–streptomycin. Brain tissue was dissociated with a sterile pestle upon the wire mesh. The cells and media were triturated and placed into a 50 ml centrifuge tube and centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and 5 ml of RBC lysis buffer was added. The centrifugation step was repeated. Next, cells were washed with 5 ml of complete DMEM/F-12 media and centrifuged again. In the meanwhile, media was added to T-75 flasks and cells were distributed. Media was

changed every 3 days and on the 9th day of cell culture, microglia was dissociated from mixed glial cultures by mild trypsinization. Briefly, 0.08% trypsin (dilute bottle in 1:3.5 ratio) + 0.35 mM EDTA (25200-072, Life Technologies, Somerset, NJ) in Dulbecco's modified Eagle medium (DMEM/F-12 only) is applied to mixed cultures for 35 min to dissociate all cells but microglia. The trypsinization is stopped by diluting the trypsin with DMEM and immediately removing all medium on the plate. The remaining pure microglial population was incubated with 0.25% trypsin + 1 mM EDTA for 10 min, dissociated by vigorous pipetting, resuspended in culture media, and plated at 75,000/cm^2 for 24 h in DMEM/F12 supplemented with 10% FBS and 1% penicillin–streptomycin. (To remove microglia for experiments: 10 mins with full trypsin, centrifuge etc).

Co-Culture Model:

Our hypothesis was tested using an in vitro model of TBI using immortalized neurons (HT22) to validate the injury model or primary neurons (embryonic day ~E18-20) co-cultured with primary microglia prepared from post-natal day P0-4 from WT mice by standard techniques.

Once primary neurons reached confluence at day 7-10 and primary microglia were isolated at day 9, neurons and microglia were plated in a 5:1 ratio of neurons: microglia for phagocytosis experiments compared to 4 h and 8 h timepoints. This occurred following neuron injury, described below.

This co-culture system allows for the in vitro investigation of complement-mediated synaptic-microglial interaction during the neurodegenerative loss of neurons. Additionally, various aspects of the experimental design can be modified to address particular experimental questions. For example, the length of treatment and co-culture can be adjusted to better assess the time-course of an observed effect.

Glutamate Injury:

It is known that following TBI, there is a release of excess glutamate which results in glutamate excitotoxicity, specifically the buildup of extracellular glutamate causing neurotoxic activation of post synaptic NMDA receptors and metabolic energy failure [127], [128]. We have generated data by chemical injury showing that exposure of HT22 neurons to 20mM of the glutamate, at 8 hours results in approximately 80% early apoptotic cells, as well as exposure of primary neurons to 2uM glutamate.

Immunofluorescence staining and imaging:

This was used solely for validation of our glutamate injury. Neurons and microglia were fixed in 4% PFA solution for 20 minutes for immunocytochemistry. The cells were washed with PBS and permeabilized using 3% H_2O_2 followed by 0.1% Triton-X in PBS. Neurons were blocked in donkey serum (5% in PBS), washed, and incubated with the primary antibody overnight followed by washing and incubation of the fluorescently tagged secondary antibody. The primary antibodies used for staining in the first experiment were anti-NeuN (Abcam, Cat. #: ab104225, 1:200) for neurons and anti-caspase 3 (R+D, Cat. # AF835, 1:200) for apoptotic cells. Simultaneous immunostaining of caspase-3 and NeuN were determined whether C3d is strictly deposited on apoptotic neurons. High-resolution imaging was conducted using a Zeiss LSM 880 confocal microscope (Zeiss) at 40x zoom with water-media overlay and using the Z-stacking feature of the microscope. Images were deconvoluted using the ZEN 2.5 software (Zeiss) and reconstructed in 3D plane. Image analysis was performed using both ZEN (Zeiss) and ImageJ (NIH) software.

Multiparameter Flow Cytometry and Sequential Gating Analysis:

This was performed to discriminate between neurons and microglia and other glial cells, as well as for the characterization and quantification of apoptosis. First, neurons were labeled and stained with cell trace violet. A Cell trace stock solution (5mM) was prepared prior to use by

adding 20uL DMSO to 1 vial of the cell trace reagent. 1uL of cell trace stock solution in DMSO was added to each mL of cell suspension in PBS for a final working solution of 5uM. Cells were incubated at 37°C for 20 minutes, protected from light. 5x the original staining volume of complete culture medium was added and mixed (this removed any free dye remaining in solution) and incubated for 5 minutes. Cells were pelleted by centrifugation and supernatant was removed. Cell pellet was resuspended in fresh pre-warmed culture medium. The cells were incubated for at least 10 minutes before analysis (this allowed the reagent to undergo acetate hydrolysis), and then a live/dead staining solution was added to the microglia cultures in the collected co-cultures and incubated for 6 minutes at 4°C. Cells were then washed 2x with PBS. 100-150uL of the mix was added to the cell co-cultures and incubated for 20 minutes at 4°C, followed by 2 PBS washes, and then final storage in FACS buffer. Flow cytometry was conducted within 10 minutes. For flow cytometry, BV-421 (Pacific Blue) channel was used to detect the cell trace violet (CTV), with excitation wavelength of 405 nm. PE-Cy7 channel was used to detect CD45.1. Normal cells without apoptotic induction were used as control for fluorescence compensation to remove spectral overlap and determine the position of cross-over.

