MODULATION OF ASTROCYTE PHENOTYPE IN RESPONSE

TO T-CELL INTERACTION

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ABBREVIATIONS

A1	Neurotoxic astrocytes
AβH	L-aspartic acid beta-hydroxamate
APC	Antigen presenting cells
ATCC	American Type Culture Collection
BBB	Blood brain barrier
C1q	Complement Component 1 q
CCK-8	Cell counting kit 8
CCR2	CC Chemokine Receptor 2
CD95	Cluster of Differentiation 95 death receptor
CNS	Central nervous system
DAMPs	Damage associated factors in prescribed molecular patterns
DAPI	4', 6-diamidino-2-phenylindole
DIC	Differential interference contrast
ECAR	Extracellular acidification rate
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone
Foxp3	Forkhead box p3
GLAST+	Glutamate transporter aspartate positive

GM-CSF	Granulocyte monocyte colony stimulating factor
GSF	Granulocyte colony stimulating factor
H1R	Histamine 1 receptors
H2R	Histamine 2 receptors
H3R	Histamine 3 receptors
H4R	Histamine 4 receptors
HMGB1	High-motility group box 1
IC	Ischemic Cortex
IFN	Interferon
IL	Interleukin
IP	Interferon gamma induced protein
iNOS	inducible nitric oxide synthase
KC	Keratinocyte derived chemokine
KO	Knockout
LPS	Lipopolysaccharides
M1	Pro-inflammatory activated microglia
M2	Anti-inflammatory activated microglia
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MPER	Mammalian protein extraction reagent
NK	Natural killer

OCR	Oxygen consumption rate
Oct3	Organic cation transporter 3
Oligo	Oligomycin
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PD-1	Programmed death receptor -1
PD-L1	Programmed death receptor – ligand 1
PMAT	Plasma membrane monoamine transporter
PMN	Polymorphonuclear cells
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RANTES	Regulated on activation, normal T-cell expressed and secreted
STAT3	Signal transducer and activator of transcription 3
STAT3p	Phosphorylated signal transducer and activator of transcription 3
rtPA	recombinant tissue plasminogen activator
SEM	Standard error of the mean
SRC	Spare respiratory capacity
TFAM	Transcription Factor A, mitochondrial
Th	T-helper
TNF	Tumor necrosis factor
tPA	tissue plasminogen activator
TTC	Tetrazolium chloride
WT	Wild type

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PREFACE

Worldwide, stroke is the second leading cause of mortality [1]. However, in the United States, stroke is the leading cause of long-term disability and the fifth leading cause of death [1, 2]. Stroke, which disproportionately affects the aging population, can be characterized as hemorrhagic or ischemic, with ischemic stroke consisting of approximately 7/8 of all strokes [2]. Ischemic stroke occurs when there is an occlusion of blood vessels resulting in a severe reduction of blood flow to a brain region leading to glial scar formation, changes in BBB permeability, and trafficking of leukocytes into the brain [3-5]. It has been well established that astrocytes are one of the main cell types in the glial scar and are important modulators of ischemic injury interacting with leukocytes in the peri-infarct region [3, 6].

Astrocytes have been identified as cells that communicate with the cellular immune system and bridge interactions between lymphocytes and neurons demonstrating that the nervous and immune systems cross-communicate [7-9]. However, the mechanism of interaction between astrocytes and T-cells and the functional astrocytic changes this causes in the brain is unknown. Thus, there is an imperative need to understand the effect of these immune cells on brain injury. This raises the possibility that T-cells in the brain, may initiate a sequence of events which ends in changes to astrocyte function, astrogliosis, exacerbated damage, or homeostatic repair mechanisms. Previously our data showed that IL-10 was increased in the brain of mice 1 month

post experimental ischemic stroke [10], suggesting that this glial immune interaction participates in an anti-inflammatory process.

Understanding the role of T-cells post ischemic stroke may present an opportunity for novel treatments to reduce stroke risk or complications associated with recovery from stroke. Presently, the FDA has approved one drug for the treatment of ischemic stroke, a thrombolytic, known as tissue plasminogen activator (tPA) [11]. Since this drug must be used in less than 4.5 hours following stroke onset to be significantly effective, less than 2% of stroke patients are able to benefit from this therapy [11-13]. Additionally, tPA increases hemorrhagic risk [14]. Therefore, alternative stroke treatments or prophylaxis would be advantageous to lessen the severity of stroke related disability, and death. This is especially important as our aging population continues to grow.

Discovering functional changes in astrocytes in the presence of T-cells, and the mechanism of interaction between astrocytes and T-cells may be useful in finding new biological targets for therapeutic agents to prevent irreversible damage, disability, and premature death for ischemic stroke patients. The overall objective of this application, to attain my long-term goal, is to identify glial-immune interactions, which may alter astrocytic function which can be manipulated to provide neuroprotection and therefore be targeted for treatment before or after ischemia.

My central hypothesis is that T-lymphocytes in the brain crosstalk with astrocytes via direct and/or indirect contact, which increases IL-10 production, causing functional astrocytic changes, and leads to an anti-inflammatory state as supported by immunosuppressive gene expression. This hypothesis was formulated as a logical extension of previous data that showed co-localization of astrocytes and T-cells in the peri-infarct region, increased transcription of T-cell

mediators in the stroked brain, and astrocytic morphological changes in response to changes in the astrocytes' environments.

The rationale that underlies this research is that T-cell to astrocyte interactions are responsible for releasing mediators that are involved in damage and or recovery from ischemic stroke. This research may point to or identify new therapeutic targets to reduce the stroke risk and premature stroke-related disabilities and death.

CHAPTER 1

1. INTRODUCTION

1.1

GLIA-IMMUNE INTERACTIONS POST-ISCHEMIC STROKE AND POTENTIAL THERAPIES

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1.1.1 Abstract

Although the primary responsibility of the immune system has for over a century been perceived as the protector of the host against infection in the peripheral organs, we now know the immune system also plays a vital role in recovery pathways associated with central nervous system (CNS) injury. There is mounting evidence that the blood–brain barrier does not preclude the CNS from immune surveillance. Of particular interest for this review is how microglia and astrocytes interact with the cells of the immune system to modulate repair and recovery mechanisms in ischemic stroke. Our review argues that by deepening our understanding of neuroimmunity, specifically the bidirectional glial–immune cell communications, a plethora of new therapeutic targets and mechanisms may be revealed. Consequently, this review instigates novel experimental approaches to neuroimmunology and fosters a more rapid discovery process for the treatment of stroke.

Keywords: Ischemic stroke, neuroimmunology, astrocytes, microglia, histamine, T-cells, natural killer cells, monocytes, glutamate, interleukin-15, glial–immune interactions

1.1.2 Impact Statement

This article reviews glial cell interactions with the immune system post-ischemic stroke. Research has shown that glial cells in the brain play a role in altering phenotypes of other glial cells and have downstream immune cell targets ultimately regulating a neuroinflammatory response. These interactions may play a deleterious as well as beneficial role in stroke recovery. Furthermore, they may provide a novel way to approach potential therapies, since current stroke therapy is limited to only one FDA approved drug complicated by a narrow therapeutic window. Until this point, most research has emphasized neuroimmune interactions, but little focus has been on bidirectional communication of glial-immune interactions in the ischemic brain. By expanding our understanding of these interactions through a compilation of glial cell effects, we may be able to pinpoint major modulating factors in brain homeostasis to maintain or discover ways to suppress irreversible ischemic damage and improve brain repair.

1.1.3 Introduction

The brain has long been considered a site of immune privilege established by the presence of the blood brain barrier (BBB) and lack of conventional lymphatic vessels. It was thought that the BBB barricaded peripheral immune cells from extravasating into the cerebrum [15, 16], however, recent findings in animals and humans have indicated that immune cells can enter the CNS and may contribute to immune surveillance in the brain [3, 8, 9, 17-20].

The interactions between glial cells and immune cells in the brain have opened the floodgates for a new area of research that is deepening our understanding of immunity in neuroscience and how the two systems play a role in regulating each other. Furthermore, this area of research is still in its infancy and has the potential to present us with novel therapies for ischemic stroke [3, 8, 9, 21]. Post-ischemic stroke, immune cells infiltrate the brain and interact with glial cells [3, 21-23]. Multiple points of contact and communication between glial cells of the nervous system and various immune system cells or markers have been identified [3, 22-24]. The role of immune cells in the brain as it relates to the progression of cerebral infarction or repair and recovery mechanisms remains unresolved. The immune cell to brain cell interaction has received notable attention for providing evidence that an innate immune response exists between peripheral immune cells such as neutrophils and brain cells [16, 25, 26]. On the other hand, there has been a more limited amount of research delving into the role of the adaptive immune response between immune cells and glial cells. Nevertheless, there is mounting evidence that adaptive immune cells indeed impact ischemic stroke outcome [27], which provides the rationale to extend the focus of this research to include studying the interaction of adaptive immune cells and glial cells. Here, we review the interaction between glial cells and the immune system in brain ischemia, examine the

pro- and anti-inflammatory effects, and potential therapies to reduce damage caused by cerebral infarction.

1.1.4 Stroke, Neuroimmunity, and Current Therapy

Stroke is a disease associated with aging. It is the fifth leading cause of mortality and the leading cause of long-term disability in the United States [1, 2]. Globally, it is the second leading cause of death [1, 28]. In the United States, by 2050; the population over age 64 will approach 84 million people [29]. This is nearly a two-fold increase for this age group, as compared to 2012 [29]. Therefore, as the aged population increases, it is expected that the incidence of stroke will also rise. Of the two types of stroke, hemorrhagic and ischemic stroke, ischemic stroke predominates and consists of approximately 87% of cases; hence this review will focus on ischemic stroke [1].

Ischemic stroke is characterized by occlusion of an artery that restricts cerebral blood flow causing oxidative stress and glucose deprivation in the damaged brain region and triggering necrosis and apoptosis leading to functional deficits [30]. When cells die they release damage associated factors in prescribed molecular patterns (DAMPs) [31]. DAMPs such as high-motility group box 1 (HMGB1), Transcripton Factor A, mitochondrial (TFAM), and ATP are biomolecules that trigger activation of a non-infectious inflammatory response. HMGB1 and TFAM have been shown to play roles in upregulation of cell adhesion molecules and drive immunogenic cell death [32]. Beyond this brief introduction, DAMPs are a complex topic meriting a review of its own and is therefore beyond the scope of this mini review. Nevertheless, these sets of events can stimulate toll-like receptors, which in turn activate a signaling cascade leading to production of pro-inflammatory mediators that recruit immune cells from the periphery to the brain [30].

Post-ischemic stroke, macrophages, neutrophils, B- and T-cells of the immune system enter the brain to engage in bidirectional communication with glial cells [9]. This glial-immune interaction was likely intended to be a protective mechanism to maintain homeostasis and prevent invaders in the CNS, although when over activated it appears to contribute to the damaging affects seen in ischemic brain injuries [9]. Hence, inflammation and immunity play an undeniable role in the pathogenesis of stroke [33].

Recent research has rebutted the common mantra that the brain via protection of the BBB was held on a pedestal of immune privilege. The immune system has been shown to play a role in a variety of CNS diseases including multiple sclerosis, psychiatric disorders, and stroke [24, 32]. In stroke, signaling generated by cerebral ischemia may activate the immune system, contribute to tissue damage and neuroinflammation, and may be responsible for changes in BBB permeability, leading to activation of macrophages and mast cells thereby causing the release of pro-inflammatory cytokines and histamine [5, 24, 34]. Pro-inflammatory mediators are known to play a role in leukocyte trafficking making it possible for peripheral monocytes, neutrophils, natural killer cells, or lymphocytes to infiltrate the brain [3, 4, 22, 25].

A non-specific defense mechanism which is initiated immediately or within hours by the presence of an antigen is known as the innate immune response [9]. This first line of defense against pathogens involves phagocytes such as monocytes, macrophages, microglia, and neutrophils. The initial insult to the brain during ischemia involves the disruption of the BBB sending signals for upregulation of adhesion molecules on endothelial cells to promote extravasation of neutrophils and monocytes into the brain [9]. Neutrophils are considered first responders of the immune system as they are triggered within minutes of ischemic insult [9, 25].

Following the first response, blood monocytes migrate to brain tissue becoming active macrophages as second responders [9].

The adaptive immune response is a more complex delayed antigen-specific.response involving T and B cells which are used to provide memory for subsequent exposure to an antigen. This response involves a specific antigen which is recognized and processed prior to presentation on antigen presenting cells (APCs). APCs of the peripheral immune system present antigens on their major histocompatibility complex (MHC) to T-cells causing T-cells to become activated [35, 36]. MHC II is found on the surface of both astrocytes and microglia [11, 23, 37]. Therefore, it is possible that astrocytes and microglia are the important regulators of ischemic injury, activating T-cells in a cascade that mimics adaptive immunity in the periphery.

Fostering our understanding of the intricate relationship between the immune system and glial cells post-ischemic stroke is essential to unveiling novel immunomodulating therapies that may reduce or prevent irreversible brain damage associated with ischemia or other brain injuries. Therefore, there is an urgent need to develop alternative therapies that may work alone or in conjunction with the current limited treatment, recombinant tissue plasminogen activator (rtPA). This would expand treatment modalities blocking the deleterious effects of stroke via an alternate pathway. Presently, rtPA a thrombolytic, is the only drug approved by the Food and Drug Administration (FDA) for the treatment of ischemic stroke [11]. While this drug is valuable if used within the narrow therapeutic window of 4.5 hours, this time limitation allows only about 2% of stroke patients to benefit from this infusion [11-13]. Beyond this short treatment window, rtPA can increase hemorrhagic risk [14]. Thus, finding alternative routes to prevent and treat ischemic stroke by using combination therapies or by lengthening the therapeutic window is crucial to prevent stroke related disability, premature death, and enhance recovery.

In this review two main types of glial cells will take center stage: microglia and astrocytes. The justification for this emphasis is that these cells have been identified as key CNS cells important in modulating interactions between neurons and peripheral immune cells and have characteristics themselves that mimic immune cells in the periphery. Understanding the function of each glial cell in its physiological and pathological conditions and how it interacts with immune cells during ischemic stroke may lead to effective therapies modifying the post-stroke immune response. The subsequent discussion will be outlined first by glial cell and second by innate and then adaptive immune interactions so as to follow the natural course of events.

1.1.5 Microglial Cells

Microglia, characterized as the resident macrophages of the CNS, actually enter the CNS after birth linking the CNS to the immune system, however the exact function of microglia during immune challenged states continues to be up for debate [9, 38, 39] . Much like macrophages acting as first responders in the peripheral immune system, microglia cells become activated and respond within hours following brain damage [40, 41], suggesting that this cell type is also a component of the innate immune system in the brain. Currently, two activation states have been proposed: classically activated microglia (M1) and alternatively activated microglia (M2) [42]. For ease of discussion, the M1 and M2 terms will be used, however it should be acknowledged that the M1/M2 nomenclature is under refinement as it has been recognized that microglial phenotypic switching of M1 and M2 states exists on a continuum with more than two polarization states [43, 44].

Lipopolysaccaraides (LPS), a microbial endotoxin used to simulate neuroinflammation, has been shown to exacerbate infarct volumes playing a role in stroke severity [45] and worsening neurological outcomes post-stroke. In contrast, repeated or single administration of LPS prior to stroke, termed LPS pre-conditioning, has demonstrated a shift from an early pro-inflammatory M1

microglial phenotype in association with an elevation in inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNFα) to a later anti-inflammatory M2 phenotype associated with an increased arginase-1 and interleukin 10 (IL-10) [46, 47] lending to LPS pre-conditioning as neuroprotective following stroke [48, 49].

Animal and human studies have shown that aging plays a role in microglial phenotype specificity, whereby M1 over M2 microglial phenotypes are selected in response to immune challenges [42]. Consequently, this may suggest that the aged brain is compromised by its inability to respond to stressors such as injury or ischemia due to a pro-inflammatory mechanism overriding the repair mechanism provided by M2 microglial cells [42]. Additionally, aged mice demonstrate a reduction in $Iba1^{+}$ cortical microglia, which cluster in an uneven distribution pattern [47]. Dystrophic changes in microglia have also been reported in 68 year old human subjects compared to 38 year olds [50]. Since ischemic stroke is a condition disproportionally affecting the aged population it is possible that this senescent selectivity could alter signaling that impacts the glial-immune interactions during ischemia [1, 51]. Microglia are known to be cells that respond to environmental stimuli, however it was reported that the microglial response to micro-laser lesion injury has a reduced reaction speed in aged 26-27 month old compared to 3 and 11-12 month old Iba-1-EGFP transgenic mice [52]. Aged microglia in humans and the retina of aged mice demonstrated slower acute response time, processing motility in response to laser induced injury, and reduced dendritic arborization compromising the ability of aged microglia to survey the environment [53, 54]. Furthermore, in mice microglia express receptors which when activated induces pro-inflammatory signaling cascades, stimulating microglia and leading to upregulation and secretion of pro-inflammatory markers [55]. Additionally, microglia express inhibitory receptors to prevent overactivation of inflammation and to reduce injury-driven inflammatory

response [56-58]. With age, microglia have impaired phagocytosis and inhibitory receptors are deficit in their ability to maintain microglial quiescence [59]. Therefore, since response to injury, and receptor function of microglia alters with age, it is likely that these cells when aged may not be able to adapt to their environment and interact in the same capacity as non-aged microglia interfering with the ability of the microglia cells to provide neuroprotection.

