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The Inflammatory Response Initiated by the Spleen to Ischemic Stroke

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The Inflammatory Response Initiated by the Spleen to Ischemic Stroke

by

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A dissertation in partial fulfillment
of the requirements for the degree of
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Dedication

This dissertation is dedicated to my family for all their love and support, thank you.

Acknowledgments

I would like to thank and acknowledge all the individuals that have provided support throughout this endeavor. First, I would like to thank my mentor Dr. Keith Pennypacker for his guidance and intellectual support that has provided me with a strong foundation for a career in research. I would also like to thank Dr. Alison Willing for all of her intellectual collaborations. While this is an individual work, I would like to recognize all the individuals that contributed intellectually and helped with data collection on this project, including: Dr. Christopher Leonardo for his intellectual contributions to aim 1 and training in the middle cerebral artery occlusion procedure that was important for the completion of all aims of this project; Dr. Stanley Benkovic for the help with some of the histology in aims 1 and 3; Dr. Aaron Hall for his intellectual contributions to aims 1 and 2 and training in the splenectomy procedure; and all other laboratory personnel, including Lisa Collier and Cortney Chapman, for their technical contributions to the project and their support. Finally I would like to thank all of my family and friends who have supported me throughout this academic pursuit.

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Abstract

The peripheral immune system plays a role in delayed neural injury after stroke. This response originates from the spleen as splenectomy prior to middle cerebral artery occlusion (MCAO) in rats significantly reduces infarct volume in the brain. This research is based on the hypothesis that inhibiting the splenic response will reduce neurodegeneration after stroke. Studies in animals have implicated lymphocytes as the immune cell type that is detrimental following MCAO. Interferon gamma (IFN γ) has been identified as a pro-inflammatory cytokine that is also detrimental following stroke. IFN γ is important because it activates microglia and macrophages in a pro-inflammatory nature that increases neural injury following stroke. Therefore IFN γ was examined in the brain and the spleen following MCAO. IFN γ protein was elevated at 24 h in the spleen and at 72 h in the brain post MCAO. Microglia/macrophages become maximally activated at 72 h in the brain after MCAO. Splenectomy decreases the levels of IFN γ in the brain following MCAO. Systemic administration of IFN γ reversed the protective effects of splenectomy.

The cellular response to MCAO was examined next because of the difference in time between the spike in IFN γ in the spleen and the delayed increase in the brain. The cellular response from the spleen was studied by labeling splenocytes five days prior to MCAO with a fluorescein dye. Tissues were examined 48 and 96 h post MCAO or sham MCAO for fluorescence. These cells were released from the spleen into circulation at 48

h post MCAO and migrated to the brain where the cells produced IFN γ at 96 h post MCAO.

IFN γ appears to play a role in the splenic response to stroke. One protein that is up regulated by cells that have been activated by IFN γ , interferon-inducible protein 10 (IP-10) is part of the inflammatory cycle driven by IFN γ . IP-10 recruits more IFN γ producing T helper (Th) cells to the site of injury. IP-10 has the unique ability to attract Th₁ cells, the pro-inflammatory Th cells, and inhibit Th₂ cells, the anti-inflammatory Th cells. This leads to more IFN γ production as IFN γ is the signature cytokine of a Th₁ response. IP-10 is significantly increased in the brain at 72 h post MCAO, similar to IFN γ expression. In the spleen IP-10 increased at 24 h and remained elevated out to 96 h following MCAO. IFN γ signaling was inhibited by utilizing an IFN γ neutralizing antibody administered beginning 24 h post MCAO. The IFN γ antibody treated group had decreased infarct volumes, IP-10 levels in the brain, and appeared to have decreased T cells in the ipsilateral hemisphere at 96 h post MCAO.

Following ischemic stroke splenocytes are released into circulation and migrate to the brain. They release IFN γ to activate microglia/macrophages in a proinflammatory phenotype causing an increase in IP-10 levels. IP-10 then potentiates the Th₁ driven inflammation which inhibits the Th₂ response. The elevated levels of IFN γ increase neural injury following MCAO. Blocking IFN γ selectively blocks the inflammatory facet of the immune response to reduce stroke induced neurodegeneration. This leaves the other immune responses intact and able to contribute to tissue repair, regeneration, and able to respond to infections. Selectively inhibiting IFN γ signaling is a promising stroke therapeutic.

Background and Significance

Stroke:

Stroke Pathology

Strokes are caused by a disruption of blood flow to the brain, which results in brain damage to the areas supplied by the effected blood vessel. Loss of blood flow can result in two different types of stroke: ischemic stroke, caused by a clot, or hemorrhagic stroke, intracerebral hemorrhage (ICH), caused by the rupture of a blood vessel. Ischemic strokes account for 87 percent of all strokes and can be caused by two different mechanisms embolism or thrombosis. Cerebral embolisms occur when a blood clot forms at the point of occlusion in the vessel, whereas cerebral thrombosis occurs when a clot from another area of the body travels to the brain and becomes lodged in the occluded blood vessel. Occlusion of the larger vessels in the brain can have catastrophic effects because large areas of the brain are affected.

Stroke is the fourth leading cause of death in the United States, 129,000 deaths in 2011, and leading cause of disability. Approximately 795,000 strokes occur each year, of which 185,000 are recurrent strokes. There is a 40% increase in risk of stroke in the first five years following an initial stroke. Usually recurrent strokes are more disabling and have higher mortality. In 2010 there were seven million stroke survivors over age 20 in the United States. While stroke is down to the fourth leading cause of death from the third leading cause, it still remains the leading cause of disability. The reason stroke is the

leading cause of disability is because 50% of patients experienced some hemiparesis, 30% were unable to walk without assistance, 26% were dependant on assistance with activities of daily living, 19% had aphasia, 35% had depressive symptoms, and 26% were in nursing homes. This leads to a high financial burden on society as stroke resulted in \$73.7 billion in total direct and indirect costs in United States in 2010 (Roger et al. 2012).

Risk Factors

There are several risk factors for stroke that can include medical conditions, life style choices, genetic factors, and a person's family history. Common risk factors for stroke include high blood pressure, high cholesterol, diabetes, atherosclerosis, atrial fibrillation, smoking, alcohol consumption, physical inactivity, sleep apnea, and obesity. The risk for stroke is also higher in individuals over the age of 55. Men have a higher risk of stroke compared to women. Non-Caucasian individuals have a higher rate of stroke than Caucasians. Individuals with a family history of stroke are at a higher risk and people who have had a previous stroke or transient ischemic attack (TIA) are at risk of having another stroke. TIA greatly increases a person's risk of stroke as 40% of people with TIA have a stroke, 5% within 2 days and 10-15% within 3 months of the initial TIA (Roger et al. 2012).

Treatment

Recombinant tissue plasminogen activator (rTPA) is the only FDA approved treatment for ischemic stroke, with a treatment window of 4.5 h, which allows only 3-5% of patients to be eligible to receive treatment. Additionally, rTPA can cause transformation of an ischemic stroke into a hemorrhagic stroke. Currently there are no pharmaceutical treatments for hemorrhagic stroke, only strategies to manage the symptoms. There are

extensive exclusion criteria for the use of rTPA in order to minimize the risk for bleeding or hemorrhagic transformation. Recombinant TPA is contraindicated in individuals with ICH, intracranial or intraspinal surgery within the last three months, history of previous stroke or ICH, uncontrolled blood pressure (>185 mm Hg systolic or >110 mm Hg diastolic), individuals over the age of 75, seizure at the onset of stroke, active internal bleeding, arteriovenous malformation, aneurysm, intracranial neoplasm, the use of oral anticoagulants, heparin administration within the previous 48 h, or a platelet count <100,000/mm³. Individuals with National Institutes of Health Stroke Scale (NIHSS) scores greater than 22 at presentation are excluded (Genentech 2011), as individuals with NIHSS scores >20 are at a higher risk of ICH (Adams et al. 2003). Diabetic individuals and individuals with hyperglycemia have an increased risk of ICH with thrombolytic therapy (Martini and Kent 2007).