Imaging Flow Cytometry and Gating Strategy:

Imaging flow cytometry was performed on an ImageStream X Mark II operated by INSPIRE software (Amnis Corporation). CD45 (PE-Cy7) was recorded and collected with a 740-800 nm filter (channel 6). Cd11b (BV-711) was recorded and collected with a 660-740 nm filter (channel 11) and cell trace violet (BV-421) was recorded and collected with a 425-505 nm filter (channel 7). Brightfield images were collected in channel 1 (camera 1) and channel 9 (camera 2). A total of 1000 events were collected for each sample. Unstained controls and single stained controls were also collected (CTV only, Cd11b only, and CD45 only stained cells) at the same

settings, to develop a compensation matrix for removing spectral overlap of the dyes from each of the channels. Data was analyzed using IDEAS Application v 6.2 software (Amnis Corporation). In each experiment, a compensation matrix was created using single color controls and was applied to all files. Image-based gating was performed as follows: Focused cells were obtained by gating on objects with high gradient RMS (root mean square for image sharpness) values, a measure of the amount of change of pixel values above background in the image. Objects with small RMS are generally out of focus, whereas those with large RMS demonstrate a sharpness in the images. Single cells were identified by gating on objects with high aspect ratio and low area. The internalization wizard was used to create an analysis for measuring the internalization of neurons (CTV) by microglia (CD45), which represented phagocytosis.

Statistical Analysis:

We used 3 experiments per study and 3 replicates for each cell culture for robustness. All quantifications were automated via macros in ImageJ and Zeiss Zen software to eliminate bias. Group comparisons were performed using standard t tests when comparing 2 groups or ANOVA with Bonferroni for multiple comparisons. Data was presented as mean ± standard error of two or more independent experiments. Data was analyzed as parametric measure unless tests for normality and homogeneity of variance fails; non-parametric testing was used.

Results

Glutamate Injury in HT22 Neurons:

Mouse hippocampal neuronal HT22 cells were used to validate the efficacy of our excitotoxic injury prior to administration to the primary neurons. Differentiation renders susceptibility to excitotoxicity in HT22 neurons [141] and we show differentiated HT22 neurons with B27 media represent a better model of hippocampal neurons than undifferentiated. A dose response curve was generated using the glutamate concentrations of: 2um, 20um, 2mM, and

20mM. Our preliminary data showed that 20mM glutamate administration over the period of 8 hours created ~80% of early apoptotic cells. We wanted to further validate this concentration as the optimal excitotoxic injury by analyzing both C3 and caspase3 expression (*Figures 1-2*).

C3 Expression is Significantly Elevated using 20mM Glutamate:

Neurons were exposed to either WT or C7ko serum (C7-/-) to determine the impact of C3d deposition we needed a source of complement without causing cell lysis. Neurons were stained for C3 and the % fluorescent pixel intensity of C3 expression was elevated using 20mM glutamate vs uninjured neurons in WT serum (1.5%; $p<0.0001$). Similarly, C3 was substantially increased using 20mM glutamate vs 2mM glutamate in WT serum (1.5% vs 0.4%; $p<0.0001$). Similar % fluorescent pixel intensity expression occurred when the HT22 neurons were placed in C7ko serum (*Figure 1*).

Caspase3 Expression is Increased using 20mM Glutamate:

Similar as above, HT22 hippocampal neurons were stained for anti-caspase3, an apoptotic marker, and the % fluorescent pixel intensity of caspase3 expression was significantly increased using 20mM glutamate vs uninjured neurons in WT serum (2.8% vs 0.1%; $p<0.0001$). Comparably, caspase3 was enhanced using 20mM glutamate vs 2mM glutamate in WT serum (2.8% vs 0.5%; $p<0.0001$) (*Figure 2*). These results are expected as caspase 3 is an apoptotic marker, which indicates 20mM glutamate injury is the optimal concentration.

Glutamate Injury in Primary Neurons:

Based off our HT22 data, we used a similar dose response curve, and as expected there was a significant decrease in NeuN positive cells as the glutamate concentration increased. Multiple experiments confirmed 2uM glutamate as the optimal concentration in primary neurons (*Figure 3*). This was the concentration used for our flow cytometry co-culture experiments.

Immunofluorescence staining and imaging was also used as validation of our glutamate injury in primary neurons (*Figure 4A-C).*

Microglia Phagocytosis of C3 Expressing Injured Neurons:

Immunofluorescence staining and imaging was also used as validation of feasibility of our primary microglia cultures (*Figure 5A-B*). Primary neuron and primary microglia purity was validated with multiparameter flow cytometry, with cell trace violet (CTV) staining for neurons and CD45 for microglia. An initial phagocytosis assay was performed and also validated by flow cytometry, and our data showed at a 4-hour timepoint, there was ~2.6% microglia phagocytosis of neurons compared to ~17% at a 8-hour timepoint. Based off this data, we proceeded with our final primary neuron and microglia co-culture experiments using imaging flow cytometry. The gating strategy (*Figure 6*) of our Cd11b+ (microglia) and CTV+ (neurons) is demonstrated to represent the % of phagocytosis in our final cell population. Here, we compared an uninjured neuron microglia co-culture to our glutamate (2uM) injured neuron microglia co-culture. A staurosporine (1uM) injured neuron microglia co-culture was used as a positive control. This allowed us to study the phagocytosis of neurons by microglia, identify cell populations, and determine phagocytosis of CTV+ neurons by quantifying the percentage of internalized particles. We found in our staurosporine injured coculture there was a 5.41% of phagocytosis of neurons by microglia compared to a 6.36% phagocytosis in the glutamate injured coculture condition. Representative images for each cell population (*Figure 7A-B*) were demonstrated, along with the internalization of CD45/CTV, which visually represents the % of phagocytosis of neurons by microglia.