1.1.5.1 Microglia: The innate immune response - Interactions with Monocytes

Under normal physiological conditions, monocytes are formed in the bone marrow, after which they extravasate through blood vessel walls to mature into macrophages or dendritic cells in the tissues. Upon extravasation macrophages act as APCs in the periphery, phagocytizing debris, producing and releasing cytokines, and are first responders against foreign invaders.

Unlike microglial cells which are activated within hours post-brain ischemia, monocyte-derived macrophages are recruited to the ischemic region within 3 to 7 days post ischemic stroke [7]. The anti-inflammatory monocyte-derived macrophage subset, $Ly6C^{low}$, has not been thoroughly investigated in ischemic stroke, but depletion of this macrophage subtype did not affect brain injury nor increase infarct size in one study [60]. On the other hand, $Ly6C^{hi}$ monocytes, also known as inflammatory monocytes which are recruited to sites of inflammation [61], express CC chemokine receptor 2 (CCR2), which plays a role in the ability of monocytes to extravasate and migrate into the brain following ischemic insult [62]. Hence it is not surprising that $Ly6C^{hi}$ monocytes were elevated in the blood and brain post-stroke [22, 63]. Depletion of these monocytes or their inhibition by INCB3344 which binds to CCR2, lead to increased infarct volumes, and decreased M2 polarization resulting in a more damaging state and poorer functional outcomes, suggesting that monocytes play a role in reducing damage in brain ischemia [22, 63].

Manipulation of the M1 to M2 phenotype opens the possibility of targeting microglial activation states as potential therapeutic targets for neuroprotection. This is; however, a challenging endeavor as disease, medications, age, gender, or other environmental stressors may alter microglial activation states.

1.1.5.2 Microglia: The innate immune response - Interactions with Histamine

Histamine is a widespread neurotransmitter and neuroimmune modulator in the brain with functions in the CNS and the periphery, playing roles in immune system regulation and brain disorders. Mast cells in the periphery and brain release a variety of mediators, including histamine, which is activated or released by neuropeptides, cytokines, serotonin or histamine itself [64]. In the brain, approximately 50% of histamine is derived from non-neuronal cells and changes in stress, behavior, or endocrine fluctuations can modify histamine production [24]. Furthermore, microglia have also been identified as histamine producing cells [65].

In addition, microglia express all known histamine receptors [66]. Specifically, histamine can stimulate microglia [67] via histamine 3 receptors (H3R) to produce increased amounts of pro-inflammatory cytokines, which regulate microglial chemotaxis and phagocytosis in the brain [24]. Histamine has also been shown to trigger microglial phagocytosis via histamine 1 receptors (H1R) and reactive oxidation species production via H1R and histamine 4 receptor (H4R) [67, 68]. Histamine plays a role in multiple physiological functions; including interleukin 1 beta (IL-1β) and TNFα cytokine induced neuroinflammation as well as inhibition of LPS induced IL-1β on microglia at H4R [24, 66]. Changes in TNFα, IL-1β, microglial activation, and apolipoprotein E related Cluster of Differentiation 95 death receptor (CD95) expression on T-cells contribute to neuroimmune dysregulation and modify cell recruitment [24]. Therefore, this may impact CNS recovery or damage from ischemia.

1.1.5.3 Microglia: The adaptive immune response - Interactions with T-cells

The cell-mediated adaptive immune response is characterized by a delayed antigen specific immune response. B and T lymphocytes play a pivotal role in cell-mediated immunity and thus play a secondary role in response to brain ischemia. While there has been little focus on the effects of B-cells in the ischemic brain, there has been recent work on the effects of T-cells. In the periphery, T-cells respond to specific antigens by releasing pro- or anti- inflammatory cytokines via regulatory T-cells (Treg), and CD4+ T-helper cells or by destroying virally infected cells themselves via CD8+ cytotoxic T-cells.

In one animal study, Forkhead box P3 $(Foxp3)^{+}$ Treg cells present in the rat cerebrum inhibited the LPS induced M1 pro-inflammatory response of microglia, implying that certain subsets of T-cells may inhibit neuroinflammation by interacting and possibly altering microglial phenotypes which may also indirectly affect astrocyte reactivity [19].

Crosstalk between APCs expressing programmed death receptor-1 (PD-1) and programmed death-ligand 1 (PD-L1) on $CD8^{+}$ T-cells, including those that infiltrated the brain during CNS injury, has been shown to cause T-cell exhaustion or suppression. One study demonstrated that activated microglia, and astrocytes express programmed death-ligand 1 (PD-L1), therefore it is not surprising that *in vitro* co-cultures of these cell types combined with blockade of PD-1 to PD-L1 communication caused increased production of T-cell interferon gamma (IFNγ) and interleukin 2 (IL-2) [23]. These findings point out a potential area to target glial immune interactions in developing therapies to reduce effects of CNS insult including stroke.

1.1.6 Astrocytes

Astrocytes, another type of glial cell, and the most abundant cell residing in the CNS, have diverse morphology and can be classified into two main groups in the cortex: fibrous (elongated) astrocytes, or protoplasmic (radial) astrocytes [69]. Fibrous astrocytes, in the white matter, tend to be in close proximity to myelinated axons and oligodendrocytes [69]. Protoplasmic astrocytes, located in the grey matter, interact directly with neurons, blood vessels, [69] and participate in the formation of the BBB, making them a prime target for immune cell exposure. Following ischemic stroke, the BBB becomes permeable, increasing the likelihood of glial immune interactions [10]. One-month post-ischemic stroke, T-cells were found in close proximity to active astrocytes in the ischemic region [10]. Astrocytes, once thought to be passive support cells for neurons, [70] are now known to respond to CNS insults, whereby they may undergo morphological and functional changes referred to as reactive gliosis [71]. Astrocyte reactivity is a way of maintaining homeostasis in the CNS and works as a defense mechanism to limit damage caused by ischemic stroke. On the other hand, it can also hinder recovery systems in the brain. Recently, reactive astrocytes have been categorized into A1 or A2 cell types. This nomenclature is a morphological distinction and may or may not reflect a functional distinction, nonetheless these terms will be used for the sake of simplicity. The A1 astrocytes upregulate complement cascade genes thought to play a role in CNS damage and the A2 neuroprotective astrocytes upregulate neurotrophic factors [44]. LPS-induced classical activation of microglia caused the release of interleukin 1 alpha (IL-1α), and TNF, which when combined with complement component 1q (C1q) to instigate astrocyte reactivity, steered astrocytes to a neurotoxic (A1) state [44]. A recent study showed that LPS directly added to astrocyte culture media was insufficient to drive astrocytes to the A1 state,

and this was confirmed by measuring the upregulation of astrocyte genes leading to the production of neurotoxins that are lethal to neurons following CNS damage [44].

Therefore, mechanisms involved in regulation of astrocytes and astrogliosis are of particular interest as they may provide another avenue for drug treatment to reduce post-ischemic stroke damage. Astrocytes are brain cells that bridge interactions between lymphocytes and neurons and communicate with immune system cells via cytokines [3, 32].

1.1.6.1 Astrocytes: The Innate Immune Response - Interactions with Neutrophils

Polymorphonuclear cells (PMNs) are the most abundant leukocyte and generally the first immune cell to be recruited to sites of inflammation, however their function is at least partially determined by direct or indirect interactions with astrocytes [25]. For the purpose of this review, direct contact refers to cell-to-cell communication via touching, such as through cell receptors, while indirect contact refers cell-to-cell communication through non-touching means, such as cytokine secretion. PMNs isolated from C57BL/6 mice were placed in primary astrocyte cultures at a 1:1 ratio [25]. Direct and indirect astrocyte contact to PMN contact, resulted in attenuated PMN apoptosis, enhanced phagocytosis and decreased degranulation. However differences between indirect and direct contact emerged demonstrating that direct astrocyte to PMN contact resulted in increased pro-inflammatory cytokine expression, and decreased respiratory burst, while indirect contact encouraged PMN necrosis and increased respiratory burst [25]. The complexity of the interaction between PMNs and astrocytes warrants further investigation since it could be important in the innate immune response and be a target to reduce neuroinflammation and transmigration of other leukocytes into the brain during stroke.

1.1.7.1 Astrocytes and the Innate Immune Response - Interactions with Histamine

Astrocytes have been receiving increasing attention for their role as neuroinflammatory modulating cells, and histamine has been shown to play a part in astrocyte function including: neuroprotective effects on astrocytic cell damage as a potent anti-inflammatory mediator, immune response via regulation of innate and acquired immunity, energy metabolism, homeostasis, and neurotransmitter clearance [72-74]. Current research has identified three histamine receptors (H1R, H2R, and H3R) [74] that are expressed on astrocytes indicating the importance of histamine in astrocyte function. Additionally, primary human astrocytes express organic cation transporter 3 (Oct3) and plasma membrane monoamine transporter (PMAT) on their cell surface [75-77]. This signifies the importance of histamine transport in astrocytes since both of these transporters are responsible for histamine reuptake. Nevertheless, little data is available describing the immunomodulatory role of histamine on astrocytes, and even less is understood within the pathology of ischemic stroke.

Only one study evaluated the outcome of disruption to the histamine transporter, Oct3 in the re-uptake of histamine and its effects on ischemic brain damage and regulatory T-cells (Tregs) [78]. In this study, an adult male 10 and 12-week-old homozygous Oct3 knockout (Oct3 KO) and wild type (WT) mouse model was used and mice either received a 1-hour middle cerebral artery occlusion (MCAO) or sham [78]. Tetrazolium chloride (TTC) staining showed a reduced infarction volume in Oct3 KO mice compared to controls following MCAO [78]. Histamine levels in the ischemic cortex (IC), during occlusion and 3 and 24 hours after reperfusion were increased in the Oct3 KO mice compared to sham controls, however this histamine difference was not seen between groups pre-ischemia [78]. Oct3 KO mice had higher serum histamine levels 24 hours after reperfusion than WT mice [78]. Following reperfusion, all brain tissue samples taken

from Oct3 KO and WT mice showed elevated levels of monocyte chemoattractant protein 1 (MCP-1) and interleukin (IL-6) cytokine production, however the level of MCP-1 and IL-6 in the IC was remarkably higher in WT compared to Oct3 KO mice [78].

Media from primary cell cultures of astrocytes, microglia, and bone marrow derived macrophages had increased levels of IL-6, MCP-1, and TNFα when cells were activated with Lipid A [78]. Proportions of type 1 (Th_1) and type 2 T-helper (Th_2), and Tregs did not vary between Oct3 KO and WT mice, before MCAO, but, Treg proportions increased in Oct3 KO mice post reperfusion. L-histidine, when injected into both groups, did not change the proportion of Treg cells in Oct3 KO mice but it did increase the Treg cell proportion in WT [78]. In turn, Oct3 disruption may inhibit uptake and clearance of histamine in the brain.

Since Tregs are recruited by histamine [79], elevated levels of histamine will lead to higher levels of Tregs, which play a neuroprotective immunomodulating role during cerebral ischemia. Future research should investigate the role of Tregs and their migration to and in the brain, as well as targeting Oct3 as a possible therapy for cerebral ischemia. Disruption of Oct3 via increased histamine and Tregs ameliorates damage caused by ischemic brain injury [78]. Furthermore, since microglia are surveillance cells in the brain, changes in the environment such as ischemic conditions may regulate astrocyte functions which may have an impact on transporter or histamine receptor expression thereby altering interactions with histamine or recruitment of T-cells complicating repair mechanisms post-stroke.

1.1.6.3 Astrocytes: The Adaptive Immune Response - Interactions with T-cells and NK Cells

In severe oxidative stress, astrocytes, which normally play a role in removal of glutamate from extracellular fluid [80, 81], stop glutamate clearance [82]. Excessive glutamate can inhibit glutamine synthetase causing intracellular glutamate accumulation in astrocytes and inducing

reactive astrogliosis [83]. *In vitro* studies demonstrated that the addition of T-cells to astrocyte cultures rescues astrocytes from impairment by oxidative stress endowing a neuroprotective astrocyte phenotype [82, 84]. The replacement of T-cells with IL-2, or IFNγ cytokines in oxidative stress induced cultures, caused increased glutamate clearance, while cultures without cytokines, or those with interleukin 4 (IL-4) did not [82]; implying that Th1 T-cells are the primary neuroprotective T-cells [82] and that T-cell subtypes may have different effects on neuroprotection [85]. When neurons were cultured in media derived from oxidatively stressed astrocytes there was a significant reduction of neuron apoptosis [82]. This protective effect was further enhanced when medium from T-cells was co-cultured at a 1 to 1 ratio with astrocytes under oxidative stress [82]. By adding a glutamate uptake inhibitor, L-aspartic acid β-hydroxamate (AβH), to astrocyte T-cell co-cultures, the neuroprotective effect was blunted demonstrating that T-cell to astrocyte interactions via glutamate clearance are neuroprotective and further understanding of this interplay may help identify strategies to treat ischemic brain injury [82].

Additionally, it has been shown that trans-presentation of interleukin 15 (IL-15), a pro-inflammatory cytokine, by astrocytes [86, 87] to T-cells and NK cells exacerbates brain damage post-ischemic stroke [3]. Glutamate aspartate transporter positive ($GLAST^{+}$) astrocytes isolated from lesion areas 24 hours after reperfusion from male wild type (WT) C57BL/6 mice after right MCAO, demonstrated an approximately 38% higher level of IL-15 in the cell lysate compared to the sham (no MCAO) mice. Also it was determined that the largest amount of IL-15 was produced by astrocytes in the brain of both mice and humans rather than by neurons or microglia cells, revealing that IL-15 from astrocytes is a key regulator in inflammation[88] post-ischemic stroke [3]. These same researchers demonstrated that their transgenic IL-15 mouse model had increased infarct volumes and neuro-deficits compared to the WT mice [3]. Furthermore, CD8+ T-cells, and

NK cells were observed to be co-localized with astrocytes in peri-infarct regions [89] 24 hours after stroke and these leukocytes had increased expression of the T-cell and NK cell activation markers CD69, NKG2D, IFNγ in transgenic mouse brains, but not in their spleens compared to WT [3].

In addition, *in vitro* experiments showed that placing an insert which separated $CD8^+$ T-cells or NK cells from astrocytes in co-cultures decreased or inhibited activation of T-cells and NK cells from transgenic IL-15 mice indicating that direct cell-to-cell contact of immune cells and astrocytes was necessary for a pro-inflammatory response [3].

1.1.7 Pro-inflammatory Cytokine Link CNS to Immune System

As previously mentioned, pro-inflammatory cytokines and chemokines are released by both microglia and astrocytes. Hence it is important to note that pro-inflammatory cytokines are an obvious link between cells in the CNS such microglia and astrocytes and immune system cells such as T-cells.

Pro-inflammatory cytokines are up-regulated in the infarcted area, urine, or serum post-ischemic stroke [27, 90-95]. The acute expression of cytokines varies in mice and humans [96] (granulocyte colony stimulating factor (G-CSF) was elevated in mice but not humans) [4]. It is noteworthy that the following acute cytokines were elevated post-ischemic stroke in both mice and humans: granulocyte monocyte colony stimulating factor (GM-CSF), IL-6, interleukin 12 active heterodimer (IL-12(p70)), interferon gamma induced protein 10 (IP-10), keratinocyte derived chemokine interleukin 8 homologue (KC/IL-8), MCP-1, macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein 1 beta (MIP-1β), regulated on activation, normal T cell expressed and secreted (RANTES), TNFα [4, 24]. Several weeks post-ischemic stroke during the stage of liquefactive necrosis, the inflammatory response is dampened. Several cytokines

remain chronically elevated during this period: IL-6, and MCP-1[4]. Additionally, there was a chronic T-cell response in the stroked regions of both mice and humans, however activated T-cell subtypes varied depending on mouse strain [4, 97]. The $CD4^+$ Th_1 response was higher in C57BL/6 mice, while Th_2 responses were higher in BALB/c mice [4]. Furthermore, chronic cytokine and chemokine differences were not remarkably different in 18-months-old versus 3-months-old C57BL/6 mice, while T-cell infiltration was blunted in the younger mice [4]. Other studies have shown a different profile when comparing similar age discrepancies, with 18 month old mice versus 9 to 12 week old mice exhibiting an increased pro-inflammatory cytokine profile in the aged mice (TNF-α, iNOS, and IL-6) and anti-inflammatory profile in the young mice (TGF-β, IL-4, IL-10) post MCAO [98]. Lastly, recurrent stroke mildly increased cytokine response [4]. This data calls to question the use of *in vivo* models since the inflammatory response post-ischemic stroke appears to vary based on an individual's genetics and previous exposure to environmental factors [4].

1.1.8 Potential Therapies

When it comes to drug development for ischemic stroke, the present trend is to investigate combination therapies, which use rtPA alongside immune modulators to reduce ischemic damage. This may be a reasonable approach since ischemic stroke likely involves several mechanisms integrating cells from multiple organ systems. Potential pharmacological therapies that target glial immune interactions are summarized in Table 1.

Minocycline, a bacteriostatic antibiotic, may have an effect on microglial phenotypic changes [99, 100]. Several human and animal studies have indicated that minocycline is responsible not only for aiding in a M1 to M2 shift but also selectively inhibits the M1 phenotype [32, 101], suggesting that minocycline may provide benefits to ischemic stroke patients and

perhaps may be given prophylactically [102, 103]. In addition, it is most likely that minocycline alone is not responsible for microglial phenotypic changes [104], but rather a multitude of factors contribute to its potential drug efficacy such as stress, genetics, and gender.