The therapeutic window for rTPA treatment in ischemic stroke is narrow at 4.5 h making it very difficult for stroke patients as many do not recognize the need for treatment within that time frame. Stroke patients must first identify that they are having a stroke and then they must seek medical treatment. After getting medical attention, the possibility of a cerebral hemorrhage must be ruled out as rTPA can only be used for embolic strokes. Once all these steps are performed the individual may still be ineligible to receive rTPA due to the strict inclusion criteria for the use of rTPA.

Animal Models of Stroke

There are different types of experimental models of both ischemic strokes and ICH. One model of ICH involves injecting bacterial collagenase type IV directly into the striatum of animals. The collagenase disrupts the integrity of the basal lamina which leads to leaky blood vessels and a brain bleed (MacLellan et al. 2010). Ischemic strokes can be further

divided into two types of ischemia that can cause neural injury: global ischemia and focal ischemia. Global ischemia occurs when there is loss of blood flow to the whole brain such as during a myocardial infarction (MI) when blood flow to the whole body is stopped. Focal ischemia only affects certain areas of the brain and can be caused by a blood clot occluding a blood vessel. In animal models, global ischemia can be produced by temporarily stopping the heart which stops blood flow to the entire body including the brain. Focal cerebral ischemia can be performed to create an embolic stroke. While models of embolic stroke can be caused permanently by cauterizing a cerebral artery, most involve the middle cerebral artery (MCA), as the MCA is the most commonly occluded vessel in people. This method involves performing a craniotomy to visualize the cerebral vasculature and creates a small focal cortical lesion. Another method of creating a permanent occlusion is by injecting a clot into the common carotid artery, which becomes lodged in the smaller vasculature. One advantage to this procedure is that the animal is awake at the time of occlusion and it is a useful model to study thrombolytic therapies. However, this model is not highly reproducible as the clot can become lodged in numerous different arteries. A minimally invasive procedure to induce focal cerebral ischemia involves photochemical thrombus formation by systemically administering the dye Bengal Rose. A blood clot forms when a laser is positioned against the skull and illuminated. The resulting focal infarct is a small cortical lesion without a penumbra or area of salvable tissue. One of the most commonly used experimental stroke models is the intraluminal filament model, also referred to as the middle cerebral artery occlusion (MCAO). This model involves inserting an embolus, a monofilament, into the external carotid artery and advancing the embolus to the origin of the MCA occluding blood flow to MCA territory. The monofilament can be tied off creating a permanent occlusion (pMCAO) or removed after a period of time to create an

ischemic reperfusion injury, also referred to as a transient occlusion (tMCAO) (for a comprehensive review, see (Braeuninger and Kleinschnitz 2009)).

The Spleen and Ischemic Injuries Including Stroke:

The Immune System

The immune system is made up of a network of organs and cells which come together to form the functional unit. The two primary organs of the immune system are the bone marrow and the thymus. The bone marrow is the germinal center for all blood cells and the site of B cell maturation. T cells leave the bone marrow and mature in the thymus. Secondary peripheral immune organs include the spleen and lymph nodes. These two organs are areas where immune cells pool together and these sites are areas of filtration and immune surveillance. This filtering allows the immune system to quickly mount an immune response to a pathogen that is found in systemic circulation.

The immune system can be divided into two categories: the innate immune system and the adaptive immune system. Each system has its own set of specialized cells and performs specific functions in the clearance of pathogens, tissue recovery from injury, and surveillance for tumor cells. The innate immune system is made up of neutrophils, monocytes, and natural killer cells (NK cells). These cells are the first responders to an immune challenge. Neutrophils are phagocytic cells that primarily engulf and kill evading or dying cells. Monocytes are known as antigen presenting cells (APCs). Cells of the monocytic lineage include macrophages, dendritic cells, and tissue specific macrophages including microglia (brain) and Kupffer cells (liver). These cells phagocytize pathogens and present what they find to T cells via the major histocompatibility complex (MHC) II. MHC II is found only on APCs and presents T cells with antigens, where MHC I is expressed on all cells and displays self peptides which

allow T cells and NK cells to determine if a cell should be present in the body. Intracellular pathogens will have their peptides expressed in MHC I which will trigger a cytotoxic response from NK cells or T cells. NK cells are cells of lymphocytic origin but they are able to recognize infected cells or tumor cells and generate a cytotoxic response without becoming activated by another cell. NK cells are able to induce apoptosis in virally or bacterially infected cells or malignant cells without any signals besides the activation of their receptors. Unlike the adaptive immune system, which can take days to weeks to generate a response to a pathogen, the innate immune system can generate an immediate response.

The adaptive immune system consists of lymphocytes, T cells and B cells. The adaptive immune response takes days to weeks to initiate due to the highly specific nature of the receptors on T cells and B cells. These B and T cell receptors are very specific for their epitope on a particular antigen. These cells must come across their specific antigen to become activated and generate a response. B cells can generate a response without the signaling of other cells, although T cells can play a role in influencing antibody production. Activated B cells go on to produce antibodies directed against their specific antigen. T cells need to be presented with their antigen by APCs. The response generated by a T cell depends on the type of T cell. Cytotoxic T cells, or CD8⁺ T cells, recognize MHC I on cells and will initiate apoptosis in cells which are not presenting self peptides. Cells which are expressing pathogen peptides in MHC I will trigger a CD8⁺ T cell response if the pathogen peptide is the specific antigen for that T cell. The other subset of T cells is the T helper cells (Th cells), CD4⁺ T cells; of which there are many branching Th cell subsets. The two major Th responses are the Th₁ response and the Th₂ response. These two responses oppose each other. A Th₁ response is considered pro-inflammatory and is directed against intracellular pathogens, including bacteria and

viruses, while the Th₂ response is considered anti-inflammatory, is directed at helminths and is responsible for generating allergic reactions. The main function of Th cells is to control or influence the immune response by secreting effector molecules or cytokines. Th cells can orchestrate an immune response by activating different cells of the innate immune system, cause isotype switching in B cells or recruit specific immune cell subsets to the area of inflammation. A special subset of Th cells is T regulatory (T_{reg} cells) cells, which are responsible for stopping an immune response. These cells are important in ensuring the immune response does not cause additional injury to the surrounding tissue and in helping to decrease inflammation.

Splenic Physiology

The spleen is a highly vascularized secondary peripheral lymphoid organ. The spleen has many functions including clearing dying red blood cells, removing hemoglobin from circulation, removal of bacterial pathogens from circulation, controlling iron homeostasis, and regulating the immune response and B cell antibody production. The spleen is divided into the red pulp and the white pulp. The white pulp is made up T cell zones, or periarteriolar lymphoid sheaths, and B cell follicles. The red pulp contains B cells, NK cells, and monocytes/macrophages that are in close proximity to the vasculature. This allows monocytes/macrophages to filter the blood for dying red blood cells, hemoglobin, and antibody covered bacterial pathogens. Plasma cells or antibody producing B cells are the specific type of B cells found in the red pulp. This location in the spleen allows for rapid delivery of antibodies into circulation (Mebius and Kraal 2005). NK cells in the red pulp resemble NK cells found in circulation (Witte et al. 1990). The white pulp is split into two areas, one for T cells and another for B cells. The T cell zones allow naïve T cells to be in close proximity to the arteriole blood supply. In addition to T cells, dendritic cells are present in the T cell zones to present naïve T cells with antigens the dendritic cells

find in the blood. Once activated, T cells leave the spleen and initiate an immune response. Specialized macrophages are found in the marginal zones of the white pulp which recognize both bacterial and viral blood borne pathogens. These cells are important in secreting cytokines and chemokines that influence T and B cells. Polyclonal expansion of B cells and isotype switching occur in the B cell follicles. Due to the close proximity of the cells within the white pulp, T cells can influence B cell isotype switching (Mebius and Kraal 2005).