Discussion

We investigated the cooperative role of microglia and complement in neuronal degeneration after traumatic brain injury using an in vitro model. These findings demonstrate

that C3 activation leads to a deposition of C3d opsonins on the surface of neurons. This leads to engagement of brain microglia thus inducing upregulation and activation of phagocytic machinery and a subsequent activation of pro-inflammatory signaling that stains both microglia activation and complement breakdown over time. We have established a working primary neuron and primary microglia coculture and microglial phagocytosis assay which will lay the groundwork for future in vivo experiments by using precisely defined environments to investigate the complement mediated phagocytosis of neurons/and or synapses after chemical injury to the brain. Future experiments using immunofluorescence or imaging flow cytometry will better define the complement-microglia interaction as well as the mechanism of phagocytosis, internalization, and lysosomal localization of synaptic markers. This can be accomplished by comparing injured to uninjured primary neurons co-cultured with CR3ko microglia as well as comparing injured to uninjured primary neurons co-cultured with CR4ko microglia, as this mechanism is still not well defined and is poorly understood.

To conclude, we note that there are limitations to this study. In our imaging flow cytometry experiments, we note that the total % phagocytosis of neurons by microglia is lower than expected. This is likely due to the primary neuron population not containing solely pure primary neurons, but other glial cells. This is evident in Figure 7A-B, where CD45 and Cd11b is expressed in all cell types, indicating the primary neuron population most likely includes microglia. For future experiments, the use of neuron (HT22) and microglia (SV2) cell lines would better validate the purity of these cell cocultures in imaging flow cytometry experiments.

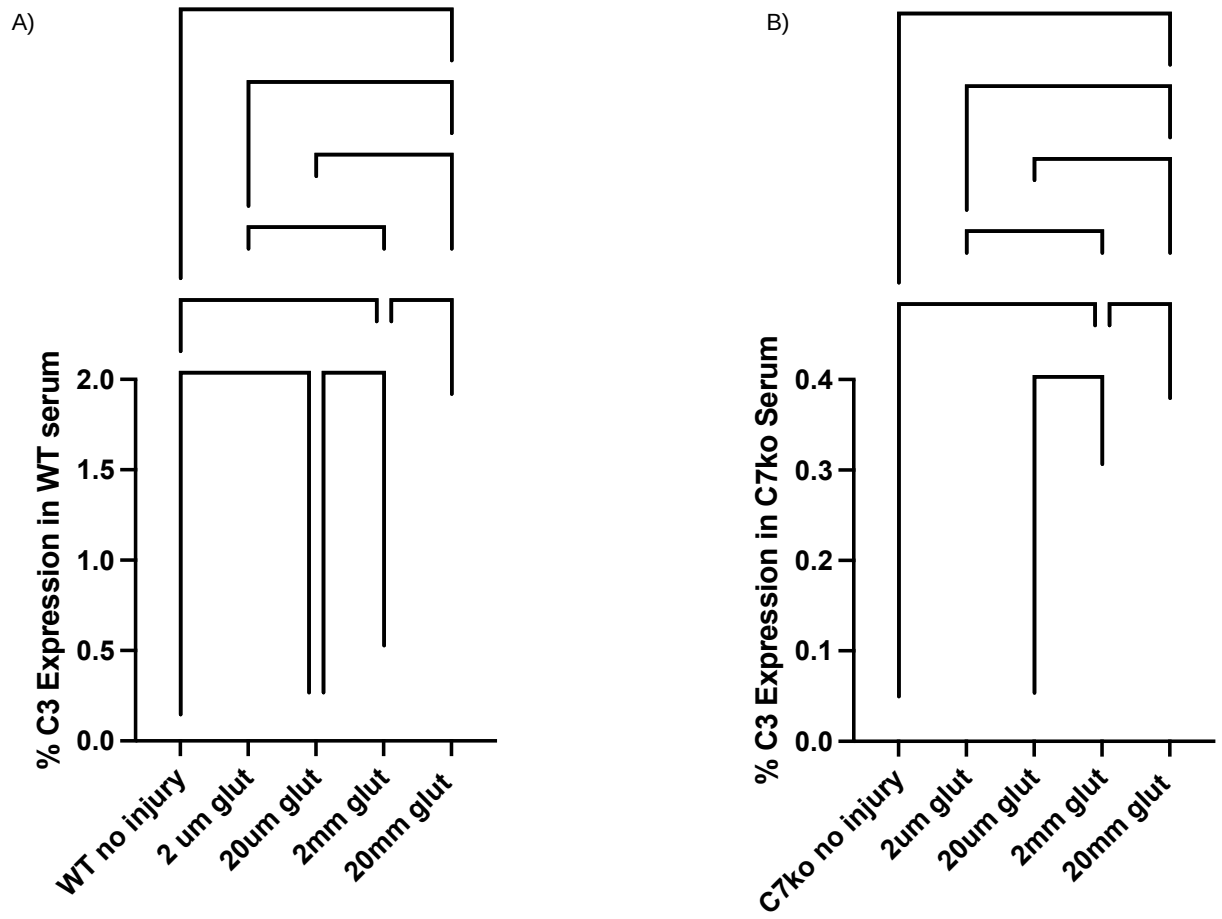


Figure 1: C3 expression in HT22 cells placed in WT serum (A) or C7ko serum (B). C3 expression was quantified by demonstrating the percent fluorescent pixel intensity of the fluorophore. % Fluorescent Pixel Intensity = corrected % total fluorescence (pixel^2) by subtracting out background signal. A dose response curve was generated using the glutamate concentrations of 2um, 20um, 2mm, and 20mm. Error Bars = mean ± SEM. One- way ANOVA with Bonferroni correction for multiple comparisons. ****P<0.0001