Blocking the interaction of astrocytes trans-presenting IL-15 to T-cells and NK cells with antibodies resulted in decreased infarct volume in transgenic IL-15 mice to wild type levels. This is encouraging as it suggests that inhibition of the interaction between astrocytes and the immune system cells may reduce ischemic damage post stroke [3]. Other ways of blocking the damage caused by IL-15 were implemented by downregulation of the IL-15R, and by inhibiting direct contact between astrocytes and T-cells or NK [3, 86].

Mast cells and disruption of Oct3 during stroke increases histamine release and that may target microglia in the brain at H4R receptors as well as increase migration of Tregs to regions of brain ischemia. Consequently, histamine and interactions with Oct3 on astrocytes should be investigated as a possible pharmacological therapy for cerebral ischemia since increased histamine and Treg T-cells ameliorates recovery from damage caused by ischemic brain injury [78].

In addition, regulation of inflammatory markers and genes are important neuroinflammatory targets. Recent reports have pointed to the following potential determinants in regulating immune related pathologies: mitochondrial factors causing systemic inflammation related to injury, the NOTCH1 pathway associated with M1 macrophage activation and inflammation, tristetraprolin, an anti-inflammatory protein encouraging decay of pro-inflammatory mediators, release of cytokines from neutrophils, and sestrins, antioxidant proteins involved in Toll-like receptor pro-inflammatory signaling [24]. There is a network of immune signaling pathways, as well as neurologic and endocrine factors that may be webbed together in stroke pathologies [24]. The complexity of these interactions suggests that neuroinflammation and

neuroprotective effects are intertwined, indicating that a delicate balance between systems is essential to avoid neurological miscommunication. Therefore, drugs targeting immune pathways may have unintended adverse effects and should be used with caution.

1.1.9 Conclusions

The dialogue between glial and immune cells is complex as illustrated in Figure 1. Much of the data reported from these studies remains controversial, due to limitations in inconsistent or non-standardized *in vitro* or incompatible *in vivo* models as was demonstrated by changes in cytokine production between different strains of mice [4]. Additionally, with microglia, macrophage, and astrocyte phenotypes changing based on a multitude of environmental factors, it is difficult to confirm the role of different immune cells during cerebral ischemia or control for various environmental factors which may work together to create the final pathology. For instance, microglia can shift from neurotoxic to neuroprotective phenotypes, which can dictate alterations in cytokine levels and can also influence the function of astrocytes.

In *in vitro* studies the ratio of glia to immune cells appears to effect ischemic damage and repair and leads to inconsistent findings between researchers. Studies have reported varied ratios of T-cells to astrocytes in co-cultures. Higher 3:1 T-cell to astrocyte ratios may be neurotoxic while a lower 1:1 ratio may be neuroprotective [82]. Furthermore, *in vitro* models do not take into consideration damaged astrocytes at the site of injury, which may be unable to respond when injured. For example, injured astrocytes may not uptake T-cell derived glutamate, which may ultimately lead to neurotoxicity. Moreover, the method used to induce oxidative stress in many studies may not be an adequate representation of ischemic stroke in an *in vivo* model.

In addition, attention should be paid to the threshold ratio of immune cells responsible for adequate activation or phenotypic alterations in astrocytes or microglia. Still, little is known about

oligodendrocytes, B-cells, and dendritic cells and their role in glial immune interactions during ischemic stroke. Current research suggests that oligodendrocytes are resistant to ischemic insult [105], although little to no research has investigated their role in interacting with cells of the immune system. Similarly, B-cells to not exhibit a pathophysiological role in cerebral ischemia, as depletion of B-cells did not alter infarct volume or influence stroke outcomes [106]. Moreover, it is possible that only a small subset of T-cells, such as the γδ T-cells, engage in stroke recovery, while another group of immune cells are part of a signaling pathway inducing a negative response. Understanding these layers of interactions will help researchers develop specific drug targets with the fewest side effects.

Despite these challenges, researchers should not be discouraged. Abundant data supports cross-talk between immune signals and glia. Researchers should continue to sort out the individual components of CNS and immune cell interactions. The first step is discovering the signaling pathways that intertwine the CNS with the immune system to help regulate and reduce damage. Implications for this research extent far beyond stroke pathology, to include neuroinflammatory mechanisms that influence aging, cellular metabolism, and insulin resistance.

AUTHORS' CONTRIBUTIONS

JH and SY evaluated the literature, drafted, and edited the manuscript. JH prepared the table and figure.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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1.1.10 Table 1.1

Potential Therapies to Target Glial-Immune Interactions Post-Ischemic Stroke

Therapy	Known Use	Target/Action	Overall Effect
Minocycline **[99-104]**	Bacteriostatic antibiotic	Microglia Selectively inhibits M1 phenotype	Anti-inflammatory Neuroprotective
PD-L1:PD-1 pathway **[23]**	Not currently used as a therapy	Inhibits $CD8^+$ T-cell activation and cytokine production	Neuroprotective
Anti-$CD8^+$ and/or Anti-NK1.1 antibodies Anti-IL-15 antibodies **[3, 86]**	Not currently used as a therapy	Blocks interaction of astrocyte transpresentation of IL-15 to T-cells and NK cells	Decrease infarct volume
L-histidine/histamine **[24, 66-68, 72-75, 77, 78]**	Likely has significant side effects; not in use	Microglia and astrocytes Prevent disruption of Oct3 Increase migration of regulatory T-cells to ischemic regions	Decrease infarct volume Neuroprotective Anti-inflammatory

1.1.11

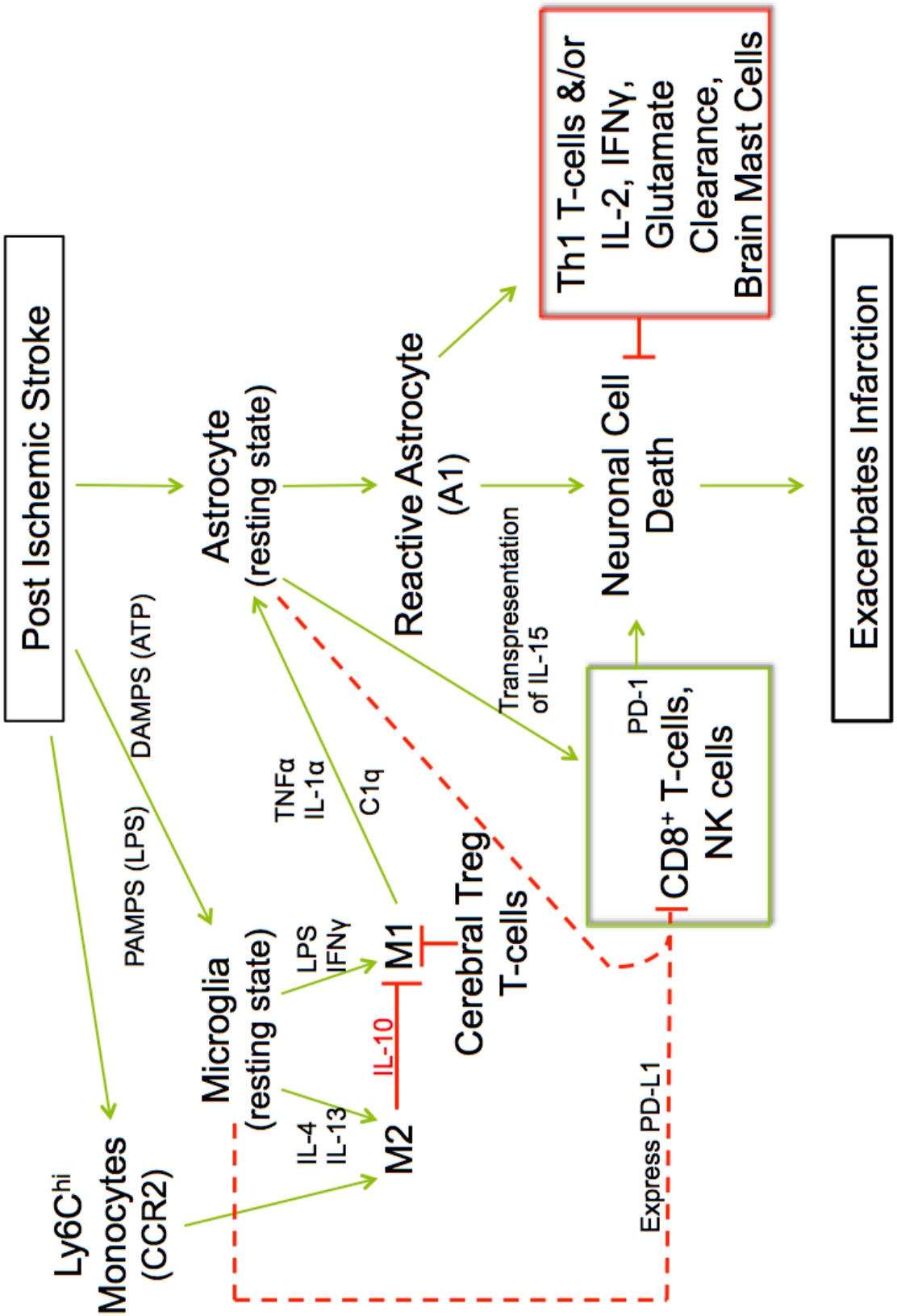
Post Ischemic Stroke
Ly6C^hi Monocytes (CCR2)
PAMPS (LPS)
DAMPS (ATP)
Microglia (resting state)
IL-4
IL-13
M2
IL-10
LPS
IFNγ
M1
TNFα
IL-1α
C1q
Astrocyte (resting state)
Reactive Astrocyte (A1)
Transpresentation of IL-15
Cerebral Treg T-cells
Express PD-L1
PD-1
CD8+ T-cells, NK cells
Neuronal Cell Death
Th1 T-cells &/or IL-2, IFNγ, Glutamate Clearance, Brain Mast Cells
Exacerbates Infarction

Figure 1.1 Glial immune interactions. Following ischemic-stroke signaling mediators trigger glial and immune cell responses leading to inhibition or stimulation of neuronal cell death. These mediators include pathogen associated molecular patterns (PAMPS), lipopolysaccharides (LPS), damage associated molecular patterns (DAMPS), cytokines: interleukin 4 (IL-4), interleukin 13 (IL-13), interleukin 10 (IL-10), interleukin 15 (IL-15), interleukin 1 alpha (IL-1α), interferon gamma (IFNγ), tumor necrotic factor alpha (TNFα), complement component 1q (C1q), and interleukin 2 (IL-2). When microglia are activated by PAMPS or DAMPS they change from a resting state to anti-inflammatory state (M2) or pro-inflammatory state (M1), respectively. Microglia in the M1 state can activate resting astrocytes causing their phenotypic shift into reactive astrocytes, resulting in neuronal cell death and exacerbating infarct damage. Microglia in the resting state which express programmed death-ligand 1 (PD-L1) can inhibit $CD8^+$ T-cells by binding to their programmed death-1 (PD-1) receptor. Activation of astrocytes post-ischemic stroke leads to trans-presentation of IL-15, natural killer (NK) cells, and $CD8^+$ T-cells which facilitate neuronal cell death. Monocyte activation following infarction leads to a dominant M2 microglial/monocyte phenotype resulting in the release of anti-inflammatory cytokines. Helper T-cells (Th1), IL-2, IFNγ, glutamate clearance, and mast cells in the brain have been shown to play neuroprotective roles to inhibit damaging effects of ischemic stroke. Arrows [79] represent stimulation. Lines with blunted ends [107] represent inhibition. The dashed red line represents inhibition of the PD-L1: PD-1 pathway only. See text for details.

1.2

COLOCALIZATION OF CD4+ AND CD8+ T-CELLS WITH ASTROCYTES *IN VIVO*

Figure From

EXPERIMENTAL ISCHEMIC STROKE INDUCES LONG-TERM T CELL ACTIVATION IN THE BRAIN

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1.2.1 INTRODUCTION

By the 1950s, the brain was considered a site of immune privilege, but in the last few decades this has been challenged. The brain is no longer considered a site of immune privilege evidenced by functional lymphatic vasculature, leukocyte trafficking to the brain including T-cells which contribute to immune surveillance and their ability to interact with glial cells [23, 108, 109]. Recent findings in animals and humans have indicated that immune cells can enter the CNS and may contribute to immune surveillance in the brain [3, 8, 9, 17-20]. Furthermore, when the brain's BBB is disrupted, such as during stroke, it leads to increased infiltration of immune cells into the brain allowing bidirectional communication between astrocytes and T-cells [23, 108, 109].

Astrocytes are brain cells that bridge interactions between lymphocytes and neurons and communicate with immune system cells via cytokines [3, 32]. Following ischemic stroke, the BBB becomes permeable, increasing the likelihood of T-cell interactions [10]. This research investigates the migration and infiltration of T-lymphocytes in regions of cerebral infarction and examines the interaction between T-cells, and astrocytes within the stroked mouse brain. It has been established that post-ischemic stroke, T-cells enter the brain, in a complex pathway responsible for both the progression of cerebral infarction as well as repair and recovery mechanisms [15, 16]. Hence, understanding the role of T-cells post ischemic stroke may present an opportunity for novel treatments to reduce stroke risk or complications associated with recovery from stroke. To date, no studies visualized colocalization of astrocytes with T-cells in the peri-infarct region at up to one month post experimental ischemic stroke in mice.

1.2.2 Materials and Methods

1.2.2.1 Immunofluorescent staining

Previously experimentally MCAO adult male C57/B6 mice (3-month-old) purchased from Charles River (Wilmington, MA, USA) were used. Mice were anesthetized by isoflurane inhalation and were intra-cardiacally perfused with 20 ml of 10% formalin. Five-micron paraffin-embedded brain sections were prepared and were incubated with antibodies against 2 m g/ml CD4 (eBioscience), CD8 (eBioscience), GFAP (Santa Cruz Biotechnology) and MAP-2 (Millipore) at 4^{o}C overnight. Sections were then incubated with 5 mg/ml of Alexa Fluor 488-conjugated goat anti-rat IgG and/or Alexa Fluor 594-conjgated goat anti-mouse IgG or Alexa Fluor 594-conjgated goat anti-rabbit IgG (Invitrogen). Sections were observed on an Axio Observer Z1 fluorescent microscope (Zeiss).

1.2.3 Results

CD4+ and CD8+ T-cells are co-localized with astrocytes in the peri-infarct region of the brain post experimental ischemic stroke (Figure 2) [10]. Immunohistochemistry demonstrated that infiltrating brain CD4+ and CD8+ T cells were predominantly located in the peri-infarct region with extensive reactive astrocytes indicated by strong GFAP staining at one month after ischemic stroke. Fewer CD4+ and CD8+ T cells were found in the normal brain region distal to the infarct lesion where less reactive astrocyte and more MAP2 positive neuron was observed.

1.2.4 Figures and legends

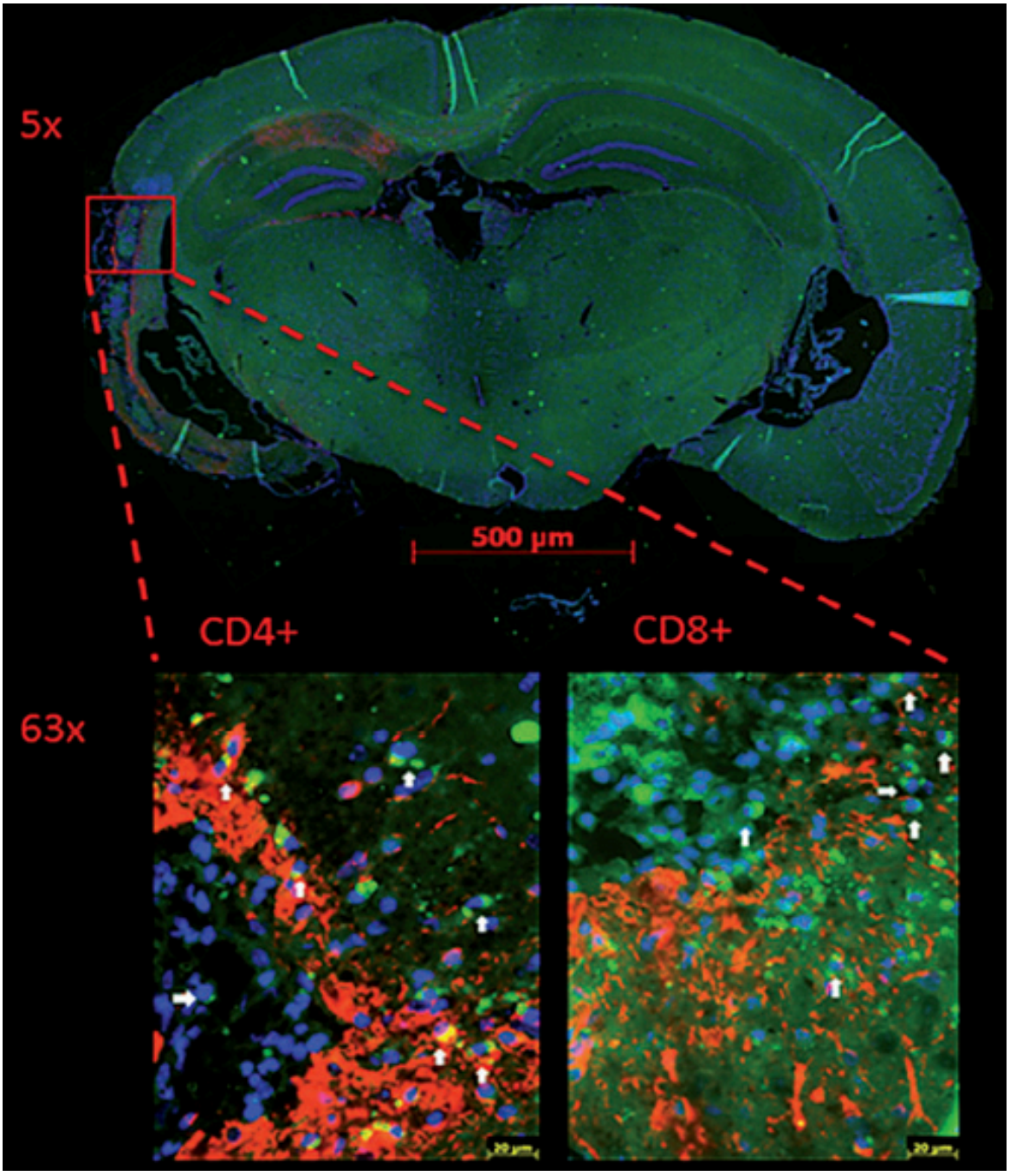

Figure 1.2 Co-localization of T-cells with astrocytes in the ischemic brain. Representative fluorescent microscopy (5x and 63x) of immunofluorescent staining of CD4+ and CD8+ T cells, and reactive astrocytes (GFAP, red) in the ischemic brain at one month after ischemic stroke. Green: CD4+ and CD8+. Blue: DAPI. Red: GFAP. Arrows indicate CD4+ and CD8+ T cells.