The splenic capsule is made up of smooth muscles that express α_1 adrenergic receptors. Activation of the α_1 receptors leads to contraction of the smooth muscles and a decrease in spleen size. In addition to being an immune cell reservoir, the spleen is also reservoir for red blood cells. During times of physical stress, the spleen has been shown to contract and release red and white blood cells into systemic circulation (Bakovic et al. 2005; Bakovic et al. 2003). The spleen is the largest reservoir of undifferentiated non-tissue specific monocytes and, in humans, contains half of the monocytic cell population. These cells have been shown to be released following MI and have detrimental effects on the damaged tissue as well as prevent tissue healing (Swirski et al. 2009).

The Role of the Spleen in Ischemic Injuries

The spleen is large reservoir of immune cells that can generate pro-inflammatory responses to various ischemic injuries. Splenectomy is protective in other ischemic injuries including the liver, intestines, kidneys, and heart. Removal of the spleen immediately prior to ischemic/reperfusion (IR) injury of the liver results in decreased levels of alanine aminotransferase levels (ALT) a biochemical markers of liver injury, and decreased hepatocellular injury. Additionally splenectomy reduced the number of

polymorphonuclear cells in the liver following liver IR (Okuaki et al. 1996). A later study found that splenectomy prior to hepatic IR reduced the elevated levels of two liver enzymes associated with liver damage, ALT and aspartate aminotransferase (AST), as well as tumor necrosis factor alpha (TNF α), and myeloperoxidase (MPO) activity in the liver, which is a marker of the presence of neutrophils. In addition, hepatic IR is associated with injury to other organs including the kidneys, lungs, and intestines and splenectomy reduced cell apoptosis and caspase 3 signaling in all four organs (Jiang et al. 2007). Intestinal IR has been linked to acute lung injury. Kupffer cells in the liver become activated in intestinal IR. Blocking Kupffer cell activation using gadolinium chloride or splenectomy prior to intestinal IR resulted in significantly decreased levels of TNF α , interleukin 6 (IL-6), MPO, and malondialdehyde assay (MDA), a marker of lipid peroxidation, in the lungs. Polymorphonuclear leukocyte (PMNL) counts were also significantly decreased in the lungs in the gadolinium chloride and splenectomy groups. Gadolinium chloride treatment and splenectomy did not reduce the levels of any of the outcome measures down to sham IR operated groups (Savas et al. 2003). Kupffer cells also play a detrimental role following renal IR. Gadolinium chloride was administered or splenectomy was performed prior to renal IR. Both the gadolinium chloride and splenectomy groups had decreased histopathological changes compared to the renal IR group. In addition, serum ALT, AST, BUN, and creatinine levels were significantly increased in the renal IR group but significantly decreased in the gadolinium chloride and splenectomy groups. Tissue levels of MDA, MPO, and lactate dehydrogenase (LDH), a marker of cell death, were significantly elevated in the renal IR groups and significantly decreased in the treatment groups. Renal IR also decreased glutathione (GSH) levels and Na⁺/K⁺ ATPase activity while treatment with gadolinium chloride or splenectomy restored the activity of these two proteins (Kara et al. 2009). The studies on intestinal IR and renal IR both concluded that splenectomy was protective because of

the removal of a large reservoir of monocytes. Monocytes have also been found to play an important role in exacerbating injury following myocardial infarction (MI). Sequestering splenic monocytes in the spleen with enalapril, an angiotensin-converting enzyme (ACE) inhibitor, or splenectomy prior to experimental MI decreased inflammation and infarct size (Leuschner et al. 2010).

Monocytes have been shown to play a detrimental role in ischemic pathology in many organs. As the spleen contains a majority of the monocytes in the body, this suggests these cells are responsible for IR organ damage. This has led to the conclusion that the spleen is an important mediator of post IR injury tissue damage. Additionally blocking Kupffer cell activation with gadolinium chloride was as efficacious as splenectomy in renal and intestinal IR. These studies indicated that the spleen activates Kupffer cells in a pro-inflammatory state that increases tissue damage following IR injuries. Kupffer cells, like microglia, are tissue-specific macrophages. If the spleen causes Kupffer cell activation following IR injuries to the kidney and intestines, then the spleen might be negatively influencing microglia in the same manner, which would increase neural injury following ischemic brain injury.

Removal of the spleen is also protective in pMCAO, tMCAO, ICH, and traumatic brain injury (TBI) (Ajmo et al. 2008; Jin et al. 2013; Lee et al. 2008; Das et al. 2011; Li et al. 2011; Walker et al. 2010). Splenectomy prior to pMCAO in rats decreases infarct volume and the number of neutrophils and activated microglia in the brain (Ajmo et al. 2008). In mice, splenectomy prior to tMCAO decreased infarct volume, brain IFN γ levels, and did not increase post stroke infections (Jin et al. 2013). Brain water content was significantly lower in splenectomized animals compared to intact animals prior to ICH (Lee et al. 2008). Splenectomy immediately after TBI was found to decrease neural injury in two

different models of experimental TBI (Walker et al. 2010; Das et al. 2011; Li et al. 2011), which creates areas of ischemia from vessel damage and edema following TBI. As an alternative to splenectomy, irradiation of the spleen 4 h post tMCAO decreases infarct volume in rats similar to the effects of splenectomy prior to pMCAO. Splenic irradiation causes a temporary decrease in splenocytes which does not result in wide spread immunosuppression (Ostrowski et al. 2012). These experiments all demonstrate that the spleen plays an inflammatory role in brain injuries and ischemic injuries to other organs. Further investigation is needed to determine the mechanisms by which the spleen is inflammatory following ischemic injuries.

Spleen Size Decreases following Permanent and Transient MCAO

The spleen has been found to decrease in size following pMCAO in rats (Vendrame et al. 2006) and tMCAO in mice (Offner et al. 2006b). The spleen transiently decreases in size from 24 to 72 h following pMCAO in rats (Seifert et al. 2012). The transient changes seen in pMCAO are likely due to the catecholamine (CA) surge which occurs following damage to the insular cortex, an area mainly perfused by the MCA. Activation of the α_1 adrenergic receptors on the splenic smooth muscle capsule results in contraction of the splenic capsule, which leads to the decrease in spleen size. Administration of prazosin, an α_1 adrenergic receptor blocker, prevents the decrease in spleen size following pMCAO (Ajmo et al. 2009). Spleen size has also been inversely correlated with infarct volume in rats following pMCAO, with smaller spleen sizes correlating with larger infarcts (Vendrame et al. 2006). The splenic response in mice following tMCAO appears to be different from the response observed in rats following pMCAO. The spleens of mice continually decrease in size following tMCAO out to 96 h. This decrease in spleen size appears to be due to apoptosis of the spleen and a loss of the germinal B cell centers. The only immune cell population that has been shown to decrease in number following

tMCAO in mice are B cells (Offner et al. 2006b). There are several reasons that could explain the observed differences in the splenic response to MCAO. The studies were performed in different animal species, mice compared to rats, and the observations were made in two different injury models, transient versus permanent MCAO. There are many factors that could result in the differences seen in mice and rats following MCAO. The only way to determine how the spleen responds to stroke is to study stroke patients.