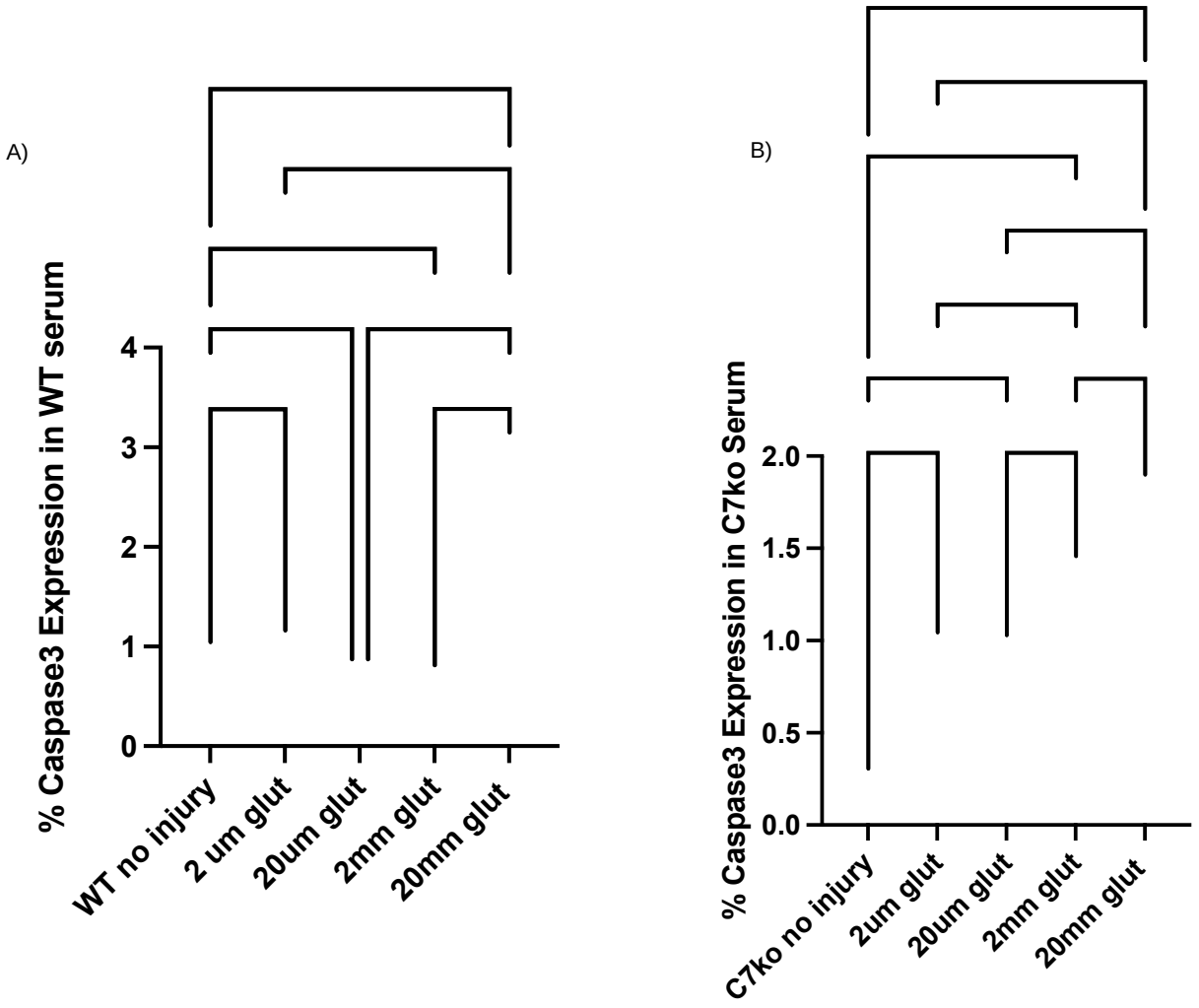


Figure 2: Caspase 3 expression in HT22 cells placed in WT serum (A) or C7ko serum (B). Caspase3 expression was quantified by demonstrating the percent fluorescent pixel intensity of the fluorophore. % Fluorescent Pixel Intensity = corrected % total fluorescence (pixel^2) by subtracting out background signal. A dose response curve was generated using the glutamate concentrations of 2um, 20um, 2mm, and 20mm. Error Bars = mean ± SEM. One- way ANOVA with Bonferroni correction for multiple comparisons. ****P<0.0001