1.2.5 Discussion

One month post-ischemic stroke, T-cells were found in close proximity to active astrocytes in the ischemic region [10]. Immunohistochemistry demonstrated that brain invading $CD4^+$ and $CD8^+$ T-cells were predominately in the peri-infarct zone with extensive reactive astrocyte evidenced by strong GFAP staining at the chronic stage after ischemic stroke. The predominate localization of $CD4^+$ and $CD8^+$ T cells in the peri-infarct region where reactive astrogliosis developed after ischemic stroke indicates that the infiltrating $CD4^+$ and $CD8^+$ T cells after ischemic stroke may play a role in the repairing process after ischemic stroke.

Understanding of the crosstalk between the T cells and the brain cells is still rudimentary. Our study warrants future research to determine the interaction between infiltrating brain T cells and the innate CNS immune system which may lead to significant insight for the understanding of neural repairing after ischemic stroke. More specifically, this provides rationale to investigate whether co-localization of T-cells with astrocytes may impact cytokine production.

CHAPTER 2

MODULATION OF ASTROCYTE PHENOTYPE IN RESPONSE TO T-CELL INTERACTION

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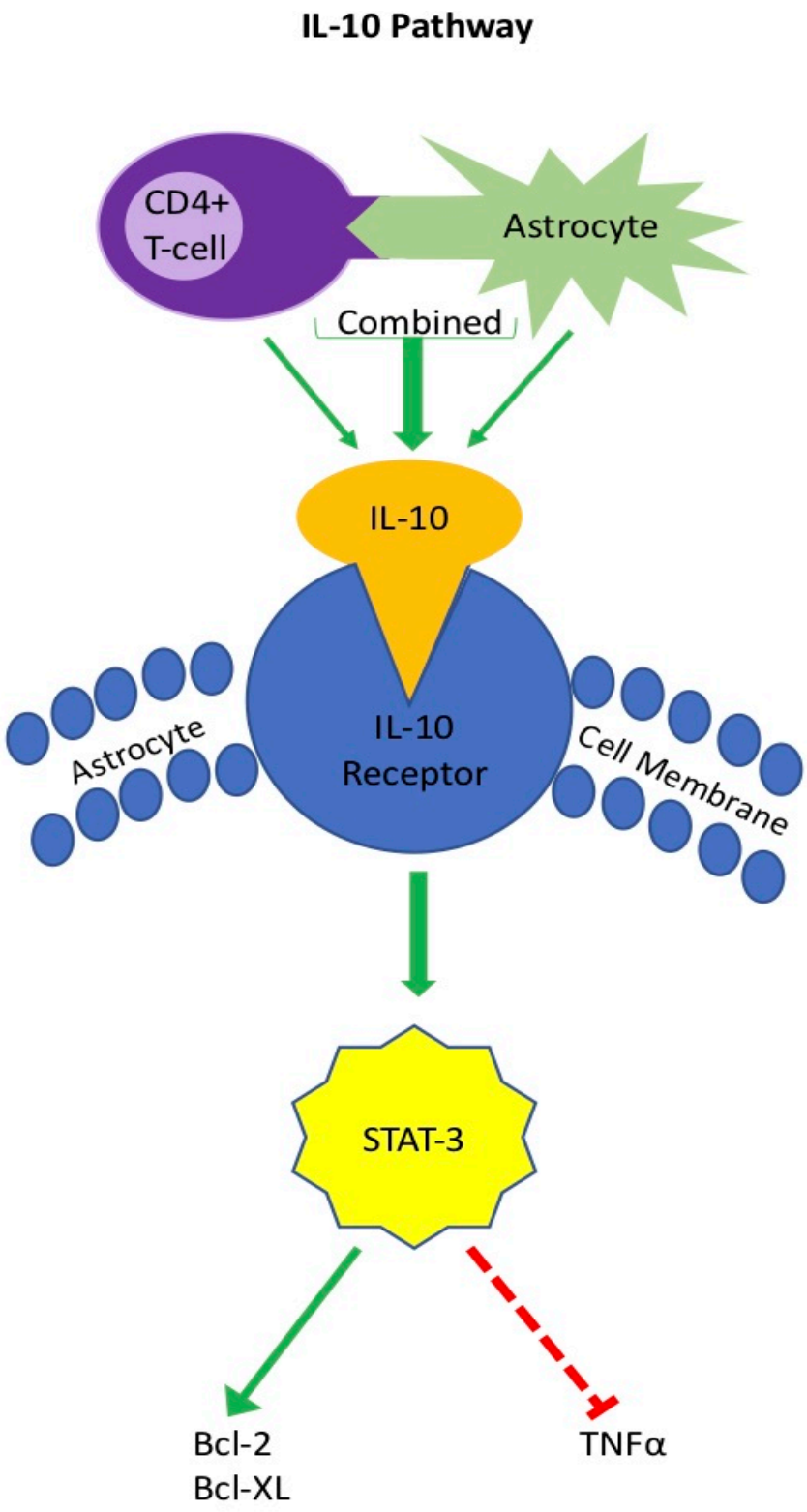
2.1. Abstract

We determined that T-cell astrocyte interaction modulates interleukin-10 (IL-10) production from both cell types. The impact of IL-10 on astrocytes was compared to IL-10 generated from T-cell-astrocyte interactions *in vitro*. We demonstrated that T-cells directly interact with astrocytes to upregulate gene expression and secretion of IL-10, confirmed by elevated STAT3p/STAT3 expression in astrocytes. IL-10 increased astrocytes proliferation. In addition, IL-10 treatment and CD4+ co-culture shifts primary astrocytes toward a more energetic phenotype. These findings indicate that direct interaction of CD4+ T-cells with astrocytes, activated the IL-10 anti-inflammatory pathway, altering astrocyte phenotype, metabolism, and proliferation.

Keywords: Interleukin-10; Astrocytes; CD4+; T-cells; STAT3; neuroinflammation

2.2 Figures

Figure 2.1 Graphical Abstract.



2.3 Introduction

Classically, the brain has been described as an immune privileged organ separated from the peripheral immune system. There is increasing evidence that the brain is not devoid of peripheral immune cells. Immune cells can enter the brain via the lymphatic vessels, interacting with neurons and glial cells [23, 110], playing vital roles in immune surveillance [109, 111] and bi-directional communication between the immune system and central nervous system (CNS) [108, 112-114].

T-cells have been identified as resident cells in the brains of humans and rodents [19, 115]. Previous research from our laboratory has showed that experimental ischemic stroke increased levels of T-cells in the brain, which have close interaction with astrocytes in the peri-infarct region. Furthermore, these T-cells increased gene expression and produced pro- and anti-inflammatory cytokines *in vivo*, including interleukin-10 (IL-10) [10]. Astrocytes which have demonstrated their ability to act as antigen presenting cells in the brain [116], play critical roles in brain homeostasis and contribute to neuroimmune communication [3, 32, 71, 117, 118]. Studies have demonstrated that IL-10 increased when microglia and T-cells were in direct contact. IL-10 levels in T-cells-microglia co-cultures were reduced in a concentration dependent manner when CTLA-4 Fc was added to the culture media [113]. T-cell IL-10 production was modulated when astrocytes and T-cell interacted and induced upregulation of CTLA-4 levels in T-cells after 24 h in co-culture [114]. Therefore, we reasoned that astrocyte T-cell interaction may have an impact on astrocyte phenotype and metabolism.

IL-10 is recognized as an anti-inflammatory cytokine which regulates immune cells in the periphery, suppressing excessive inflammation [119, 120]. Recently IL-10 has been identified in

its ability to balance pro-inflammatory immune response in the brain [120, 121]. It has been previously reported that IL-10 is released from several cell types, including astrocytes and T-cells, when immune challenged as occurs during traumatic brain injury and experimental ischemic stroke [10, 122-124]. Mounting evidence suggests that the role of IL-10 in neurons is to induce an anti-apoptosis response during brain injury [120, 124-126]. However little if any attention has been given to the potential regulatory role of IL-10 on astrocyte proliferation and metabolism. The goal of this investigation was to determine the interaction of T-cells and astrocytes and the involvement of IL-10 in *in vitro* conditions. Here, we provided novel evidence to support that astrocytes interact with T-cells via direct cell-to-cell contact, increasing IL-10 gene expression and production leading to alterations in astrocyte's metabolic phenotype.

2.4. Materials and methods

2.4.1 Astrocyte cell-line and primary astrocyte cultures

The astrocyte cell-line was purchased from American Type Culture Collection (ATCC): C8-S (Astrocyte type II clone) ATCC CRL-2535™ mus musculus. Adult (3–4 months old) C57BL6 mice were purchased from Jackson Laboratory. Breeding pairs of C57BL6 mice were established from which neonatal C57BL6 mice (postnatal day 1–3) were obtained for primary astrocyte cultures. Primary astrocytes were prepared as previously described [127, 128] with the following modifications. Mouse pups were anesthetized by hypothermia and decapitated. Under aseptic conditions the meninges were removed, and the CE cortices were dissected. CE tissue was digested in TrypLE, (Sigma) at 37°C for 15 min. A single homogenous cell suspension was made by repeated gentle pipetting of the digested CE tissue through different sized pipet tips. The cell suspension was strained through a 40 μ size cell strainer and cell counted with a hemocytometer.

Cells were seeded into poly-L-lysine coated 10 cm diameter tissue culture plates (Genesee Scientific) with high glucose Dulbecco's Modified Eagle's Medium, (DMEM with 4500 mg/l Glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, Thermo Scientific) containing Streptomycin (10,000 µg/ml)-Penicillin (10,000 units/ml) and cultured in a TC incubator at 37°C with 5% CO_2 for two weeks. Once the culture plates became 90% confluent, the plates were shaken for 48 h in a CO_2 TC incubator at 37°C to eliminate microglia and other cell contaminants. The primary astrocytes were then transferred into new plates and incubated media as described above for 2 days before the media was removed, the cells were washed twice with sterile PBS, and the PBS was replaced with fresh high glucose DMEM (4500 mg/l Glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, Thermo Scientific) containing 2% of heat inactivated Fetal Bovine serum (FBS) and Streptomycin (10,000 µg/ml)-Penicillin (10,000 units/ml). All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center (UNTHSC).

2.4.2 Magnetic beads and isolation of T-cells

T-cells were sterilely isolated from spleens of 3-4 month-old female C57BL6 mice. Mice were anesthetized using inhaled 2% isoflurane. Spleens were gently strained through 40 µ cell strainers to prepare a homogenized tissue suspension, red blood cell (RBC) were lysed with lysis solution (10x concentration: NH_4Cl 8.02 gm, $NaHCO_3$ 0.84 gm, EDTA 0.37 gm in 100 ml deionized sterile water) to remove RBCs. T-cells were separated from all white blood cells using Dynabeads™ Untouched™ Mouse T-Cells Kit, Dynabeads™ Untouched™ Mouse CD4 Cells Kit, and Dynabeads™ Untouched™ Mouse CD8 Cells Kit per

manufacturer's instructions (Invitrogen). All T-cells added to astrocyte cultures were added at a 1:1 ratio of astrocytes to T-cells.

2.4.3 Co-culture treatments

Primary astrocytes or C8-S astrocytes were co-cultured with either recombinant mouse IL-10 (at 0.0-or 1000 pg/mL, KingFischer) containing high glucose DMEM (4500 mg/l Glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, Thermo Scientific) containing 2% of heat inactivated Fetal Bovine serum (FBS) and Streptomycin (10,000 µg/ml)-Penicillin (10,000 units/ml)) media or T-cells (CD4+, CD8+, or pan T-cells) at a 1:1 ratio. Anti-CTLA-4 antibody (Santa Cruz) was added to T-cell astrocyte co-cultures at 14.8 µg/ml. The recombinant mouse IL-10 was suspended in PBS with 0.1% BSA per manufacturer's instructions. IL-10 neutralizing antibody (R & D Systems) was added to T-cell astrocyte co-cultures at 10 ng/ml. In the cell co-culture insert experiments, primary astrocytes or T-cells were placed on the bottom surface of 24-well plates (Grenier). The second cell type was added to 24-well inserts (Grenier Bio-One Thincert 24 well 0.4 µm) for close proximity incubation of cells.

2.4.4 Cell Collection

T-cells floated in culture and laid gently on astrocytes by 48 h in culture, while astrocytes adhered to the bottom of the wells. Tapping the plate gently caused the T-cells to float off the astrocytes. The supernatant containing T-cells was pipetted gently twice and collected. T-cells were separated from the supernatant by centrifugation. Astrocytes were washed once with PBS and then TrypLE (Gibco) was placed in the wells to release astrocytes from the wells' surfaces.

Astrocytes or T-cells were analyzed via PCR IL-10 gene expression. The supernatant from the cultures were used to quantify IL-10 protein via ELISA.

2.4.5 Microscopy

Astrocytes were seeded at 100,000 cells/well in a 24-well plate (Grenier) and co-cultured with or without T-cells at a 1:1 ratio. Differential Interference Contrast (DIC) images were taken with a Zeiss Axio Observer Z1 microscope.

2.4.6 RNA isolation and RT-PCR

Total RNA was extracted from cells using a RNAeasy mini kit (Qiagen). RNA was reverse transcribed to cDNA using Superscript Vilo Master Mix (Invitrogen) following manufacturer's instructions. Quantitative Real-time (QRT)-PCR was performed using a BioRad CFX96 detection system. In brief, experimental cDNA was amplified by real-time PCR where a target cDNA (IL-10 or TNFα) and a reference cDNA (β-actin) were amplified simultaneously using an oligonucleotide probe with a 5′ fluorescent reporter dye (SYBR-green). Fluorescence was determined on a BioRad CFX Manager Software. Data was analyzed using the comparative threshold cycle (ΔΔCT) method and results are expressed as fold change. Primer sequences were as follows: β-actin Forward (5' to 3') CTGTCGAGTCGCGTCCA and Reverse (5' to 3') ACGATGGAGGGGAATACAGC, IL-10 Forward (5' to 3') AGGCGCTGTCATCGATTTCT and Reverse (5' to 3') ATGGCCTTGTAGACACCTTGG, TNF-α Forward (5' to 3') ATCGGTCCCCAAAGGGATGA and Reverse (5' to 3') ACAGGCTTGTCACTCGAATTTTG.

2.4.7 IL-10 ELISA analysis

Concentration of IL-10 was determined from conditioned media using the ELISA MAX Mouse IL-10 ELISA kit (Biolegend) according to the manufacturer's instructions. In brief, 96-well enzyme immunoassay plates were coated with anti-mouse IL-10 capture antibodies and incubated overnight at 4°C. Samples and IL-10 standards (0–10,000 pg/ml) were added and incubated for 2 h at room temperature [29]. Plates were washed with sterile PBS and incubated with biotinylated anti-mouse IL-10 antibodies. Plates were washed and incubated with streptavidin-horseradish peroxidase conjugate. After 1 h incubation at RT, plates were washed and incubated with tetramethylbenzidine liquid substrate for 15 min. Reactions were terminated with sulfuric acid and absorbance was read at 450 nm using a Tecan Plate Reader F200. The assay was sensitive to 10 pg/ml IL-10.