Studies are currently being conducted which examine spleen size in stroke patients to truly understand the role the spleen plays in patients following stroke. One such study is currently being conducted and preliminary data indicates that the spleen in individuals who have suffered a stroke decreases in volume initially, < 6 h to 3 days, and slowly begins to increase in volume starting at day 4 and continuing out to 8 days following the stroke. An individual who suffered a severe stroke and ultimately died had spleen volumes that continued to decrease as their NIHSS score also progressively increased, indicating a worsening of neurological symptoms. Two other individuals who had better outcomes had spleens that initially decreased in volume and began to increase in volume as their NIHSS scores decreased. One of the two individuals had their spleen volume measured 90 days following their stroke and at 90 days the individual's spleen volume was not different from the measurement taken at discharge (Sahota et al. 2013).

Stem Cell Therapies and the Spleen

Human umbilical cord blood (HUCB) cells (Vendrame et al. 2004; Makinen et al. 2006; Zhang et al. 2011), hematopoietic stem cells (HSC) (Schwartz et al. 2008), bone marrow stem cells (BMSC) (Keimpema et al. 2009), and neural stem cells (NSC) (Lee et al. 2008) have all been shown to reduce neural injury in experimental models of stroke. Stem cells are more efficacious when administered systemically compared to

intracerebral administration. When administered systemically, stem cells migrate to the spleen (Lee et al. 2008; Schwarting et al. 2008; Keimpema et al. 2009; Vendrame et al. 2004), which may be why the cells are more efficacious via this injection route. Even NSCs migrate to the spleen following intracerebral hemorrhage and are not as efficacious when combined with splenectomy. NSCs were found to be in direct contact with CD11b⁺ splenocytes (Lee et al. 2008). This suggests that part of the neuroprotection provided by NSCs involves interacting with the spleen. HUCB cells are another cell type that has been shown to interact with splenocytes. Systemic administration of HUCB cells 24 h post pMCAO results in altered splenic T cell responses to concavalin A. Splenic T cells had decreased cell proliferation and decreased production of inflammatory cytokines TNF α and interferon gamma (IFN γ) with an increase in the production of the anti inflammatory cytokine interleukin 10 (IL-10). HUCB cells also prevent the decrease in spleen size seen at 48 h in rats. This effect is thought to be mediated by HUCB cells sequestering immune cells in the spleen following MCAO, preventing their release into systemic circulation (Vendrame et al. 2006). This set of experiments suggests stem cell therapies work in part by modulating the immune response to stroke, specifically at the level of the spleen.

The Initial Cascade of Neural Death following Stroke

The hypoxic and glucose deprived environment that develops following ischemic stroke leads to cellular dysfunction and cell death through necrosis or apoptosis. In an attempt to keep up with the high energy demands in the brain, neural cells switch to anaerobic cellular respiration. Cell membranes become damaged from the resulting build up of reactive oxygen and nitrogen free radicals, which leads to cellular edema and necrosis. Additionally, as neurons and astrocytes die there is a release of glutamate that compromises more neurons through glutamate excitotoxicity. The activation of glutamate

receptors leads to excessive intracellular calcium release, edema, and caspase activation resulting in apoptosis (Lipton 1999). All of these mechanisms lead to early cell death in the core of the infarct, the area directly perfused by the occluded artery.

In addition to neural cell death, activation of matrix metalloproteinases (MMPs) lead to the opening of the blood-brain-barrier (BBB). Shortly after occlusion the BBB is broken down transiently by MMP-2. Later, at 48 h post MCAO, up regulation of MMP-9 leads to a prolonged disruption of the BBB (Candelario-Jalil et al. 2009). This break down in the BBB allows neural antigens into the peripheral circulation. The leaky BBB contributes to enhanced neural injury by increasing edema as intracranial pressure builds from the influx of excess fluid. This BBB dysfunction also allows the immune system to come in contact with these neural antigens and generate an immune response to the brain.

The Peripheral Immune Response to Stroke:

Cellular Response

The CNS has a structurally different network of capillaries that are different from the rest of the body. Within the CNS, the endothelial cells closely control which substances or cells can enter the brain or spinal cord. The specialized endothelial cells have extracellular tight junctions connecting neighboring cells together and these cells are closely associated with a basement membrane and extracellular matrix. The absence of fenestrations in the endothelial cells and reduced pinocytotic activity also contribute to the protection of the brain by the cerebral vasculature. This restricted access to the brain created by these cells is known as the BBB (de Vries et al. 1997). The BBB protects the brain from exposure to anything harmful in the blood. This includes protection from the peripheral immune system under normal healthy conditions. Generally, the only immune cells present in the brain are the endogenous macrophages, microglia. Occasionally a T

cell may enter the brain but due to the decreased expression of MHC molecules in the CNS, the T cell leaves the brain within 24-48 h (Miller 1999). This makes the brain an immunoprivileged site which is beneficial in protecting the brain from systemic inflammation. However, neural antigens can be seen as foreign to the immune system resulting in immune responses generated against neural antigens which are present in systemic circulation following brain injuries, including stroke.

The peripheral immune system, both the innate and the adaptive systems, plays an important role in the inflammatory response following ischemic brain injury. The injured cells of the CNS, in combination with glial cells which become activated after a stroke, express chemotaxic molecules that signal to the peripheral immune system that there is an injury to the brain. Various cytokines cause up regulation of vascular adhesion molecules in endothelial cells and on immune cells. This creates a leaky BBB which allows entry of immune cells into the brain (de Vries et al. 1997).

Cells of monocytic origin, CD11b⁺ cell, become activated as early 18 h and are significantly increased in number out to 96 h post tMCAO in mice (Stevens et al. 2002). It is not possible to determine the difference between microglia and peripheral monocytes/macrophages, as both types of cells express CD11b. Microglia/macrophages become maximally activated in the brain 72 h post pMCAO in rats (Leonardo et al. 2010). Neutrophils are significantly increased in the infarcted hemisphere beginning 48 h and remain elevated out to 96 h post tMCAO. As expected with an adaptive immune response T cells, CD3⁺ cells, are present in the brain starting 72 h and remain at 96 h post tMCAO (Stevens et al. 2002).

There is strong experimental evidence that peripheral immune cells, particularly lymphocytes, play a role in enhancing neural injury after an ischemic stroke. Following tMCAO, Rag^{-/-} mice which lack functional T or B cells have decreased infarct volumes compared to wild type (WT) mice. T cell^{-/-} mice, both CD4^{-/-} and CD8^{-/-}, have decreased infarct size compared to WT mice. However B cell^{-/-} mice had infarcts similar to WT mice indicating B cells play a minimal role in detrimental post stroke brain inflammation (Yilmaz et al. 2006). Additionally, severe combined immunodeficiency (SCID) mice, which lack lymphocytes, also have reduced infarcts compared to WT mice. SCID mice also have reduced cytokine levels, except for interleukin 1 β (IL-1 β), in the brain post tMCAO (Hurn et al. 2007). T cells that are primed to react with a pro-inflammatory response to myelin oligodendrocyte glycoprotein (MOG) prior to tMCAO increased infarct volumes in mice (Ren et al. 2012) or resulted in death following sensitization with myelin basic protein (MBP) prior to tMCAO in rats (Becker et al. 1997) compared to animals primed with a non-neural neutral antigen. However, T cells that are tolerized to MBP prior to tMCAO have decreased infarct volume compared to controls (Becker et al. 1997). The reaction of the immune system towards neural antigens, which is orchestrated by Th cells, can be harmful or beneficial following ischemic stroke.