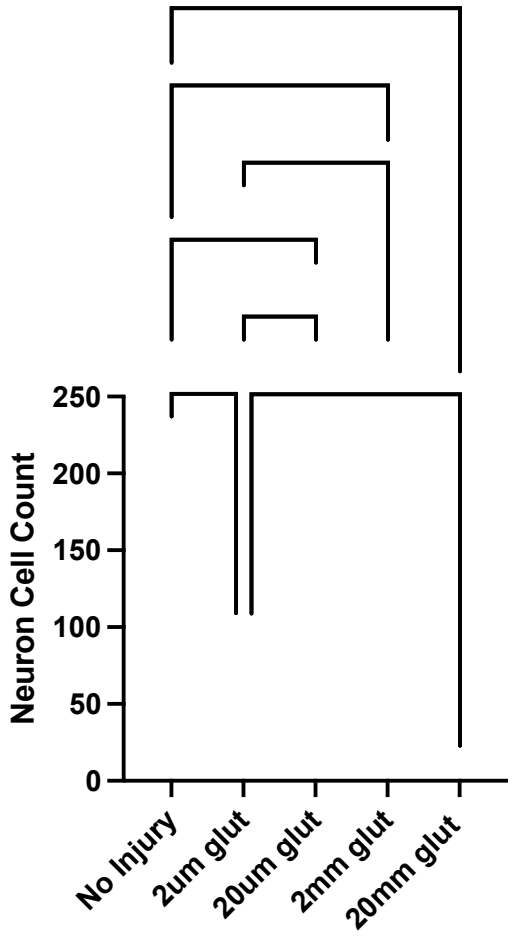
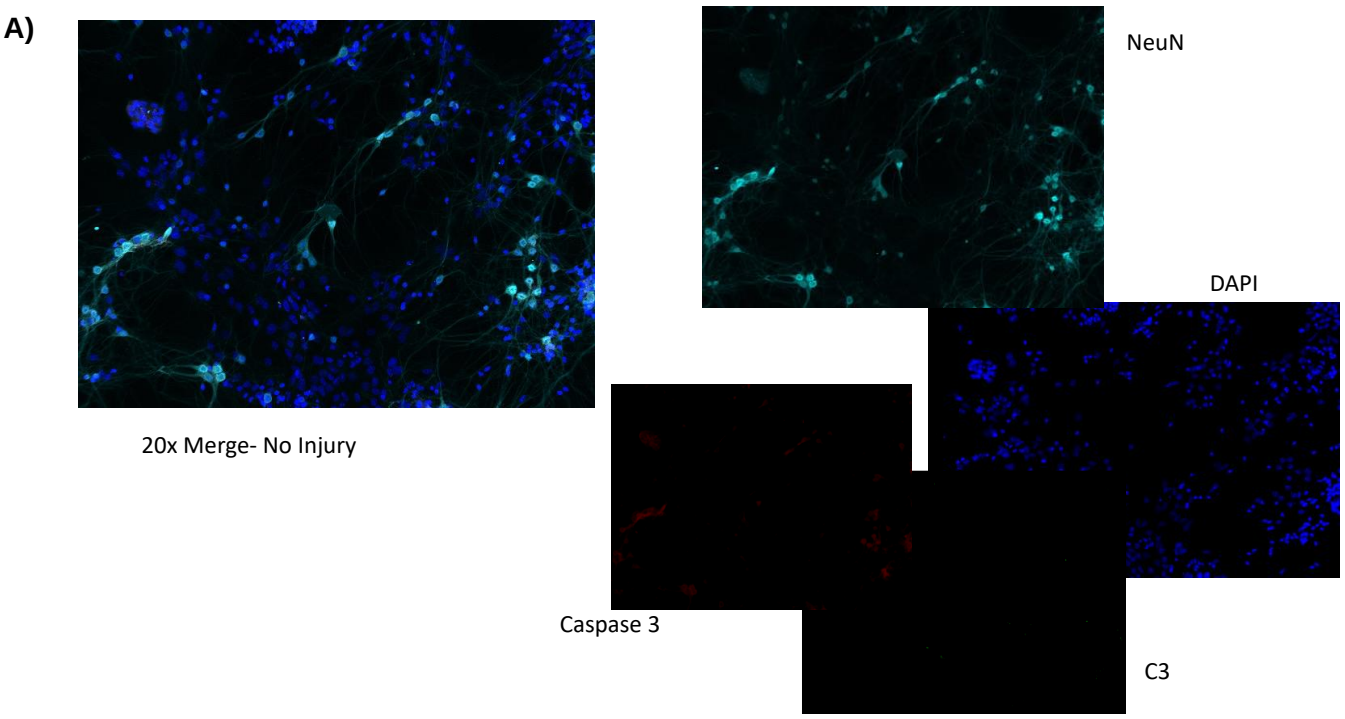


Figure 3: Neuron count of Primary NeuN+ cells
There was a decrease in NeuN+ cells as the glutamate concentration increased. Error Bars = mean ± SEM. One- way ANOVA with Bonferroni correction for multiple comparisons. ****P<0.0001


A)
NeuN
DAPI
20x Merge- No Injury
Caspase 3
C3
B)
NeuN
DAPI
20x Merge- 2um Glutamate
Caspase 3
C3