2.4.8 Astrocyte metabolism assays

Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), ATP production rates, and glycolytic production rates were determined as follows: primary CE astrocytes were seeded in a Seahorse XFe96 plate at a density of 20,000 cells/well and cultured for 2 days in high glucose DMEM (4500 mg/l Glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, Thermo Scientific) containing 2% of heat inactivated Fetal Bovine serum (FBS) and Streptomycin (10,000 µg/ml)-Penicillin (10,000 units/ml) to form a confluent monolayer. At 48 h, the 2% heat inactivated FBS high glucose DMEM media was removed and replaced with IL-10 (0.0, 1000 pg/ml, KingFischer) or CD4+ T-cells in fresh media for another 48 hours. Twenty-four hours prior to the experiment, Seahorse calibrant was added to a sensor cartridge and placed in a non-CO_2 incubator at 37 ºC. On the day of the experiment, the culture media was replaced with Seahorse

XF base media supplemented with 1 mM pyruvate, 2 mM glutamine, and 5.5 mM glucose, and incubated for 1hr in a non-TC CO_2 incubator at 37°C. Assay media was warmed and calibrated to a pH of 7.4 with 0.1 N NaOH. In the accompanying cartridge of the XFe96 plate the following drugs were prepared in Seahorse medium and loaded to conduct the Mito Stress Test assay: oligomycin, carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/antimycin to achieve the final concentrations of 1.5, 2, and 0.5 μM, respectively. In the accompanying cartridge of the XFe96 plate the following drugs were loaded for the ATP rate assay: oligomycin and rotenone/antimycin to achieve a final concentration of 1.5 and 0.5 μM, respectively. The XFe96 plate and cartridge were loaded into Seahorse Bioscience XFe96 Extracellular Flux Analyzer and OCR, ECAR, and ATP production rate, were monitored following the sequential injection of oligomycin, FCCP, and rotenone/antimycin for OCR and ECAR and oligomycin and rotenone/antimycin for ATP production with each cycle set as 3 min mix, 2 min delay, and measure for 3 minutes. Protein concentration of each sample was determined using calcein AM to normalize all data. Wave software by Agilent was used for all calculations.

2.4.9 ATP assay

Astrocytes were cultured for 24 h in a 6 well culture plate at a density 2 X 10^4 cells/well in 2% heat inactivated high glucose DMEM media (Thermo Scientific) at 37°C. Following treatment with IL-10 (0.0, 1000 pg/mL) or T-cells for 48 h. On the day of the experiment control wells were treated with oligomycin for 2 hours. Cells were trypsinized, washed twice with PBS by centrifugation in Eppendorf tubes at 400 g, and lysed with ATP assay buffer (500 mM Tricine buffer, pH 7.8, 100 mM $MgSO_4$, 2 mM EDTA, and 2 mM sodium azide, 1% Triton X-100). ATP reaction buffer (30 μg/ml D-luciferin, 20 μM DTT, and 25 μg/ml luciferase) was added to 10 μl of

cell lysate. Luminescence was measured using a Tecan Infinite F200 plate reader. The ATP values were determined from a standard curve and normalized to protein content for each sample using the Pierce 660 nm Protein Assay (660 nm absorbance).

2.4.10 Protein isolation and Capillary Western Immunoassay

Astrocytes cultured with or without IL-10 or T-cells and with or without neutralizing IL-10 antibodies added to co-cultures were collected after treatments, washed twice with PBS and placed with TrypLE (Gibco) at 37°C for 10 minutes, and collected via centrifugation and resuspended in mammalian protein extraction reagent (MPER, Thermo Fisher Scientific) with protease and phosphatase inhibitors (Sigma). Protein levels were determined using Pierce 660nm Protein Assay (660 nm absorbance). WES analysis was performed using a 12-230 kDa separation module (ProteinSimple, San Jose, CA, USA), an anti-mouse detection module (ProteinSimple) and WES system (ProteinSimple) according to the manufacturer's instructions. In brief, protein samples were diluted 10-fold in sample buffer (10x Sample Buffer from the kit Separation Module), then mixed with Fluorescent Master Mix and heated for 5 min at 95°C. The ladder, samples, antibody diluent, primary and secondary antibodies (in antibody diluent), Streptavidin-HRP, Luminal-Peroxide, and wash buffer were pipetted onto the plate of the separation module. Instrument default settings were used. WES columns were probed with antibodies for phosphorylated signal transducer and activator of transcription 3 (STAT3p) (mouse, Biolegend, San Diego CA) (WES 1:50), signal transducer and activator of transcription 3 (STAT3) (mouse, Biolegend, San Diego CA) (WES 1:50), and β-actin (mouse, Santa Cruz, Dallas TX) (WES 1:200) antibodies. Secondary detection was achieved with anti-mouse-HRP. Chemiluminescence was detected with WES column-based protein detection. The specific peaks for STAT3p, STAT3, and

β-actin were obtained from resulting electropherograms and quantified by measuring the area under the curve using Compass for SW software (Protein Simple). β-actin was used as the control to quantify protein expression and average AUC calculated as a proportion of the control. Densitometry for WES was calculated using Compass for SW software (Protein Simple).

2.4.11 Cell proliferation assay

Astrocyte proliferation was assessed by Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) which determines the number of viable cells directly related to a colorimetric reaction (formation of formazan dye). Briefly, the cells were exposed to varying concentrations of IL-10 (1000, 2000, and 4000 pg/mL) in 96-well plates for 48h, absence of IL-10 served as the control. After treatment, 10 μL of CCK-8 solution was added to each well, and the 96-well plate was incubated in a TC incubator with 5% CO_2 for 2 h at 37°C. The percent of cell proliferation was calculated based on absorbance at an optical density of 450 nm using a microplate reader (TECAN Infinite F200) according to the manufacturer's protocol.

2.4.12 Statistical analysis

Prism V7 (GraphPad Software, LaJolla CA) was used to perform the statistical analysis. Results are expressed as mean ± standard error of mean (SEM). Unless otherwise stated, for the comparison of two groups, an unpaired t-test was used to identify significant differences. When comparing multiple groups, one-way analysis of variance was used and a post-hoc Bonferroni correction was applied to identify significant differences between pairwise comparisons. Values were considered significant at p-values < 0.05.

2.5. Results

2.5.1 T-cells interact directly with C8-S astrocytes to increase IL-10 production

C8-S astrocyte cell-lines were cultured alone for 48 h and after which they were either given fresh media or co-cultured at a 1:1 ratio with pan T-cells in fresh media for an additional 48 h (Supplemental Figure 3.11A and 3.11B). C8-S astrocytes co-cultured with pan T-cells generated an approximately 10-fold increased IL-10 mRNA level compared to C8-S astrocytes alone (Figure 3.2A). The increased IL-10 gene expression was confirmed by the increase of IL-10 protein secreted into the supernatant which peaked at 48 h after co-culture (Figure 3.2B). Co-culturing of CD4+ and CD8+ T-cells with the C8-S astrocytes resulted in an approximate 40- and 3-fold increase in IL-10 gene expression, respectively, suggesting that increased IL-10 expression in C8-S cells after co-culturing with pan T-cells was primarily due to interaction with CD4+ T-cells (Figure 3.2C and 3.2D). When C8-S astrocytes were cultured with T-cells and anti-CTLA-4 antibodies, both C8-S astrocytes and T-cells isolated from the co-cultures show decreased IL-10 expression compared to co-cultured groups without the anti-CTLA-4 antibodies, implying that a direct interaction may occur between these two cell types to increase IL-10 expression (Figure 3.3).

2.5.2 T-cells interact directly with primary astrocytes to increase IL-10 production

We further confirmed the interaction of astrocytes and T-cells in primary astrocyte cultures (Supplemental Figure 3.12). Similar to the C8-S cell-line, the primary astrocytes co-cultured with CD4+ T-cells for 48 h, demonstrated an increased IL-10 expression which was partially blocked with anti-CTLA-4 antibody (Figure 3.4A and 3.4B). Separation of T-cells and astrocytes by cell culture inserts reduced expression of IL-10 from both astrocytes (Figure 3.4C) and CD4+ T-cells (Figure 3.4D).

2.5.3 The effects of IL-10 and CD4+ T-cells on primary astrocytes metabolic phenotype

With the Seahorse XFe96 we determined OCR and ECAR before and after injection of oligomycin, FCCP, and rotenone/antimycin A. We observed a significant increase in basal respiration in primary astrocytes exposed to IL-10 and a non-significant increase in basal respiration in astrocytes exposed to CD4+ T-cells (~ 14% and ~9%). A significant increase in maximal respiration was found in primary astrocytes exposed to IL-10 and CD4+ T-cells (~15 and ~18%, respectively), Increased proton leak (~17%) and ATP production (~16%) was indicated in IL-10 treated astrocytes. Spare respiratory capacity (SRC) was increased (~20%) in primary astrocytes co-cultured with CD4+ T-cells. No significant difference was observed in non-mitochondrial oxygen consumption (non-MOC) among these groups (Figure 3.5A). A significantly higher baseline and stressed ECAR (~19 and ~13% respectively) was observed in primary astrocytes cultured with CD4+ T-cells compared to primary astrocytes culture alone (Figure 3.5B). Assessment of cell energy phenotype indicated a more energetic phenotype for all groups but with more glycolytic and aerobic activity for the primary astrocytes treated with IL-10 or co-cultured with CD4+ T-cells as compared to primary astrocytes alone. (n=4-5 for all groups in Figure 3.5).

2.5.4 Effects of IL-10 and CD4+ T-cells on ATP production and metabolism in primary astrocytes

We observed increased ATP contents in primary astrocytes correlating with IL-10 dose-dependent treatment. (Figure 5A, n=4 per group). Increased ATP content was also observed in primary astrocytes co-cultured with CD4+ T-cells (Figure 5B, n=11-12 per group). Interestingly, neither treatment of IL-10 nor co-culture with CD4+ T-cells had a significant impact on basal

glycolytic, basal mitochondrial, and total ATP production rates in primary astrocytes (Figure 3.6C, n=4). Assessment of cell energy phenotype indicated a more energetic phenotype with more glycolytic and mitochondrial phosphorylation activity for primary astrocytes treated with IL-10 or CD4+ T-cells compared to primary astrocytes alone (Figure 3.6D, n=4-5).

2.5.5 Primary astrocytes treated with IL-10 or CD4+ T-cells increased the STAT3p and STAT3 signaling

Using WES (Protein Simple) we determined that there was a significant increase of STAT3p in astrocytes treated with IL-10 or co-cultured with CD4+ T-cells, which was blocked by neutralizing IL-10 antibodies (Figure 6A, n=4-5 per group and Figure 6B, n=4-8 per group). A significant increase of total STAT3 was also observed in primary astrocytes when exposed to IL-10 (1000 pg/mL) or co-cultured with CD4+ T-cells (Figure 3.8, IL-10 treated astrocytes, n=4; and Figure 8, CD4+ treated astrocytes, n=3). With the proliferation assay (CCK-8) we observed that primary astrocytes exposed to increasing concentrations of IL-10 (1000, 2000, and 4000 pg/ml) had increased cell proliferation (120%, 174%, and 214%) respectively when compared to control (no IL-10 present) (Figure 3.8D, n=7 for all groups).

2.5.6 T-cells interact with C8-S astrocytes to decrease TNFα production

C8-S astrocyte cell-lines were cultured alone for 48 h, after which they were either given fresh media or co-cultured at a 1:1 ratio with pan T-cells in fresh media for an additional 48 h. C8-S astrocytes co-cultured with pan T-cells generated a greater than 75-fold reduction in TNFα mRNA gene expression levels compared to C8-S astrocytes alone (Figure 3.10A). The decreased TNFα gene expression was confirmed by the decreased TNFα in the supernatant (Figure 3.10B).

2.6. Discussion

Our investigation demonstrates that the IL-10 pathway is implicated in the interaction between T-cells and astrocytes and that this anti-inflammatory pathway plays an important role in directing astrocyte metabolism and proliferation. These results strongly support our previous research which reported that there were increased levels of IL-10 gene expression from T-cells harvested from post-ischemic mouse brain hemispheres compared to the contralateral non-stroked hemispheres [10] and corroborates with similar findings that other cells in the brain, most notably microglia, can also interact with T-cells via direct cell contact to trigger the IL-10 pathway. Our initial data measured IL-10 production from C8-S astrocytes at 24, 48, and 72 h. Since the highest level of IL-10 production from C8-S astrocytes was at 48 h, the consecutive experiments were conducted at the 48 h time point. Other timepoints did not produce as robust of a response. It is possible that since IL-10 production increases to an apogee at 48 h, but is reduced at 72 h, that there is some form of recycling, an undiscovered mechanism of depression, or a feedback inhibition loop.

We demonstrated that IL-10 signaling is at least partially activated by direct T-cell and astrocyte interaction since blocking the cell-to-cell interaction with anti-CTLA-4 antibodies blunted IL-10 expression from both astrocytes and T-cells. Our data indicated that upregulated IL-10 expression in T-cells was blocked to control levels in the presence of anti-CTLA-4 antibody, while expression of IL-10 in astrocytes was blunted by about 50%, suggesting T-cells may induce IL-10 production in astrocytes via mechanisms independent of direct T-cell and astrocyte interaction. Separation of T-cells from astrocytes by cell inserts in co-cultures eliminated the IL-10 gene expression seen in co-cultures where cell-to-cell contact was permitted. Similarly, IL-10

is unlikely the only factor involved in the interaction between astrocytes and T-cells modulating the final outcome of astrocytes. As anticipated, there were differences of ATP contents in primary astrocytes between the IL-10 treated astrocytes and the CD4+ co-cultured astrocytes. IL-10 treatment increased ATP content in primary astrocytes in a dose dependent manner which was not replicated in primary astrocytes co-cultured with CD4+ T-cells. We reasoned that there may be additional mechanisms affecting IL-10 production and utilization when T-cells are present which may impact ATP production. Furthermore, according to our data the T-cell treatment group produced approximately 600 pg/ml of IL-10 when at a 1:1 ratio with astrocytes instead of 1000 pg/ml of IL-10 for astrocytes treatment at 1000 pg/ml. The ratio of T-cell to astrocyte interaction may vary from our experimental 1:1 ratio. This ratio was chosen based on previous data, which showed that T-cell to astrocytes at a 1:1 ratio provided neuroprotection and increased astrocyte survival [82].

Both MHC I and II are expressed on astrocytes in human and rodent brains as well as on primary astrocytes [116, 129-132]. A previous study has shown that B7 expression on astrocytes correlates with T-cell activation and cytokine production *in vitro* [133]. CTLA-4 expression has been confirmed on activated T-cells [113] and astrocytes can upregulate CTLA-4 in T-cells [114]. Furthermore, it has been demonstrated that T-cells interact with microglia to induce IL-10 production through a contact-dependent mechanism [113]. Thus, we expected that T-cells may interact with astrocytes via a similar contact-dependent mechanism. Indeed, we observed that the increase of IL-10 expression in astrocytes when co-cultured with T-cells was attenuated by anti-CTLA-4 antibodies and was totally blocked by cell culture inserts. Nonetheless, modulation of astrocyte phenotype by T-cells may not require continuous cell-to-cell contact. The production of

IL-10 from astrocytes and T-cells may alter the function of other astrocytes even without direct contact via an autocrine and/or paracrine manner.

The current study was not focused on the mechanism by which IL-10 is produced, but rather what does this elevated IL-10 do to astrocytes *in vitro.* Thus, we did not investigate CTLA-4 receptor levels on astrocytes cultured nor the percentage of cells with these receptors. Previous studies have shown that anti-CTLA-4 antibodies decreased IL-10 content in microglial T-cell cultures. Following this logic, we used anti-CTLA-4 antibodies to inhibit T-cell interaction and to assess whether it played any role in IL-10 expression. While our study showed that CTLA-4 antibodies inhibit IL-10 production, this does not mean that the cells are interacting via the CTLA-4 receptor. CTLA-4 is upregulated in T-cells in a cell contact independent manner, therefore it is possible that cell contact increases IL-10 expression and CTLA-4 receptor blockade inhibits IL-10 expression via another route [114]. Additionally, anti-CTLA-4 antibodies may have only blunted IL-10 expression by 50% because it is dose dependent or perhaps because it is one of several receptors that together reduces IL-10 levels [113]. Further research should be conducted to elucidate the exact mechanism of astrocyte T-cell interaction and receptor versus soluble factor involvement.

It is possible that IL-10 levels may fluctuate depending on the severity of the insult and at various timepoints following trauma for damage control during and after neuroinflammation. Since our previous data showed that T-cells closely interact with astrocytes in the peri-infarct region after ischemic stroke [10], it is plausible that the influx of T-cells through the BBB is playing a reparative role in ischemic stroke via IL-10 release. In addition, since we removed all other cells and factors from our *in vitro* experimentation we voided or reduced microglia, cytokines,

metabolites, and neurotransmitters that may have had a role in changing astrocytic phenotype *in vivo*. It is possible that other factors and cells or their combined effect may contribute to neuroinflammatory damage or other repair mechanisms and should be explored in future research.

There are significant differences in OCR and ECAR between IL-10 treated and CD4+ T-cell co-cultured astrocytes and between treatment groups and control. Nevertheless, our Seahorse analysis overall indicated that IL-10 treated and CD4+ T-cells co-cultured with astrocytes have a similar trend in their energetic phenotype with increased aerobic and glycolytic metabolism. Furthermore, proton leak, spare respiratory capacity (SRC), non-mitochondrial oxygen consumption (Non-MOC), and ATP production coupled to O_2 consumption are not significantly changed for both treatment groups compared to the control.

Lastly, we investigated the activation of STAT3p and STAT3, downstream signals of the IL-10 pathway [134] which inhibits TNFα and activates Bcl-2, and Bcl-XL. Our study showed that TNFα expression was downregulated in the C8-S astrocytes when exposed to pan T-cells for 48 h compared to controls. This correlates well with the increased STAT3p and STAT3 expression demonstrated when the astrocytes were co-cultured with IL-10 or CD4+ T-cells. To further confirm the effects of IL-10 activation in astrocytes we measured astrocyte proliferation subjected to increasing concentrations of IL-10 because STAT3 activates the anti-apoptotic markers which promote cellular proliferation. The CCK-8 proliferation test was the preferred method used to determine if IL-10 was causing proliferation because the kit allowed for measurement without removal of media from the well plates. For this reason, only the IL-10 treatment group was analyzed because T-cells in culture and loss of cells by washing may have altered results.