Humoral Response

Cytokines have been extensively studied following experimental stroke and in stroke patients. Most of the data regarding cytokines and stroke have been contradictory, as some cytokines have dual roles in the immune response and can be protective or detrimental depending on the circumstances. Some cytokines can be inflammatory early after a stroke, but provide trophic support to cells at delayed time points. Other cytokines can have survival or inflammatory effects depending on the receptor to which they bind. Additionally, some cytokines are elevated very early following stroke. All the above

stated examples demonstrate why no good therapeutic targets for cytokines have been developed.

In mouse models of tMCAO, TNF α , IL-1 β , and IL-10 have elevated mRNA levels 6 h post tMCAO (Chang et al. 2011; Offner et al. 2006a). All of these cytokines are elevated at a time that is outside the therapeutic window to successfully interfere with their signaling. IL-1 β mRNA expression increases early in the brain after an ischemic event and remains elevated out to 96 h post tMCAO (Chang et al. 2011). IL-1 β is expressed by non immune cells in the brain. Primary producers of IL-1 β in the brain are astrocytes and microglia (de Vries et al. 1997), which could explain why IL-1 β is the only cytokine that does not have decreased expression in SCID mice following tMCAO (Hurn et al. 2007). IL-10 is considered an anti-inflammatory cytokine and is associated with a Th₂ response. IL-10^{-/-} mice have increase infarct volumes compared to WT mice following tMCAO (Liesz et al. 2009) and mice that over-express IL-10 have decreased infarct volumes compared to controls following pMCAO (de Bilbao et al. 2009). This suggests IL-10 may play a beneficial role following brain ischemia.

TNF α is also expressed early in the brain and is primarily responsible for the activation of the immune system and recruitment of other immune cells. In addition to being expressed early, TNF α is known to have different effects following brain ischemia. TNF α has been shown to exacerbate infarct volume in both tMCAO and pMCAO in a dose dependant manner (Barone et al. 1997). However, TNF α ^{-/-} mice had increased infarct volumes compared to WT mice following pMCAO (Lambertsen et al. 2009). This suggests TNF α plays a protective role following ischemic stroke, but when TNF α converting enzyme (TACE) was inhibited, causing a decrease in TNF α production prior

to and following pMCAO, TACE-inhibited rats demonstrated decreased infarct volume and reduced neurological deficits compared to control rats (Wang et al. 2004). In addition, blocking TNF α with a neutralizing antibody injected intracerebroventricularly (i.c.v.) 15 min post tMCAO in mice decreased infarct volume, however, administration of the same antibody 3 days post tMCAO did not decrease infarct volume (Liesz et al. 2009). TNF α has also been linked to the up regulation of manganese superoxide dismutase (Mn-SOD), an important anti-oxidant enzyme that is believed to play a role in ischemic preconditioning in stroke (Hallenbeck 2002). All of the contradictory results in experimental stroke with TNF α could be due to the two different TNF α receptors and the subsequent cellular processes induced by these receptors.

The two TNF α receptors, when activated, can result in different cellular responses depending on the cell type or the presence of both receptors on the same cell. TNF α can initiate a response resulting in apoptosis and the production of cytokines or be protective by preventing apoptosis. The two different receptors, TNF α R1 and TNF α R2, result in a combination of different cellular responses. TNF α R1 has an intracellular death domain that can divert the cellular response to TNF α in a Fas-associated protein with a death domain (FADD) towards apoptosis or the binding of TNF-receptor associated protein 2 (TRAP2) which leads to the transcription of anti-inflammatory factors. FADD signals to activate caspase 8 leading to apoptosis. TRAP2 leads to the activation of NF κ B and c-JUN which induce anti-apoptotic, anti-inflammatory and cellular protective proteins (Hallenbeck 2002). TNF α R1 is expressed on all cells, while TNF α R2 is expressed only on oligodendrocytes, astrocytes, T cells, myocytes, endothelial cells, thymocytes, and human mesenchymal stem cells. TNF α R2 does not contain an intracellular cytoplasmic death domain and activation leads to the recruitment of TNF receptor-associated factor 2

(TRAF2). TRAF2 acts similarly to TRAP2 and activates NF κ B, AP1, and mitogen-activated protein kinase (MAPK). The activation of these pathways leads to inflammation, cellular proliferation, and cell survival (Speeckaert et al. 2012). In pMCAO experiments with TNF α R knockout mice, TNF α R1^{-/-} was associated with neuroprotection while TNF α R2^{-/-} was not associated with neuroprotection (Lambertsen et al. 2009). Studies using cuprizone toxicity as a model of white matter injury found that TNF α is important in the recruitment of oligodendrocyte progenitors to remyelinate axons. This TNF α signaling is mediated through the TNF α R2 (Arnett et al. 2001). The diverse effects of TNF α can be contributed to many factors including timing of TNF α signaling and the receptors it signals through, and the contradictory outcomes in animal experiments indicate TNF α is not a good therapeutic target for stroke. Additionally it is initially elevated outside therapeutically relevant time points to treat stroke.

Another cytokine that has been research extensively in experimental stroke is IL-6. One of the primary reasons a lot of emphasis has been placed on IL-6 is because it is detectable in the serum of stroke patients. Serum IL-6 levels in stroke patients have been shown to be the strongest independent predictive variable of in hospital mortality following stroke (Rallidis et al. 2006). IL-6 mRNA levels are elevated early in the brain following tMCAO in mice (Offner et al. 2006a). Despite evidence that IL-6 might be playing a detrimental role in stroke pathology, there has been very little investigation into blocking IL-6 as a therapeutic for stroke which is likely due to the confounding data from animal studies looking at IL-6 and experimental stroke. IL-6^{-/-} mice had infarcts that were not significantly different than their WT or heterozygous littermates following tMCAO (Clark et al. 2000). However, IL-6 is known to have pyrogenic activity and IL-6^{-/-} mice were found to have lower body temperatures compared to WT mice. Body temperature

is known to play an important role following any brain injury, including stroke. When IL-6^{-/-} mice had their body temperatures monitored and adjusted to match the WT mice, the IL-6^{-/-} had significantly increased infarct volumes and increased neurologic deficits compared to the WT mice following MCAO (Herrmann et al. 2003). An additional study found i.c.v. administration of recombinant IL-6 prior to pMCAO in rats significantly decreased infarct volume suggesting IL-6 is directly neuroprotective (Loddick et al. 1998). A different study using IL-6^{-/-} mice found increased infarct volumes compared to WT mice following tMCAO out to four weeks. This study suggested the loss of IL-6 was important for angiogenesis during the recovery phase following stroke (Gertz et al. 2012).

One reason for the conflicting experimental data with IL-6 could be due to the multiple functions of IL-6. Prior to being named IL-6, the 26-kDa protein was named B-cell stimulatory factor 2, IFN-β2, hybridoma/plasmacytoma growth factor and hepatocyte stimulating factor along with 26-kDa protein. Once DNA sequencing was completed it was discovered that all five proteins were the same molecule (Kishimoto 2010). IL-6 signals through one receptor that is a complex of the IL-6R and gp130. The cytoplasmic domain of gp130 contains several signaling motifs that allow IL-6 to signal through ERK or Jak₁ and STAT3 or STAT1. STAT3 and STAT1 can form homodimers or a heterodimer once activated. These different signaling pathways allow IL-6 to have diverse effects on a variety of cells and cellular functions. IL-6 is important for liver regeneration, angiogenesis, bone, cartilage, and lipid metabolism, iron homeostasis, and is known to promote cancer cell survival. The immune functions that IL-6 plays a role in include promoting neutrophil production and recruitment, enhances antibody production by B cells, and works with transforming growth factor beta (TGFβ) to increase the

production of pro-inflammatory Th₁₇ cells. Th₁₇ are thought to play a major role in autoimmune diseases (Mihara et al. 2012). IL-6 plays many different roles following stroke depending on the exact timing following the stroke, which makes targeting IL-6 after stroke extremely difficult.