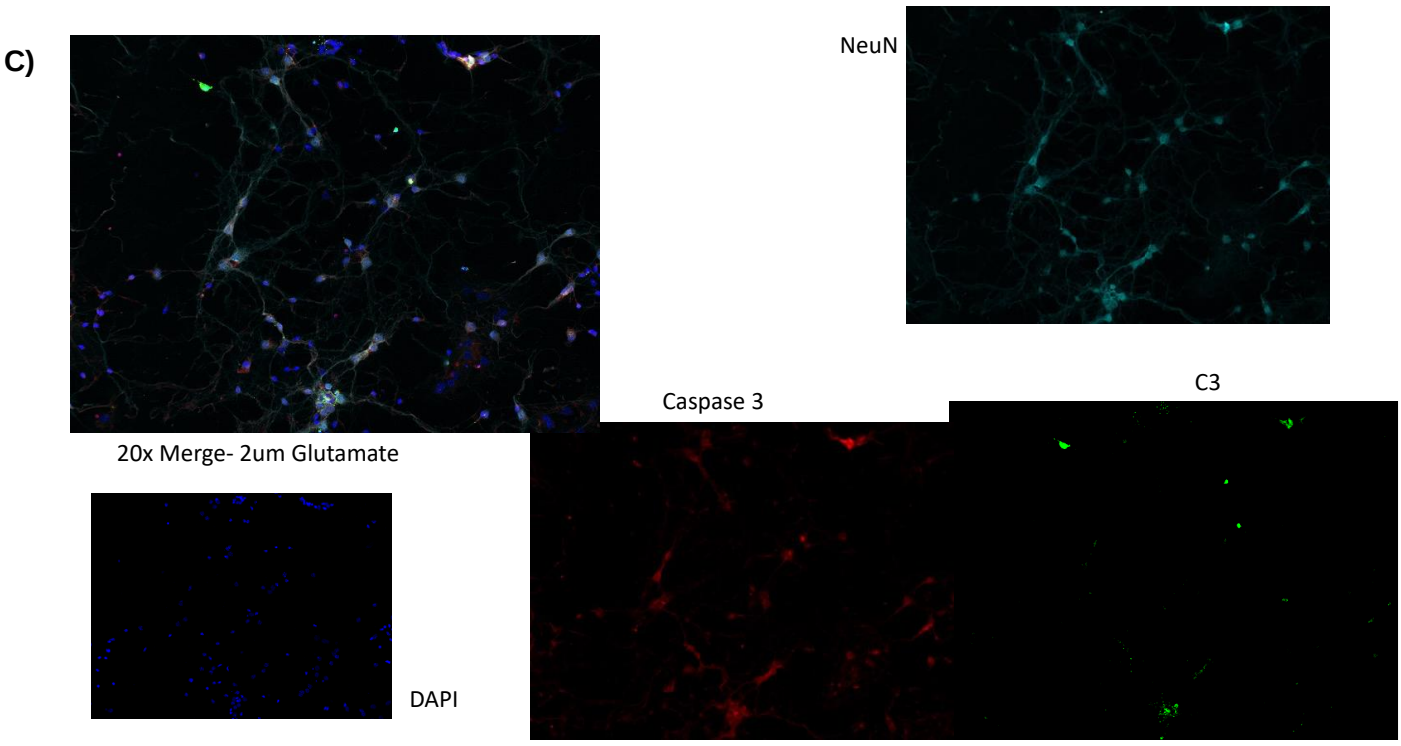


Figure 4(A-C): Immunofluorescence Images of Primary Neurons
(A) shows the 20x merge of uninjured primary neurons as well as NeuN (1:200), DAPI (1:1000), Caspase3 (1:200), and C3 (1:200) expression. (B-C) shows the 20x merge of 2uM glutatmate injured primary neurons.