In summary, our studies indicated that CD4+ T-cell interaction with astrocytes, increased IL-10 expression from both cell types and activated the STAT3p/STAT3 pathway which resulted in a decreased expression of TNFα. This interaction also enhanced astrocyte glycolysis, increased extracellular acidification and oxygen consumption rates, and shifted astrocytes to a more energetic phenotype. Altogether, these findings give insight into the roles CD4+ T-cells and IL-10 play in changes to astrocyte phenotype and metabolism and may have implications for brain injury.

Acknowledgements

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2.7 Figures and legends

Figure 2.2

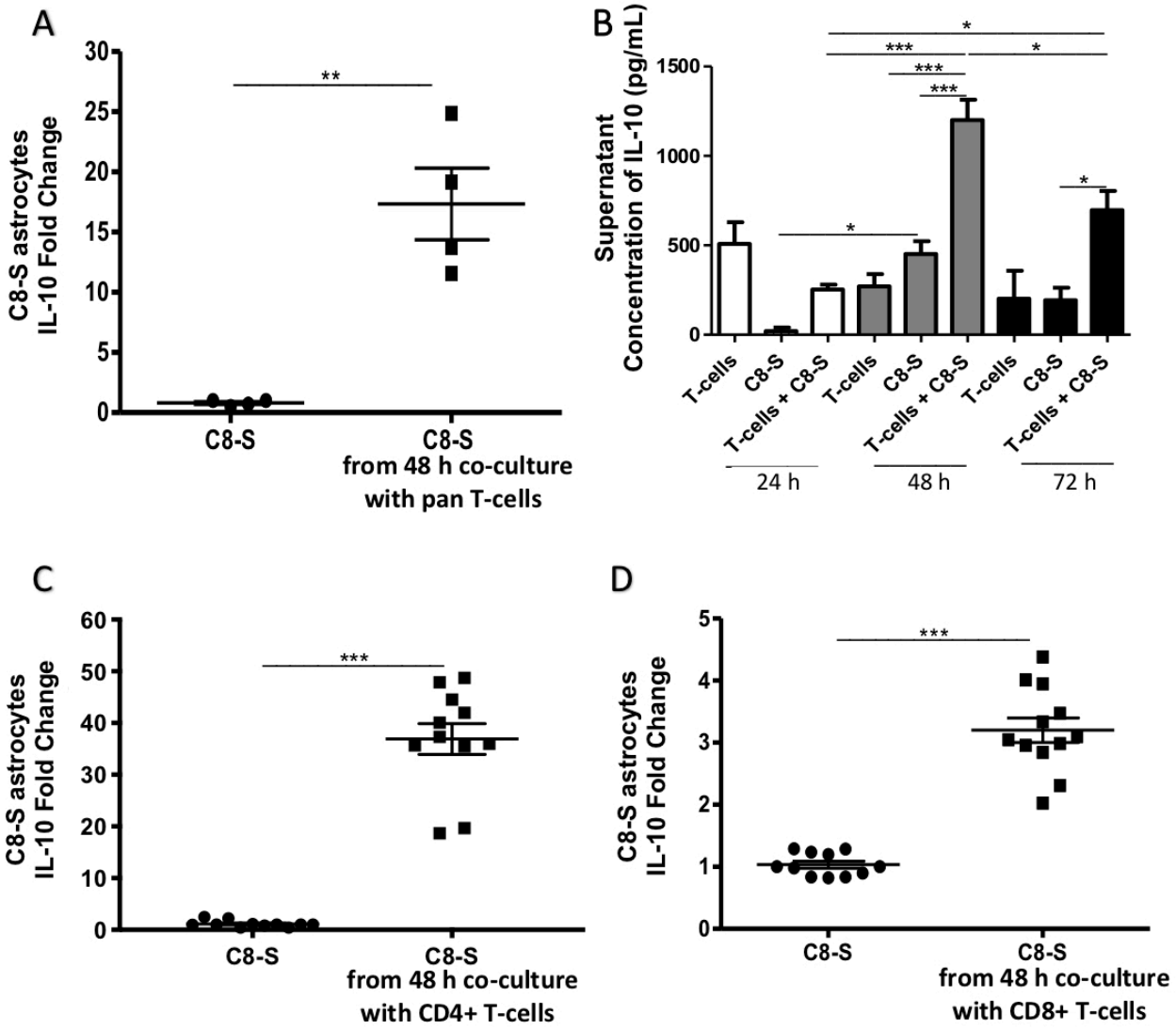
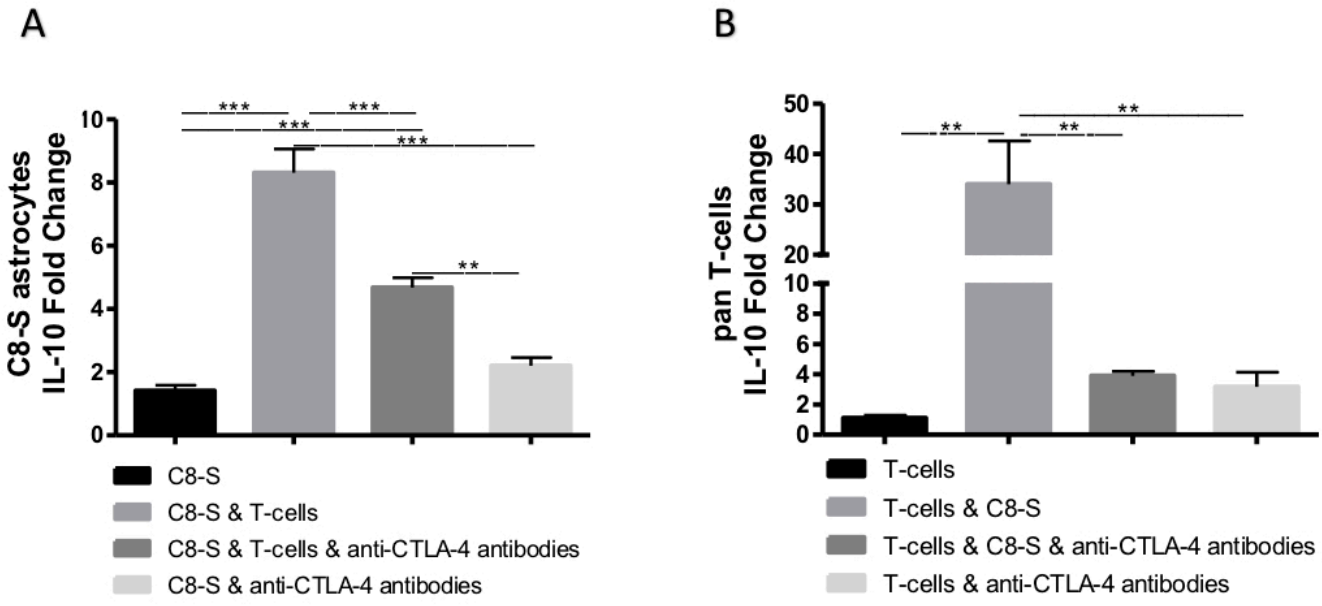


Figure 2.2 T**-cell C8-S astrocyte co-cultured 1:1 increased IL-10 Expression at 48 h.** (A) Real-time PCR analysis of IL-10 expression demonstrated an increase of IL-10 expression in C8-S astrocytes co-cultured with pan T-cells for 48 h (**$p<0.01$, n=4). (B) ELISA assay demonstrated higher levels of IL-10 protein in the supernatant of C8-S astrocyte and pan T-cell co-cultures than control cultures at 24, 48, and 72 h after culture (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n=4-10). (C) Real-time PCR analysis demonstrated increased IL-10 expression in C8-S astrocytes co-cultured with CD4+ T-cells for 48 h (***$p<0.001$, n=11). (D) Real-time PCR analysis demonstrated increase of IL-10 expression in C8-S astrocytes co-cultured with CD8+ T-cells for 48 h (***$p<0.001$, n=11-12).

Figure 2.3


A
C8-S astrocytes
IL-10 Fold Change
10
8
6
4
2
0
***
***
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C8-S
C8-S & T-cells
C8-S & T-cells & anti-CTLA-4 antibodies
C8-S & anti-CTLA-4 antibodies
B
pan T-cells
IL-10 Fold Change
50
40
30
20
10
8
6
4
2
0
**
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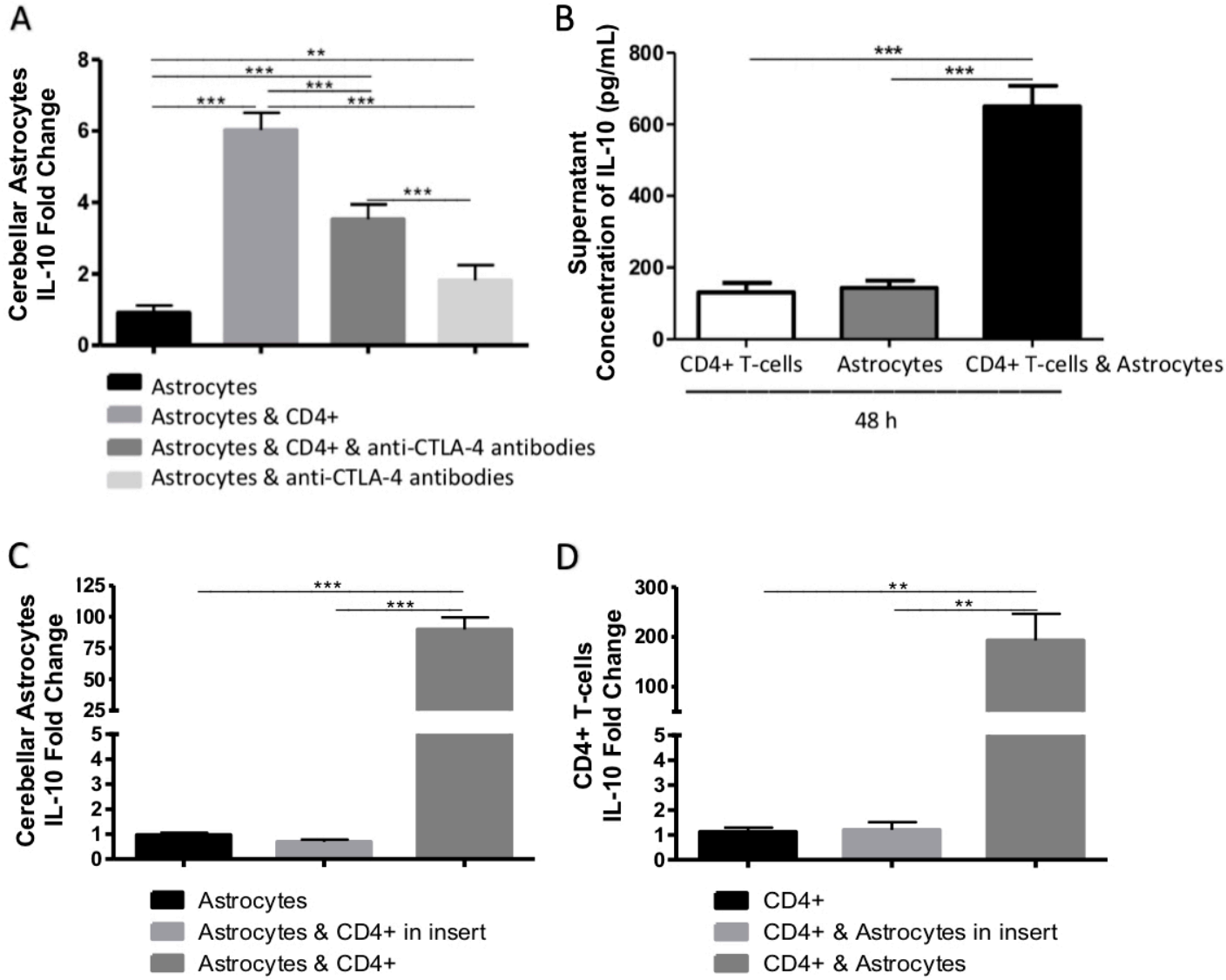
**
T-cells
T-cells & C8-S
T-cells & C8-S & anti-CTLA-4 antibodies
T-cells & anti-CTLA-4 antibodies

Figure 2.3 Direct interaction of C8-S astrocytes with pan T-cells induced up-regulation of IL-10 expression in both C8-S astrocytes and pan T-cells. (A) Real-time PCR demonstrated increased IL-10 expression in C8-S astrocytes co-cultured with pan T-cells which was blunted when anti-CTLA-4 antibodies were in the co-culture (***$p<0.001$, n=11-12). (B) Real-time PCR demonstrated increased IL-10 expression in pan T-cells when co-cultured C8-S astrocytes and was blunted when anti-CTLA-4 antibodies were in the co-culture (**$p<0.01$, n=5-8).

Figure 2.4


A
Cerebellar Astrocytes IL-10 Fold Change
8
6
4
2
0
**
***
***
***
***
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Astrocytes
Astrocytes & CD4+
Astrocytes & CD4+ & anti-CTLA-4 antibodies
Astrocytes & anti-CTLA-4 antibodies
B
Supernatant Concentration of IL-10 (pg/mL)
800
600
400
200
0
***
***
CD4+ T-cells
Astrocytes
CD4+ T-cells & Astrocytes
48 h
C
Cerebellar Astrocytes IL-10 Fold Change
125
100
75
50
25
5
4
3
2
1
0
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Astrocytes
Astrocytes & CD4+ in insert
Astrocytes & CD4+
D
CD4+ T-cells IL-10 Fold Change
300
200
100
5
4
3
2
1
0
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CD4+
CD4+ & Astrocytes in insert
CD4+ & Astrocytes

Figure 2.4 IL-10 generation from primary astrocytes CD4+ T-cell interaction. Interaction of primary astrocytes with CD4+ T-cells at 1:1 at 48 h causes up-regulation of IL-10 gene expression by primary astrocytes and increased IL-10 protein in supernatant of co-cultures. (A) Real-time PCR analysis of IL-10 expression in astrocytes increases when astrocytes are co-cultured with CD4+ T-cells (***$p<0.001$, n=6) and this gene expression is blunted when anti-CTLA-4 antibodies are in the co-culture (***$p<0.001$, n=6) versus astrocytes alone (***$p<0.001$, n=6) or with astrocytes with antibody only (**$p<0.01$, n=6). (B) ELISA assay determined higher levels of IL-10 protein detected in the supernatant of astrocytes in co-culture with CD4+ T-cells versus CD4+ T-cells alone or CE astrocytes alone co-cultures at 48 h (***$p<0.001$, n=6). Interaction of primary astrocytes with CD4+ T-cells at 1:1 at 48 h separated by cell culture inserts does not increase up-regulation of IL-10 gene expression by primary astrocytes or CD4+ T-cells. Real-time PCR analysis of IL-10 expression in astrocytes (C) and CD4+ T-cells (D) does not show a significant increase when astrocytes are co-cultured with CD4+ T-cells separated by cell culture inserts preventing cell contact between the different cell types but does show increased IL-10 gene expression in astrocytes and T-cells harvested from co-cultures with cell-to-cell contact (***$p<0.001$, **$p< 0.01$, n=5).