Out of numerous studies on cytokines in animals and stroke patients, there has yet to be a therapeutic developed for stroke. Many cytokines are part of the innate immune response and increase rapidly following stroke, while other cytokines have dual roles following stroke or serve a potentially protective function. One cytokine has not been extensively studied and could have the promise of providing a delayed therapeutic option that is strictly pro-inflammatory. IFN γ is the signature cytokine of the adaptive immune Th₁ response.

The Importance of Interferon Gamma Signaling following Stroke:

Interferon Gamma and its Receptors

IFN γ is a pleiotropic cytokine that can affect cellular processes ranging from immune cell function to playing a role in vascular leukocyte adhesion. The 34-kDa homodimer is the biologically active form of IFN γ (Boehm et al. 1997). The half-life of IFN γ in the blood is 1.1 min if it is not bound to heparin or heparin sulfate, and when bound the half-life is increased to 99 min. When IFN γ is injected into the bloodstream, 90% of the protein is proteolytically cleaved at the carboxyl-terminal within 5-10 min rendering it inactive. The other 10% becomes bound to a heparin molecule, increasing its half-life (Lortat-Jacob et al. 1996). The biologically active form of IFN γ binds to the IFN γ receptor complex that is comprised of two pairs of transmembrane proteins which signal through the Janus kinases (Jaks), and signal transducers and activators of transcription (STATs). Of the

two different proteins that make up the IFN γ receptor, the IFN γ R $_1$, or the α -chain, is the part of the receptor that binds IFN γ . Following binding of IFN γ the α -chain dimerizes with another α -chain. Then, the second protein IFN γ R $_2$ or the β -chain dimerizes with another β -chain to the complex. The β -chain is primarily involved in signaling, whereas the α -chain is primarily involved in binding IFN γ . Jak $_1$ is associated with the α -chain and Jak $_2$ is associated with the β -chain. Once IFN γ binds, the Jaks become phosphorylated and phosphorylate STAT1 α that homodimerizes and translocates to the nucleus to affect transcription. The IFN γ receptor is expressed on every cell in the body but its density varies from cell type to cell type with immune cells, monocytes in particular, having the highest expression.

The primary producers of IFN γ are T cells, CD4 $^+$ Th $_1$ cells, CD8 $^+$ T cells, and NK cells. IFN γ production is induced by IL-12, IFN α , and even by IFN γ through positive feedback. IFN γ induces the production of more IL-12 by macrophages which further increase IFN γ production. In addition to increasing its own expression, IFN γ causes increased expression of proteins involved in the generation of reactive oxygen species (ROS), chemotaxis of more immune cells to the site of injury, up regulation of MHC molecules, and induces isotype switching in B cells. Indirectly, there is also an increase in cellular adhesion molecules on endothelial cells by IFN γ through up regulating chemokines, including monocyte chemoattractant proteins (MPCs), monokine induced by gamma interferon (MIG), interferon-inducible protein 10 (IP-10), IL-8, and interferon-inducible T cell α chemoattractant (I-TAC). With the recruitment of additional immune cells to the site of injury, IFN γ also primes T cells and cells of monocytic origin towards a pro-inflammatory phenotype. IFN γ and its effector molecules prime naïve Th cells to develop into Th $_1$ cells, blunting the response of other Th subsets. Microglia/macrophages

become activated in towards a pro-inflammatory state by the up regulation of ROS production and MHC II expression. The primary role $IFN\gamma$ plays in the immune response is to increase resistance to bacteria and viruses, particularly against intracellular pathogens. To obtain this immune response, the primary target cells of $IFN\gamma$ in the immune system are macrophages. These cells are also the primary producers of all the chemokines like IP-10, MIG, and I-TAC.

The Detrimental Role of $IFN\gamma$ following Ischemic Brain Injury

There are several data in animals and stroke patients indicating $IFN\gamma$ plays a detrimental role in stroke pathogenesis. $IFN\gamma$ mRNA is up regulated 2 days post pMCAO in the brain of rats (Li et al. 2001). Additionally, $IFN\gamma^{-/-}$ mice have decreased infarcts compared to WT mice and the $IFN\gamma^{-/-}$ mice infarcts are comparable to $Rag^{-/-}$ mice (Yilmaz et al. 2006). Delayed administration of antibodies directed against $IFN\gamma$ decreased infarct volume when injected i.c.v. 3 days, but not early, post tMCAO (Liesz et al. 2009). In addition, indirect blocking of $IFN\gamma$ is neuroprotective. Administration of an anti CD49d (VLA-4) antibody 24 h prior to the MCAO reduced infarct volume and blocked T cells and NK cells from entering the brain following tMCAO. This prevented $IFN\gamma$ producing cells from entering the injured brain, decreased $IFN\gamma$ levels and infarct volume (Liesz et al. 2011). In stroke patients that developed an infection, particularly pneumonia, within 15 days of having a stroke had a worse outcome compared to individuals that did not develop an infection, regardless of stroke severity. Individuals that developed an infection were more likely to have a Th_1 response to myelin basic protein (MBP) and glial acid fibrillary protein (GFAP) at 90 days post stroke, and individuals that generated a higher Th_1 response to MBP at 90 days were more likely to have a poorer outcome regardless of age or baseline stroke severity (Becker et al. 2011). As $IFN\gamma$ is considered a signature

cytokine of a Th₁ response, this could implicate IFN γ as being detrimental following stroke in patients when an inflammatory T cell response is generated against brain antigens.

The Generation of an IFN γ Driven Response to Brain Antigens following Stroke

IFN γ levels in the spleen and in the brain could become increased due to the elevated levels of circulating catecholamines. Increased levels of circulating norepinephrine (NE) and epinephrine have been found in humans and rats after they experience a blockage of the MCA (Meyer et al. 2004; Cechetto et al. 1989). The additional amount of circulating CAs has been attributed to insular cortex damage, which is mainly perfused by the MCA. Damage to this region in patients has also been shown to cause sympathetic dysregulation (Meyer et al. 2004). Furthermore, NE reduces the ability of Th₁ cells to respond when activated, and this effect is thought to be mediated by the presence of β_2 adrenergic receptors on Th₁ cells (Sanders et al. 1997). However, naïve T cells also express β_2 adrenergic receptors. When naïve T cells are exposed to NE, these cells are driven to differentiate into the Th₁ phenotype. Upon reactivation, these cells express 2-4 fold more IFN γ than naïve T cells not exposed to NE (Swanson et al. 2001). This effect of NE on naïve T cells and Th₁ cells may account for the immune dysfunction to pathogens and the increased levels of IFN γ production observed in the spleen following MCAO.

In addition, the cells which are becoming activated in the presence of catecholamines are also being exposed to brain derived antigens that enter circulation following stroke (Herrmann et al. 2000; Wunderlich et al. 1999). This could lead to a Th₁ response to brain antigens, which has been shown in animal studies to result in a more severe injury

(Becker et al. 2005). This may also be mediated through IFN γ , as IFN γ is considered an initiator of a Th₁ response.