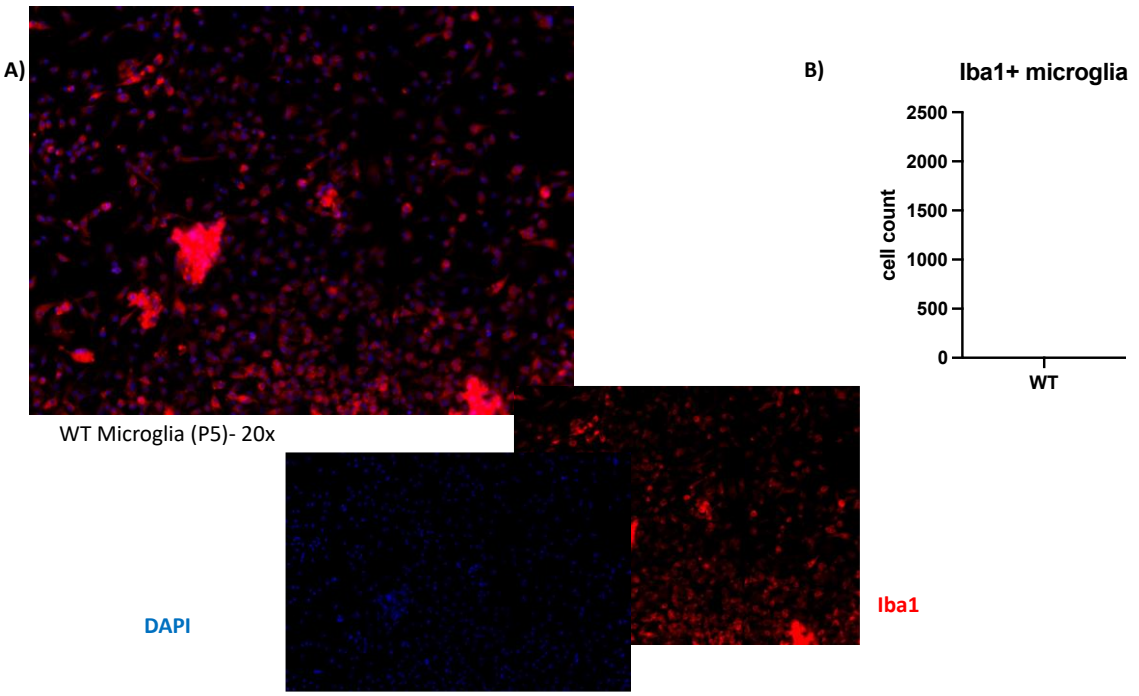
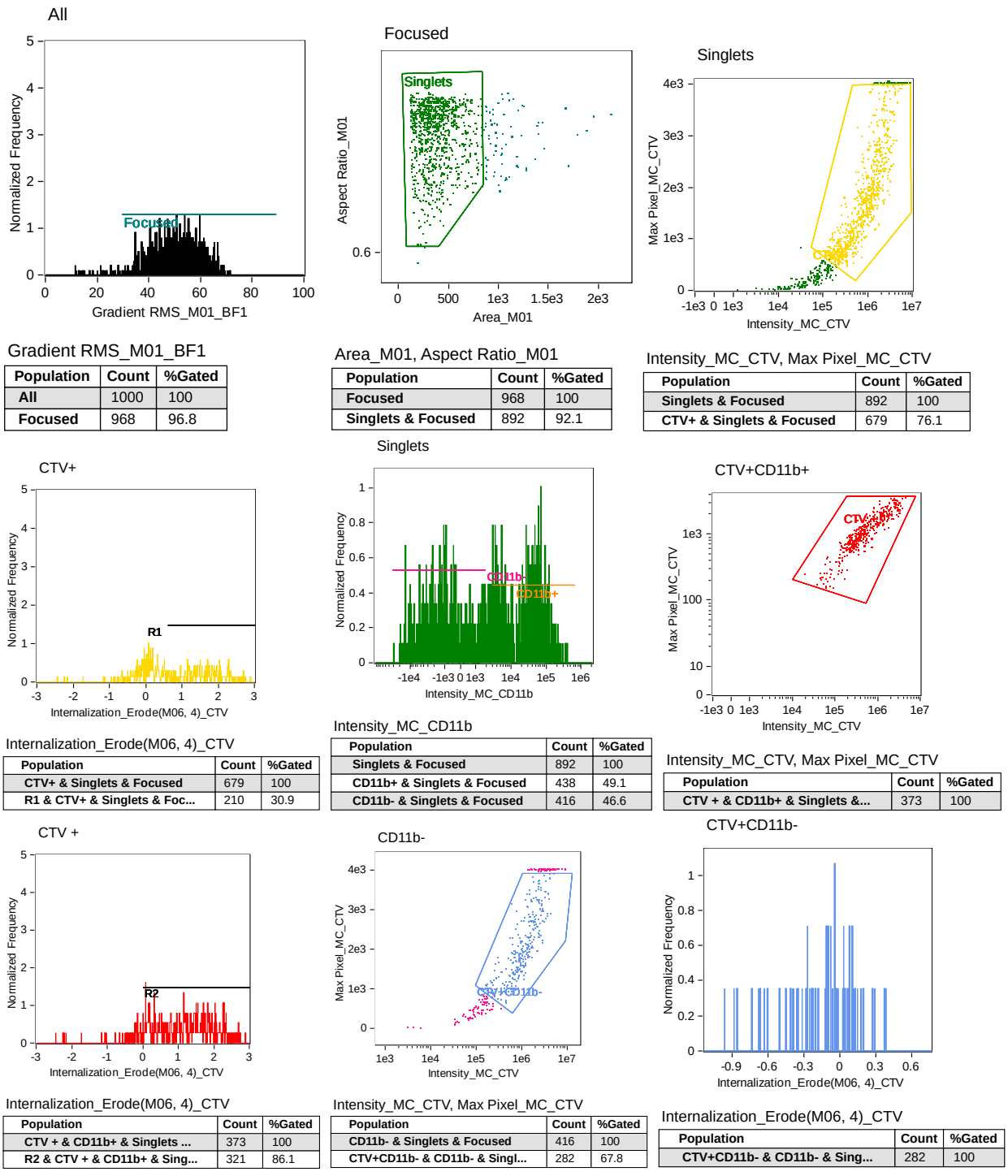


Figure 5: Immunofluorescence Images of Primary Microglia
(A) demonstrates a 20x merged image of primary microglia from WT mice at post-natal day 5. (B) represents the cell count of Iba1+ microglia in this cell isolation.



Population	Count	%Gated
All	1000	100
Focused	968	96.8

Population	Count	%Gated
Focused	968	100
Singlets & Focused	892	92.1

Population	Count	%Gated
Singlets & Focused	892	100
CTV+ & Singlets & Focused	679	76.1

Population	Count	%Gated
CTV+ & Singlets & Focused	679	100
R1 & CTV+ & Singlets & Foc...	210	30.9

Population	Count	%Gated
Singlets & Focused	892	100
CD11b+ & Singlets & Focused	438	49.1
CD11b- & Singlets & Focused	416	46.6

Population	Count	%Gated
CTV + & CD11b+ & Singlets &...	373	100

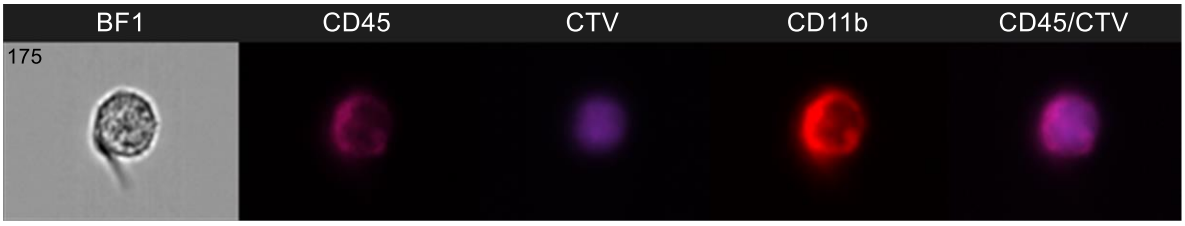
Population	Count	%Gated
CTV + & CD11b+ & Singlets ...	373	100
R2 & CTV + & CD11b+ & Sing...	321	86.1

Population	Count	%Gated
CD11b- & Singlets & Focused	416	100
CTV+CD11b- & CD11b- & Singl...	282	67.8

Population	Count	%Gated
CTV+CD11b- & CD11b- & Sing...	282	100

Figure 6: Imaging Flow Cytometry Gating Strategy of CTV+ and Cd11b+ cells (2uM Glutamate)
This gating strategy was also applied to the staurosporine (1uM) injured neurons in the primary neuron-microglia coculture.