Figure 2.5

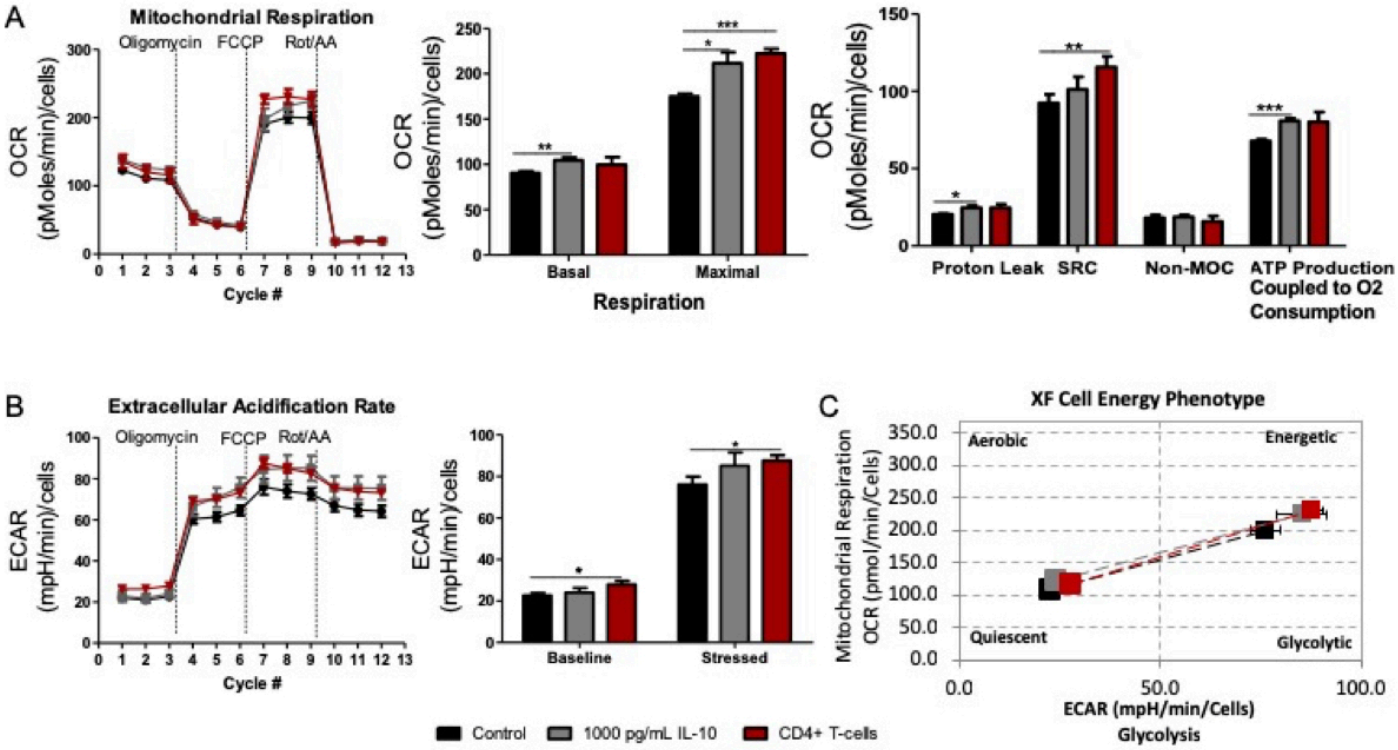
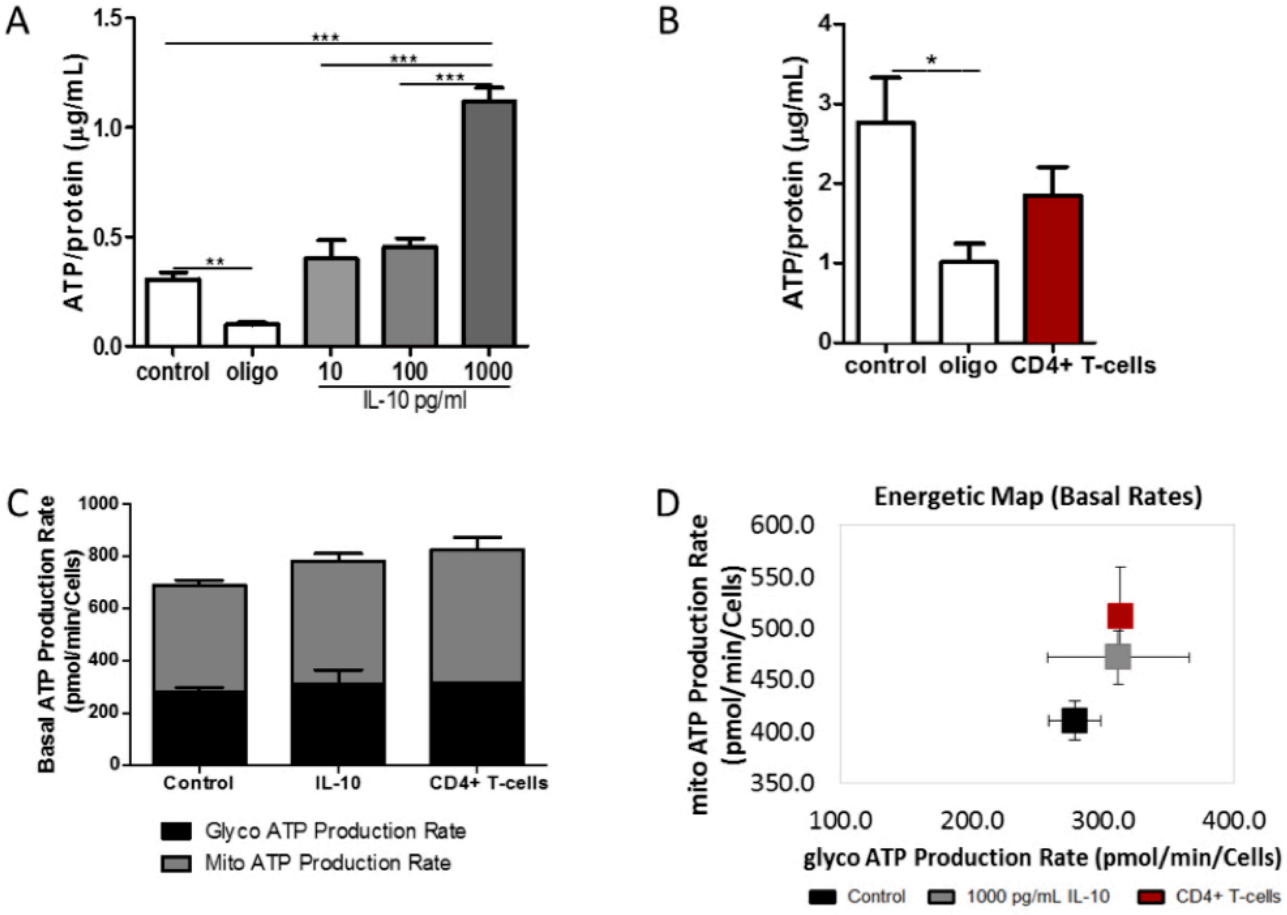

A
Mitochondrial Respiration
Oligomycin
FCCP
Rot/AA
OCR (pMoles/min)/cells)
Cycle #
Basal
Maximal
Respiration
Proton Leak
SRC
Non-MOC
ATP Production Coupled to O2 Consumption
B
Extracellular Acidification Rate
ECAR (mpH/min)/cells
Baseline
Stressed
C
XF Cell Energy Phenotype
Aerobic
Energetic
Quiescent
Glycolytic
Mitochondrial Respiration OCR (pmol/min/Cells)
ECAR (mpH/min/Cells)
Glycolysis
Control
1000 pg/mL IL-10
CD4+ T-cells

Figure 2.5 Modification of primary astrocytes metabolic parameters. Primary astrocytes co-cultured with IL-10 or CD4+ T-cells enhanced glycolysis and increased extracellular acidification and oxygen consumption rate**.** (A) Seahorse extracellular flux analysis of oxygen consumption rate (OCR); bar graphs indicate basal and maximal respiration, proton leak, spare respiratory capacity (SRC), non-mitochondrial oxygen consumption (non-MOC), and ATP production coupled to O_2 consumption. CE astrocytes exposed to IL-10 had higher basal and maximal OCR than CE astrocytes alone (*$p<0.05$, **$p< 0.01$, n=4-5). Primary astrocytes exposed to CD4+ T-cells had higher maximal OCR than astrocytes alone (***$p< 0.001$, n=4-5). (B) Extracellular acidification rate (ECAR); bar graph indicates increased baseline and stressed ECAR in primary astrocytes co-cultured with CD4+ T-cells (*$p<0.05$, n=4-5). (C) Cell metabolic potential of astrocytes alone and cultured with IL-10 or CD4+ T-cells.

Figure 2.6


A
ATP/protein (µg/mL)
1.5
1.0
0.5
0.0
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control
oligo
10
100
1000
IL-10 pg/ml
B
ATP/protein (µg/mL)
4
3
2
1
0
*
control
oligo
CD4+ T-cells
C
Basal ATP Production Rate (pmol/min/Cells)
1000
800
600
400
200
0
Control
IL-10
CD4+ T-cells
Glyco ATP Production Rate
Mito ATP Production Rate
D
Energetic Map (Basal Rates)
mito ATP Production Rate (pmol/min/Cells)
600.0
550.0
500.0
450.0
400.0
350.0
100.0
200.0
300.0
400.0
glyco ATP Production Rate (pmol/min/Cells)
Control
1000 pg/mL IL-10
CD4+ T-cells

Figure 2.6 ATP content and production Rates from primary astrocytes modified by IL-10 or CD4+ T-cells. Primary astrocytes co-cultured with different concentrations of IL-10 or CD4+ T-cells increased total ATP content when (A) astrocytes are co-cultured with 1000 pg/ml of IL-10 (***$p<0.001$, n=4), but (B) not when astrocytes are treated with CD+ T-cells (*$p<0.05$, n=11-12). Basal ATP production rates (C) are not significantly different between groups although cell energy (d) demonstrates a similar increase in ATP production in primary astrocytes treated with IL-10 and CD4+ T-cells.

Figure 2.7

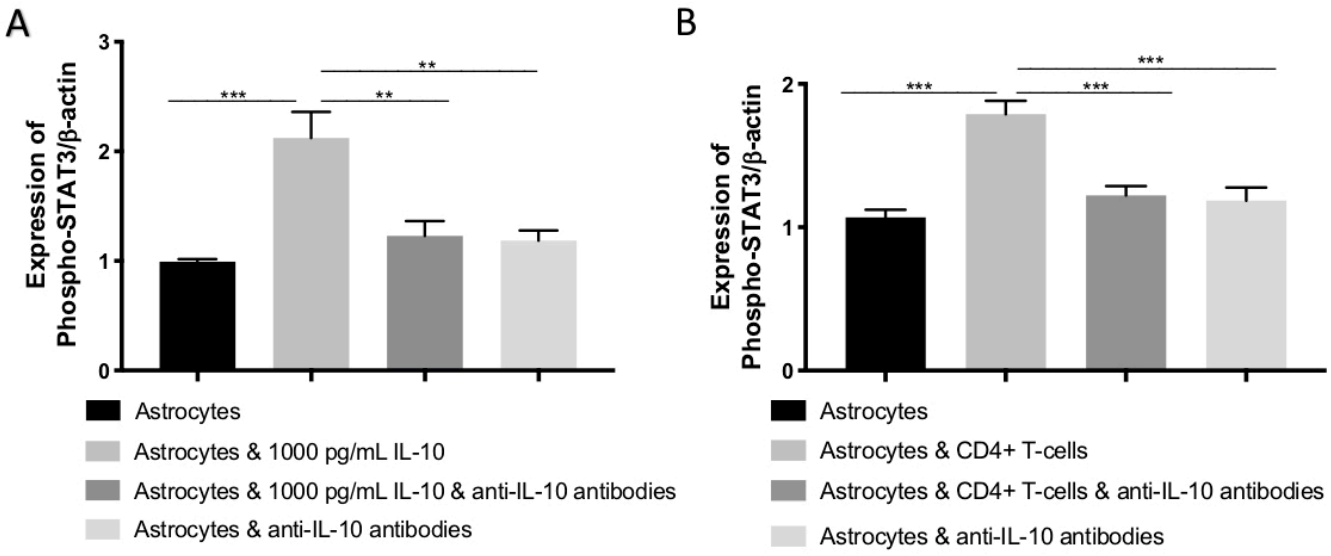
A
Expression of Phospho-STAT3/β-actin
3
2
1
0
***
**
**
Astrocytes
Astrocytes & 1000 pg/mL IL-10
Astrocytes & 1000 pg/mL IL-10 & anti-IL-10 antibodies
Astrocytes & anti-IL-10 antibodies
B
Expression of Phospho-STAT3/β-actin
2
1
0
***
***
***
Astrocytes
Astrocytes & CD4+ T-cells
Astrocytes & CD4+ T-cells & anti-IL-10 antibodies
Astrocytes & anti-IL-10 antibodies

Figure 2.7 Primary astrocytes treated with IL-10 or co-cultured with CD4+ T-cells increased STAT3p. WES analysis of STAT3p expression in primary astrocytes increases when astrocytes are (A) treated with IL-10 (1000 pg/mL) (**p<0.01, ***p<0.001, n=4-5) or (B) co-cultured with CD4+ T-cells (**p<0.01, ***p<0.001, n=4-8). Cultures containing IL-10 neutralizing antibody were not significantly different from astrocytes alone. The protein expression of STAT3p was determined by AUC measurements generated by Compass Software (Protein Simple) and □-actin used as a control.

Figure 2.8

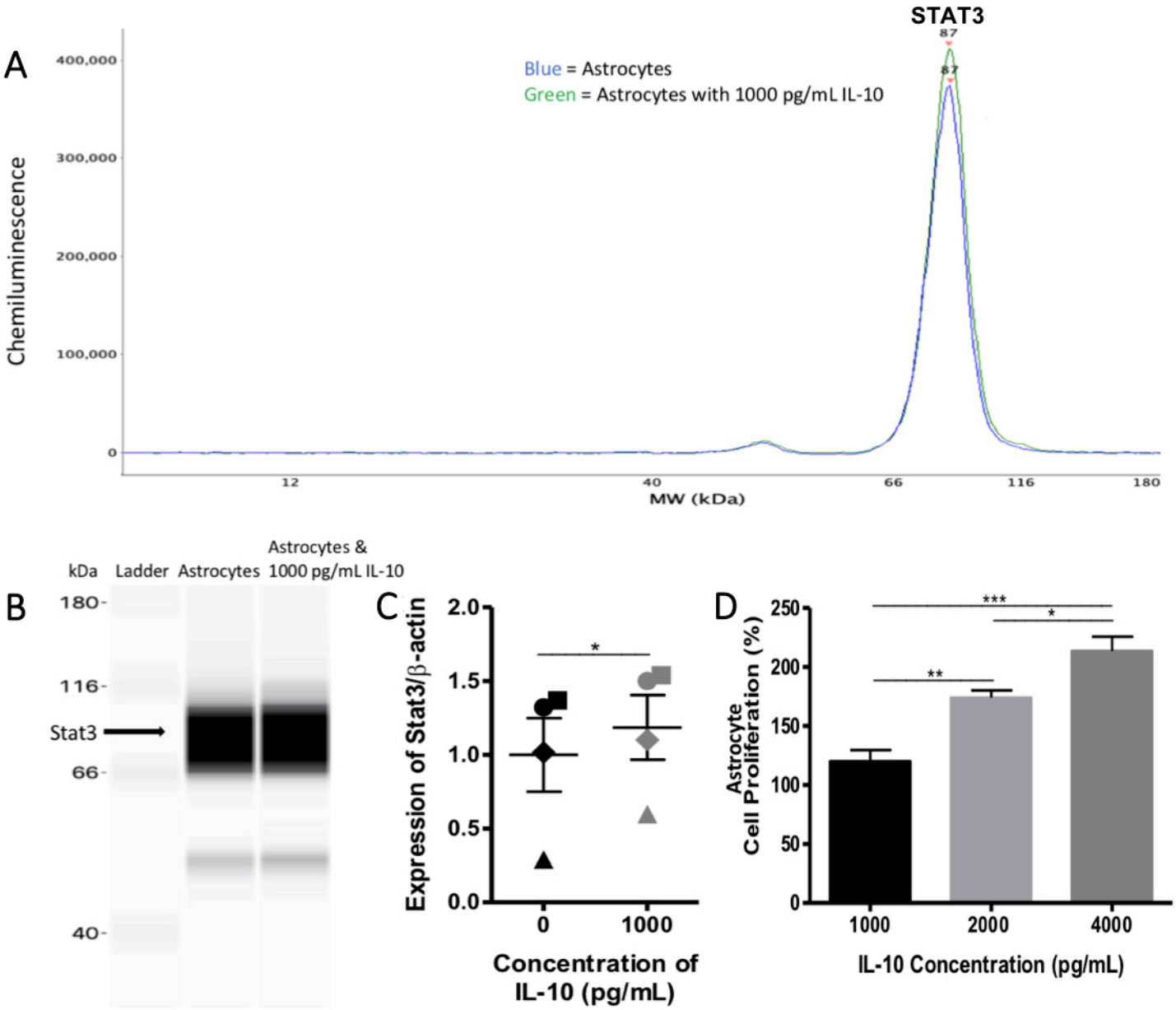
A
STAT3
87
87
Blue = Astrocytes
Green = Astrocytes with 1000 pg/mL IL-10
Chemiluminescence
400,000
300,000
200,000
100,000
0
12
40
66
116
180
MW (kDa)
B
kDa
Ladder
Astrocytes
Astrocytes & 1000 pg/mL IL-10
180-
116-
Stat3
66-
40-
C
Expression of Stat3/β-actin
2.0
1.5
1.0
0.5
0.0
*
0
1000
Concentration of IL-10 (pg/mL)
D
Astrocyte Cell Proliferation (%)
250
200
150
100
50
0
***
*
**
1000
2000
4000
IL-10 Concentration (pg/mL)

Figure 2.8 IL-10 treated primary astrocytes increased STAT3 and proliferation. WES analysis of STAT3 protein production in primary astrocytes increases when primary astrocytes are cultured with IL-10 (1000 pg/mL) (A) electropherogram view, (B) lane view. The protein expression (C) of STAT3 is upregulated in astrocytes when cultured with IL-10 (1000 pg/mL) (*$p<0.05$, n=3-4) determined by AUC measurements generated by Compass Software (Protein Simple). A paired *t*-test was used to determine significance. Pairs are represented by different shapes. The cell proliferation assay (CCK-8) (D) showed that an increased concentration of IL-10 correlated to increased proliferation (%) of astrocytes (*$p<0.05$, **$p<0.01$, ***$p<0.001$, n=7).