Interferon Gamma and the Splenic Response following Cerebral Ischemia

Interferon gamma and the spleen both have the potential to play important roles in exacerbating neural injury following ischemic stroke. The splenic response does contribute to increased neural cell death following brain injuries. Additionally, experiments have measured early IFN γ mRNA levels following experimental stroke in animals, however, no experiments have been conducted to address whether IFN γ protein expression is increased following ischemic stroke. Therefore, **the first set of experiments were designed to test the following hypotheses: 1) increased levels of IFN γ protein are found in the brain and the spleen following pMCAO, and 2) this increased level of IFN γ protein in the brain is connected to the increased levels found in the spleen and this increases neural injury.**

Splenectomy experiments have demonstrated that the splenic response to brain injuries is detrimental. Studies using stem cells to treat experimental stroke have shown that stem cells, including neural stem cells, are more efficacious when administered systemically compared to local administration. These cells have also been found in the spleen following systemic administration and that the spleen is necessary for these cells to exert all their protective effects. The spleen is a major reservoir of immune cells and peripheral immune cells have been found in the brain following stroke. **The second set of experiments were designed to test the following hypotheses: 1) labeling splenocytes *in vivo* prior to pMCAO will allow these cells to be tracked after pMCAO, 2) labeled splenocytes are found in the brain following pMCAO, and 3)**

the splenocytes in the brain will be contributing to exacerbating neural injury directly or indirectly by influencing the environment within the infarct.

IFN γ is known to induce several proteins, many of which are chemokines. IP-10 is of particular interest as it plays a role in influencing the differentiation of naïve Th cells to become Th₁ cells and is a strong chemoattractant for Th₁ cells while subsequently blocking the activation of Th₂ cells. The recruitment of more pro-inflammatory Th₁ cells would result in more IFN γ production and further activation of microglia/macrophages. This would create a feed-forward inflammatory environment in the stroke-injured brain. Blocking or interfering with IFN γ signaling could provide a targeted approach to blunting just the pro-inflammatory response and not the whole immune response that is seen with broad immunosuppressants, like cyclosporine. **The final set of experiments were designed to test the hypotheses: 1) systemic administration of an IFN γ neutralizing antibody 6 h post pMCAO will decrease infarct volume, 2) treatment with a neutralizing antibody against IFN γ will decrease the levels of IP-10 the brain and spleen, and 3) decreased levels of IP-10 will blunt the pro-inflammatory response in the brain by decreasing the number of T cells recruited to the brain following stroke.**

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Chapter 1:

The Spleen Contributes to Stroke Induced Neurodegeneration through Interferon Gamma Signaling

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Note to Reader

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Abstract

Delayed neuronal death associated with stroke has been increasingly linked to the immune response to the injury. Splenectomy prior to middle cerebral artery occlusion (MCAO) is neuroprotective and significantly reduces neuroinflammation. The present study investigated whether splenic signaling occurs through interferon gamma (IFN γ). IFN γ was elevated early in spleens but later in the brains of rats following MCAO.

Splenectomy decreased the amount of IFN γ in the infarct post-MCAO. Systemic administration of recombinant IFN γ abolished the protective effects of splenectomy with a concurrent increase in IFN γ expression in the brain. These results suggest a role for spleen-derived IFN γ in stroke pathology.

Introduction

Current clinical and animal research has shown a complex interplay between the peripheral immune system and the progression of stroke-induced neurodegeneration. The brain communicates with the immune system largely via direct innervation of the lymphoid tissues and humoral control provided by the hypothalamic-pituitary-adrenal axis (Chrousos 1995).

The spleen is a mediator of the immune response to ischemic injury in all organ systems examined. Splenectomy reduces the ischemic-induced immune response in the liver (Okuaki et al. 1996), gastrointestinal system (Savas et al. 2003), kidney (Jiang et al. 2007) and brain (Ajmo et al. 2008). These reports indicate that the presence of the spleen is necessary for promotion of the inflammatory response to ischemic injury which is responsible for delayed cellular death. Splenectomy two weeks prior to middle cerebral artery occlusion (MCAO) in the rat significantly reduces infarct volume with a concomitant decrease in the number of immune cells within the infarct (Ajmo et al. 2008). The inflammatory signal from the spleen to the ischemic brain or other organs has yet to be identified.

Many studies have attempted to decipher the immune signature for an inflammatory response to stroke (Offner et al. 2006; Liesz et al. 2009a; Ren et al. 2010; Becker et al. 2005). Many different gene knockout models of inflammatory cytokines have been

characterized in the field of stroke showing various degrees of increased neuronal death or protection (Lucas et al. 2006; Boutin et al. 2001). One study reports that the deletion of the interferon gamma (IFN γ) gene decreases brain damage after MCAO (Yilmaz et al. 2006). Moreover, when IFN γ neutralizing antibodies are infused intraventricularly three days post-MCAO this protects the brain from stroke induced injury (Liesz et al. 2009b). Also, mice with increased levels of brain IFN γ as a result of over-expression in oligodendrocytes (OL), have increased infarcts compared to wild-type mice (Lambertsen et al. 2004). IFN γ is associated with the Th₁ inflammatory response by activating cells of the monocytic lineage, microglia and macrophages. Since activation of microglia/macrophages is partly responsible for the delayed cellular damage after ischemic insult, this cytokine could play a role in the splenic response by exacerbating the inflammation associated with ischemic injury.

In the present study, we examined the expression of IFN γ after MCAO. We discovered that splenectomy reduced IFN γ expression in the brain after MCAO and that systemic administration of IFN γ reversed the protective effects of splenectomy. These findings indicate that IFN γ may be one of the inflammatory signals originating from the spleen causing a delayed inflammatory response in the ischemic brain.

Materials and Methods

Animal Care

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Male Sprague-Dawley rats (300-350g) were used for the *in vivo* experiments. Postnatal day 3 (P3) rat pups from untimed pregnant female rats were used for *in vitro* primary oligodendrocyte cell culture

experiments and prenatal day 18 (E18) rat embryos from timed pregnant females were used for *in vitro* primary neuron cell culture experiments. All rats were purchased from Harlan Labs (Indianapolis, IN), maintained on a 12 h light/dark cycle (6 am – 6 pm) and given access to food and water *ad libitum*.

Splenectomy

Splenectomies were performed two weeks prior to MCAO by making a midline skin incision at the caudal terminus of the 13th rib on the anatomical left. The abdominal wall was opened along midline and the spleen was externalized through the incision with blunt forceps. The splenic blood vessels were ligated and the spleen was removed. The incision was then closed with sutures, first closing the abdominal cavity and then the skin incision. Sham operations were also performed where the spleen was exteriorized and then reinserted into the cavity.

Laser Doppler Blood Flow Measurement

Laser Doppler was used to monitor blood perfusion (Moor Instruments Ltd, Devon, England). A hole was drilled into the right parietal bone (1 mm posterior and 4 mm lateral from Bregma), and a guide screw was set. The probe was inserted into the guide screw, and the tip of the probe was placed against the pial surface of the brain. Rats that did not show $\geq 60\%$ reduction in perfusion during MCAO were excluded from the study (Ajmo et al. 2006; Ajmo et al. 2008; Hall et al. 2009a)

Permanent Middle Cerebral Artery Occlusion

MCAO surgery was performed using the intraluminal method originally described by Longa *et al.* (Longa et al. 1989) and previously reported (Ajmo et al. 2006; Ajmo et al. 2008; Hall et al. 2009a). Briefly, rats were anesthetized, the common carotid artery

was separated from the vagus nerve, and blunt dissection was performed to isolate the internal carotid artery (ICA), and the external carotid artery (ECA). A 40 mm monofilament was introduced into the ECA, fed distally into the ICA, and advanced approximately 25 mm through the Circle of Willis to the origin of the middle cerebral artery. The filament was tied off at the internal/external carotid junction to produce permanent occlusion. The incision was then sutured closed and the rat was allowed to wake in a fresh cage. Following recovery, animals were randomly assigned into treatment groups.