A)



B)

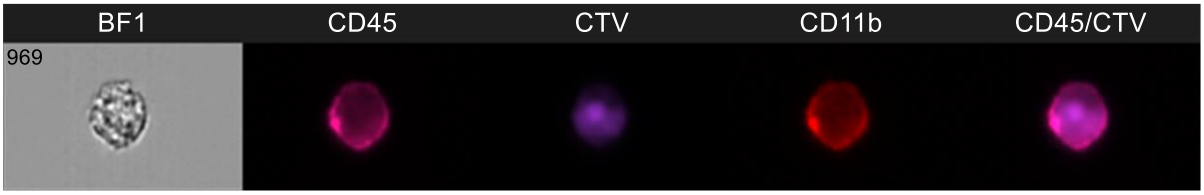


Figure 7(A-B): Imaging Flow Cytometry Image Stream Images

(A) 2uM Glutamate Injured cells represented in brightfield as well as the following channels: CD45 (microglia), CTV (neuron), Cd11b (microglia), internalization of CD45/CTV (phagocytosis).

(B) 1uM Staurosporine Injured cells represented in brightfield as well as the following channels: CD45 (microglia), CTV (neuron), Cd11b (microglia), internalization of CD45/CTV (phagocytosis).

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CHAPTER 7: CONCLUSION

The complement system plays a critical role in inflammation and modulates several downstream pathways once activated. The work in this dissertation provides a body of evidence of the complement system's role in stroke, as well as TBI, by addressing three major research needs for understanding the acute and chronic consequences of complement activation in the recovery process and how this may lead to new and more improved therapeutic strategies.

Manuscript 1 was designed to provide a synopsis of inflammatory biomarkers related to stroke and the recovery process. Our work incorporated clinical data from studies of acute ischemic stroke that analyzed the role and presence of inflammation contributing to functional recovery. Furthermore, we analyzed which inflammatory biomarkers were related to patient rehabilitation outcomes. We found more substantial evidence to support the use of inflammatory biomarkers to predict post-stroke mortality, and our findings supported the need for further investigation, using longitudinal data, of the association between inflammation and rehabilitation related recovery to determine and optimize patient outcomes.

Manuscript 2 provided evidence to include comorbidities in experimental stroke models to represent the stroke patient population more accurately to create neuroprotective therapeutics with a higher level of success in human clinical trials. Most preclinical studies do not consider these comorbidities, which may be a contributing factor to therapeutic failure in clinical trials. Our findings demonstrate that the effect of cigarette smoke exposure and aging on the rapid progression of infarct volume after stroke and the early loss of salvageable penumbra, is at least in part, complement dependent. The overall implication is that suppressing this effect may result in increasing the proportion of stroke patients eligible for revascularization treatment.

Manuscript 3 was an initial step towards investigating the role of microglia and complement in microglial degeneration after in vitro TBI, specifically in using defined conditions to investigate complement-mediated phagocytosis of neurons and/or synapses after injury to the brain. We established a working primary neuron and primary microglia coculture and microglial phagocytosis assay which will lay the groundwork for future experiments to better define the

complement-microglia interaction as well as the mechanism of phagocytosis, internalization, and lysosomal localization of synaptic markers.

7.1 Future Directions:

The collection of studies in this dissertation have provided a building block for future studies and experiments in the areas of inflammatory biomarker assessment during stroke recovery and rehabilitation, the inclusion of comorbidities in experimental stroke models, and the evaluation of the interactive role of microglia and complement in neuronal degeneration after traumatic brain injury using an in vitro model.

Most of the studies reviewed in manuscript 1 present pilot data with small sample sizes, and future studies should test whether observations in these studies are generalizable to larger patient populations. Future studies are needed to determine whether other inflammatory biomarkers would be more useful than CRP. Further research including longitudinal data is crucial to assess inflammatory biomarkers at several timepoints during stroke recovery and rehabilitation and more in-depth research is needed to define the nature and time course of the relationship during specific rehabilitation treatments, that vary in intensity. Additionally, studies need to standardize the functional status measures and inflammatory biomarkers that are reported to improve comparisons and generalizability.

A key message from the findings in manuscript 2, is the implication that complement-mediated neuroinflammation is a major contributor to exacerbation of cerebral injury after stroke, and that this complement-mediated effect is more prominent in subjects with stroke comorbidities. Using pharmacological interventions that can limit the progression of infarct and temporarily preserve the penumbra remains an unmet clinical need, and one that complement inhibition shows potential for fulfilling.

Finally, our findings from manuscript 3, indicate future experiments will better define the complement-microglia interaction as well as the mechanism of phagocytosis, internalization, and lysosomal localization of synaptic markers. Future studies should be established which compare injured to uninjured primary neurons co-cultured with CR3ko microglia as well as comparing injured to uninjured primary neurons co-cultured with CR4ko microglia. This mechanism is still not well defined and is poorly understood in the context of neurodegeneration and traumatic brain injury.

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