Figure 2.9

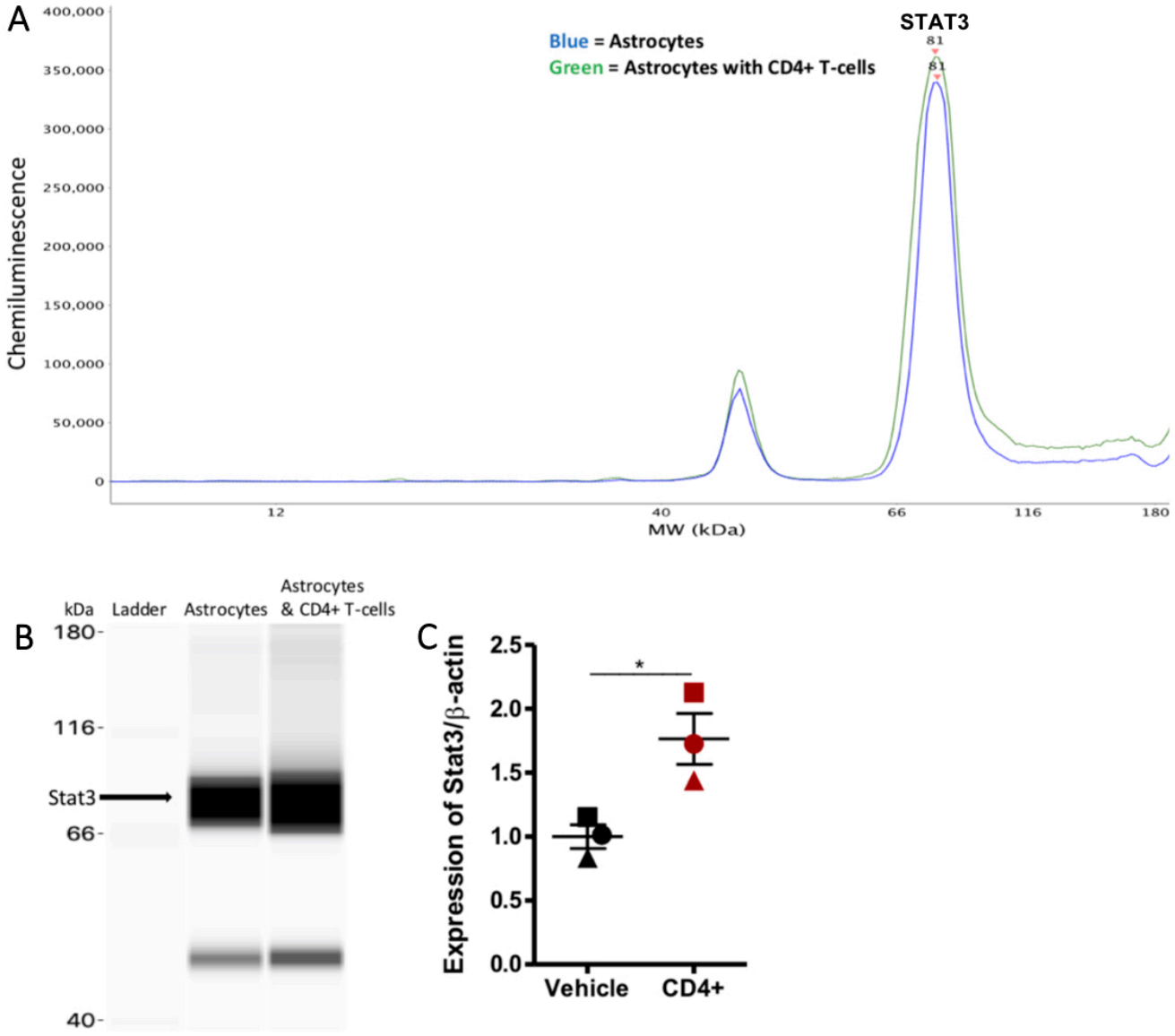
A
Blue = Astrocytes
Green = Astrocytes with CD4+ T-cells
STAT3
81
81
Chemiluminescence
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
12
40
66
116
180
MW (kDa)
B
kDa
Ladder
Astrocytes
Astrocytes & CD4+ T-cells
180
116
Stat3
66
40
C
Expression of Stat3/β-actin
2.5
2.0
1.5
1.0
0.5
0.0
*
Vehicle
CD4+

Figure 2.9 Primary astrocytes co-cultured with CD4+ T-cells increased STAT3. WES analysis of STAT3 protein expression in primary astrocytes increases when astrocytes are co-cultured with CD4+ T-cells (A) electropherogram view, (B) lane view. The protein expression (C) of STAT3 is upregulated in astrocytes when co-cultured with CD4+ T-cells (*$p<0.05$, n=3-4) determined by AUC measurements generated by Compass Software (Protein Simple). A paired *t*-test was used to determine significance. Pairs are represented by different shapes.

Figure 2.10

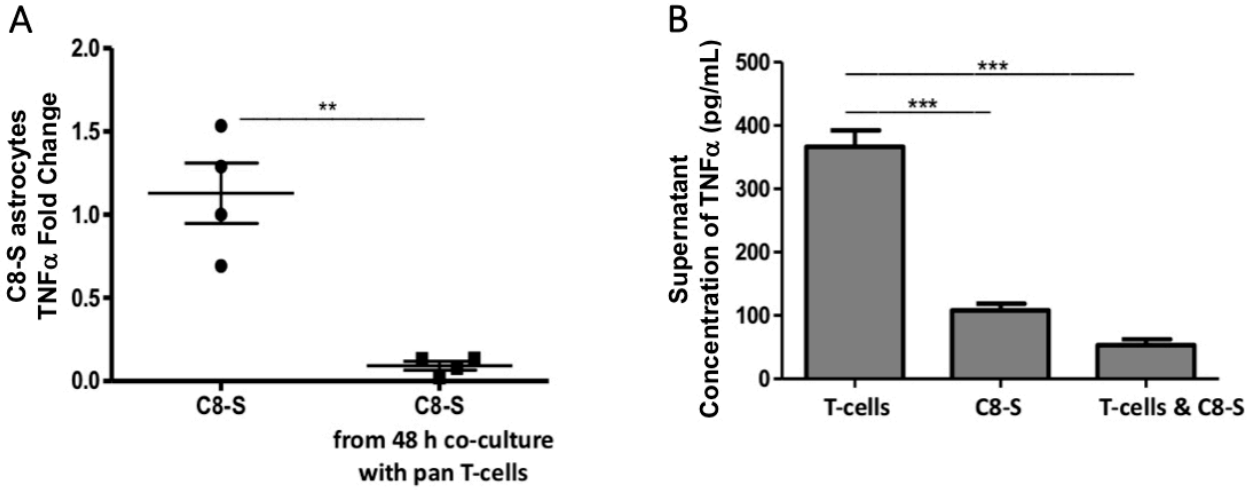
A
C8-S astrocytes TNFα Fold Change
2.0
1.5
1.0
0.5
0.0
**
C8-S
C8-S from 48 h co-culture with pan T-cells
B
Supernatant Concentration of TNFα (pg/mL)
500
400
300
200
100
0
***
***
T-cells
C8-S
T-cells & C8-S

Figure 2.10 T-cell C8-S Astrocytes co-cultured 1:1 Decreased TNFα Expression at 48 h. Real-time PCR analysis of TNFα gene expression in C8-S astrocytes (A) decreases when C8-S astrocytes are co-cultured with pan T-cells for 48 h (**$p<0.01$, n=4). (B) ELISA assay determined lower levels of TNFα protein detected in the supernatant of C8-S astrocyte pan T-cell co-cultures at 48 h (***$p<0.001$, n=3).

Supplemental Data:

Figure 2.11

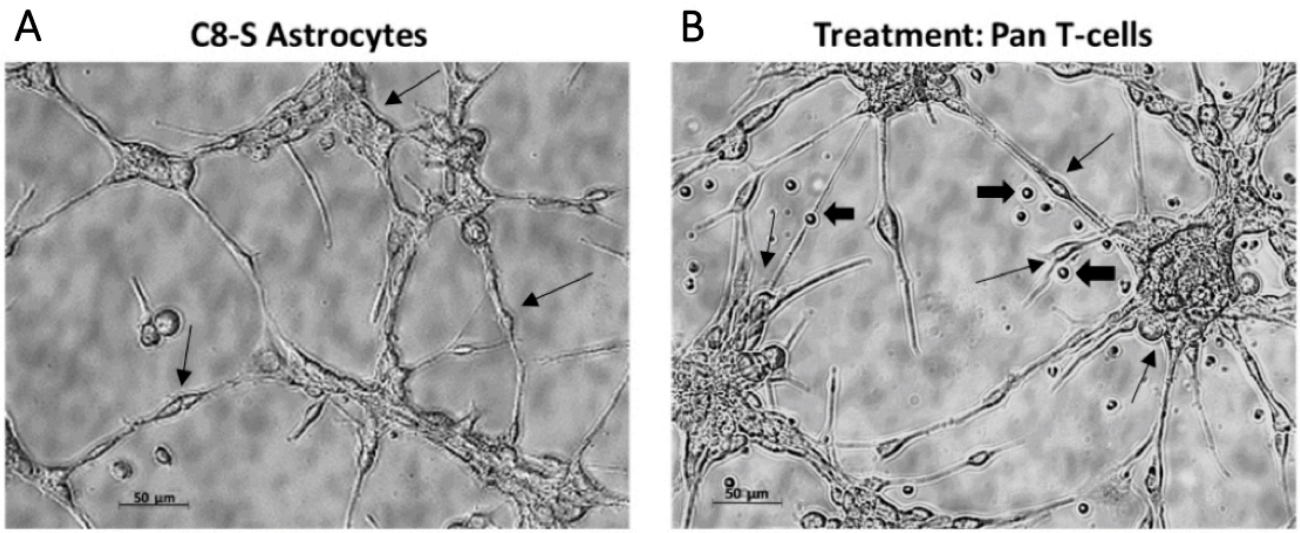

Figure 2.11 Representative **DIC images of C8-S astrocytes and C8-S Astrocytes with pan T-cells.** (A) C8-S astrocytes (←) alone at 48 h versus (B) C8-S (←) co-cultured with pan T-cells (⬅).

Figure 2.12

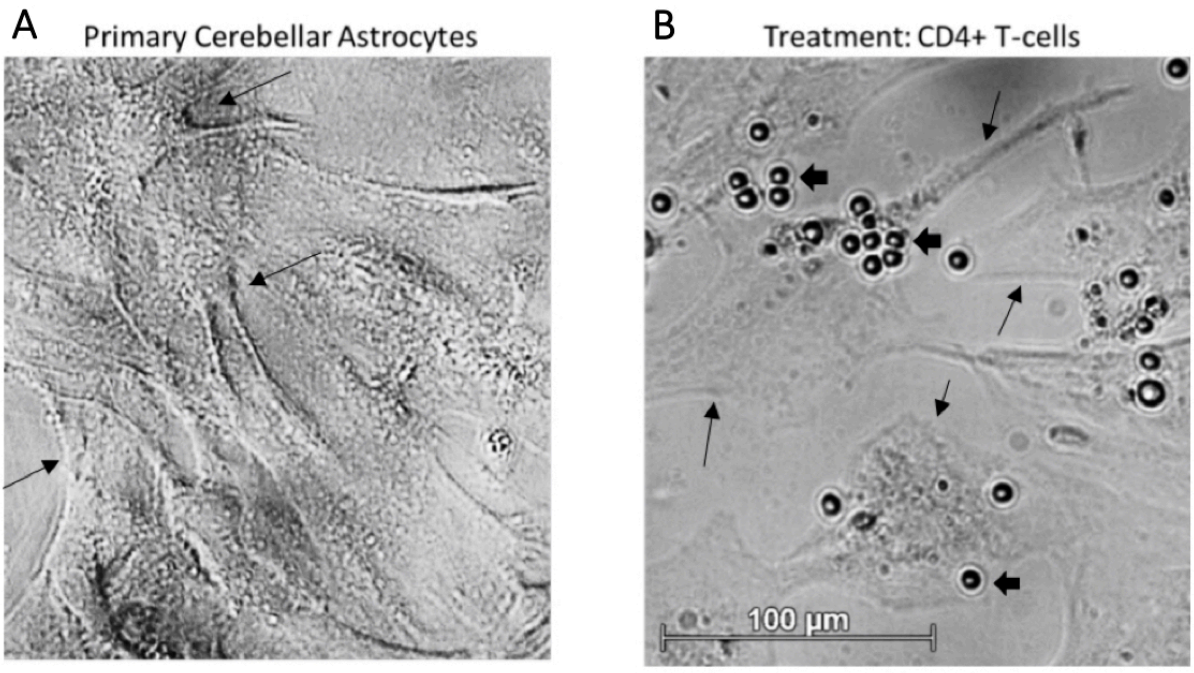
A
Primary Cerebellar Astrocytes
B
Treatment: CD4+ T-cells
100 µm

Figure 2.12. Representative DIC images of primary astrocytes and primary astrocytes with pan T-cells. (A) primary astrocytes (↙) alone at 48 h versus (B) pan T-cell (↙) to primary astrocytes (⬅) interaction.

CHAPTER 3

OVERALL DISCUSSION AND FUTURE DIRECTION

3.1 Discussion

We have made great strides in our understanding of the brain. Written history tells us that there was once a time in ancient Egypt where brains were discarded during the mummification process on the basis that it had no purpose. Then, over the course of more than 4000 years we have made enormous progress to further our neuroscientific understanding of the brain. It was not until the 1600s, through dissection of human brains, that stroke was identified as a diagnosis and it took approximately 300 more years before the term BBB was coined. Research nearly a century ago determined that the BBB is impermeable to large molecules, and while the scientific community was entrenched in the medical dogma that the brain was a site of immune privilege, established by the presence of the BBB and lack of conventional vessels [15, 16], it was soon discovered that there were areas of the brain where the BBB was missing, namely the circumventricular organs.

In the last few decades, studies have challenged the idea that the brain is a site of immune privilege and research has shown that resident T-cells reside in the brain prior to insult, and are found in higher numbers following brain injury, including in post-ischemic stroke [3, 8, 9, 17-20] whereby studies have shown that immune cells infiltrate the brain and interact with glial cells [9, 21-23]. Based on research from our lab which showed that T-cells interact with astrocytes in the peri-infarct region of the brain, even 1 month post ischemic stroke [10], and based on the fact that tPA is the only FDA approved therapeutic available as treatment for ischemic stroke [11], there was a need to further investigate the relationship between T-cells and astrocytes in the brain, as one of the many glial immune interactions, to determine what other methods of treatment may benefit stroke patients.

Treatment for stroke has been determined by our contemporaneous level of scientific understanding. Therefore, when applying the scientific method, the treatment follows our pathological understanding. Hence, the medical intervention in stroked patients is based on understanding the underlying cause of the cerebral insult as well as the technology available for treatment. Thus, the first treatment was a mechanical surgical approach, followed by a chemical drug approach almost 150 years later. As we are being pushed forward by a new cell molecular or genetics era, we are seeking other therapeutic approaches to match our understanding of stroke in present time.

My thesis, based on cell and molecular biology, is a continuation of a long and arduous tradition to understand and find new and more sophisticated approaches to the treatment of strokes. The impact of T-cells in the brain, how they interact and modulate astrocytes has not been elucidated. At one-month post-ischemic stroke there are higher numbers of T-cells in the brain of murine models [10]. Treatments to reduce the effects of inflammation in the brain have not been specifically developed and instead ways to reduce time of occlusion has been the focus of the therapeutic approach. Unfortunately, as the aged population increases, there is an expected increase in stroke, as well as other neuroimmune complications associated with age, non-optimum immune function and related diseases.

My research has several major novelties: While several studies have investigated T-cell or immune system modulation in the presence of glial cells [3, 19, 82, 113, 135] my research was the first study to investigate the modulation of astrocytes phenotype in response to the presence of T-cells, which will be helpful for future clinical studies to build a correlation between astrocyte activity and clinical outcomes such as prognosis and ability to respond to therapies. Additionally, this was also the first study to investigate mechanisms by which T-cells and astrocytes interact and

can be blocked; information from this study will be helpful in identifying potential targets for activating neuroprotective pathways or blunting inflammatory responses as neuroinflammation has been demonstrated in multiple pathologies including: stroke, multiple sclerosis, Alzheimer's disease, and Parkinson's disease. The use of animal studies to investigate the normal physiology of these interactions, primarily *in vitro* is an important first step in this endeavor.

3.2 Future Direction

Altogether, our data suggests that understanding the interaction between T-cells and astrocytes could open the door to new therapeutic strategies for stroke patients. We anticipate that by investigating the underlying mechanisms of these interactions, we will be able to enhance beneficial effects, as well as limit inflammatory damage in the ischemic region. Additionally, *in vivo* studies are necessary to determine receptors involved in glial-immune interactions, how microglia fit into the astrocyte-T-cell interaction, the effect of IL-10 released into the peri-infarct region and its impact on glial scar formation, and the impact of gender, aging, and genetics on glial-immune function.

Since this research is focused primarily on IL-10 production and the anti-inflammatory interaction that occurs between T-cells and astrocytes, future studies should investigate the potential pro-inflammatory interactions, the effect of T-cell subtypes on astrocytic function at various pre- and post- stroke time points. Also, differences in immune-glial interactions and responses should be investigated with genetically inbred or congenic mice with various major histocompatibility complexes to determine if genetic predispositions exist and age varied animal models to determine if the level of IL-10 and its receptors changes with age. Since most stroke research uses a young rodent model due to increased survivability to experimentally induced stroke,

an aged animal model could reveal new insight into the stroke mechanism. This approach could remove age related confounding results. Researchers could also use organoids made up of astrocytes and various T cells under *in vitro* controlled 3D conditions to study the effects of various cytokines, receptors and evaluate drugs of interest. Finally, *in vivo* research in animal models should be performed to obtain a more complete understanding of the cellular, molecular and genetic underlining events which precede and cause strokes as well as their potential treatment before transition into human clinical trials. Together, this thesis adds another step to the understanding of inflammatory responses in the stroked brain, with the future objective of finding a positive stroke treatment outcome.

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