Recombinant IFN γ Administration

Naïve rats were given increasing doses of rIFN γ until an observable physiological response occurred to determine the optimal rIFN γ dosage. A physiological response to the rIFN γ was determined by the presence of several characteristics: pilo erection, excessive porphyrin production, lethargy, and chills or fever. The rats were monitored every 15 min for 2 h following i.v. injections. The dosage of 20 μ g was the lowest dosage which elicited a physiological response and was used to determine the effects of IFN γ on neural injury in splenectomized and sham-splenectomized rats. The animals were injected intravenously (i.v.), via the tail vein, at 48 and 72 h post-MCAO with 0.21 ml of either 20 μ g (in ddH₂O) of recombinant IFN γ (rIFN γ) (Prospec, Rehovot, Israel) or 0.21 ml ddH₂O.

Brain Extraction and Sectioning

The animals were euthanatized with ketamine/xylazine mix, 75 mg/kg and 7.5 mg/kg respectively, intraperitoneal (i.p.) at 3, 24, 48, 51, 72 and 96 h post-MCAO, and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer.

The brains were harvested, post fixed in 4% paraformaldehyde, and immersed in 20% followed by 30% sucrose in phosphate buffered saline (PBS). Brains were frozen and sliced into 30 μm sections with a cryostat. Coronal brain sections were taken at six points from 1.7 to -3.3 mm from Bregma. Sections were either thaw mounted on glass slides or placed in Walter's Anti-freeze cryopreservative and stored at -20°C .

Fluoro-Jade Staining

Slides were stained with Fluoro-Jade, which labels degenerating neurons. This method was adapted from that originally developed by Schmued *et al.* (Schmued *et al.* 1997) and has been described by Duckworth *et al.* (Duckworth *et al.* 2005). Slides were dried, placed in 100% ethanol for 3 min, 70% ethanol for 1 min, and then ddH₂O for 1 min. Slides were oxidized using a 0.06% KMnO₄ solution for 15 min followed by three 1 min rinses with ddH₂O. Slides were stained in a 0.001% solution of Fluoro-Jade (Histochem, Jefferson, AR) in 0.1% acetic acid for 30 min. Slides were rinsed 4 times with ddH₂O for 3 min, allowed to dry at 45°C for 20 min, cleared with xylene and then cover slipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Infarct Volume Quantification

Fluoro-Jade stained tissue was digitally photographed with Zeiss Axioskop2 (Carl Zeiss INC, Thornwood, NY) microscope controlled by Openlab software (Improvision, Waltham, MA) at a magnification of 1x. Area of neurodegeneration was measured using the NIH ImageJ software. The area of the contralateral side of the brain was also measured and used to compensate for possible edema in the ipsilateral hemisphere. Infarct volumes were then calculated by the total area of ipsilateral staining divided by the total contralateral area for a given animal. Infarct quantification was only done at

96 h post-MCAO because this has been shown to be the time point at which the infarct is stable (Newcomb et al. 2006).

Immunohistochemistry in the Brain

The slides were dried at 45°C for 1 h then rinsed with PBS pH 7.4. Endogenous peroxidase activity was extinguished by incubating the slides for 20 min in 3% hydrogen peroxide. Slides were placed in permeabilization buffer containing 10% serum, 3% 1M lysine, and 0.3% Triton X-100 in PBS for 1 h at room temperature. Next, sections were incubated overnight at 4°C in a primary antibody solution (PBS with 2% serum and 0.3% Triton X-100) in a humidified chamber. Slides were subsequently washed with PBS and incubated with a secondary antibody solution (PBS, 2% serum, 0.3% Triton X-100) for 1 h. For staining with metal-enhanced 3, 3'-diaminobenzidine (DAB) visualization sections were washed in PBS (3 x 5 min) following secondary antibody solution and incubated in an avidin/biotin/horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were washed in PBS, and DAB (Pierce, Rockford, IL) was used for color development. Slides were washed thoroughly with PBS and dried for 1 h at 45°C then dehydrated, rinsed with xylene and cover slipped using DPX (Electron Microscopy Sciences).

For fluorescence staining, the same procedure was followed up to the incubation with the secondary antibody, though sections were not incubated in hydrogen peroxide. Slides were washed with PBS after secondary incubation and then cover slipped using Vectashield hard set mounting media with DAPI (Vector Laboratories). Slides were protected from light during these steps. Double-labeled immunohistochemistry, for IFN γ and immune cell surface markers, was achieved by co-incubating the slides with

primary antibodies raised in two distinct species, followed by co-incubation with secondary antibodies conjugated to distinct fluorophores.

The following primary antibodies were used: goat anti-rat IFN γ (1:200; R&D Systems, Minneapolis, MN), mouse anti-rat CD3 for T cells (1:2,000; BD Biosciences, San Jose, CA), mouse anti-rat CD161 for NK cells (1:1,000; Serotec, Raleigh, NC), mouse anti-rat CD45R for B cells (1:5,000; BD Biosciences), and mouse anti-rat CD11b for microglia/macrophages (1:3,000; Serotec). Horse anti-goat biotinylated antibody (1:300; Vector Laboratories) and Alexa-Fluor® 594 rabbit anti-goat (1:300; Invitrogen, Carlsbad, CA) secondary antibodies were used with the IFN γ antibody. Alexa-Fluor® 488 rabbit anti-mouse (1:300; Invitrogen) secondary was used in conjunction with all other antibodies noted above.

IFN γ Immunohistochemistry in the Spleen

Spleens were fixed in 4% paraformaldehyde overnight. The spleens were then placed in a solution of 20% glycerol and 2% dimethyl sulfoxide (DMSO) and embedded in a gelatin matrix using MultiBrain Technology© (NeuroScience Associates, Knoxville, TN). The block of spleens was rapidly frozen in isopentane with crushed dry ice (-70°C). Using a microtome the block was sliced into 25 μ m sections. Six consecutive sections were taken and collected in Antigen Preservation solution (50% ethylene glycol, 49% PBS pH 7.0, 1% polyvinyl pyrrolidone). The spleen sections were stained free floating in Tris-buffered saline (TBS) solutions. Endogenous peroxide activity was extinguished by treatment with 3% hydrogen peroxide for 15 min. After washing with TBS sections were incubated for 30 min in permeabilization buffer (TBS with 0.3% TritonX-100 and 10% rabbit serum). Following permeabilization, slides were incubated overnight at room temperature with primary antibody in TBS with 2% rabbit serum. The sections were

rinsed with TBS and incubated in secondary biotinylated antibody in TBS with 2% rabbit serum for 1 h. After being rinsed with TBS, sections were incubated with an avidin/biotin/horseradish peroxidase complex (Vectastain Elite ABC kit) for 1 h. Staining was visualized with DAB (Sigma-Aldrich, St. Louis, MO). The sections were then mounted on gelatinized slides, dried, dehydrated, cleared with xylene, and cover slipped with Permount (Fischer Scientific, Pittsburg, PA). The primary antibody used was goat anti-rat IFN γ (1:1,500; R&D Systems,) and the secondary antibody was biotinylated rabbit anti-goat (1:256; Vector Laboratories,).

IFN γ Immunohistochemistry Quantification

IFN γ stained tissue sections were digitally photographed with Zeiss Axioskop2 (Carl Zeiss INC, Thornwood, NY) microscope controlled by Openlab software (Improvision, Waltham, MA) at a 10x magnification. One image from each Bregma point was taken for a total of six images per brain. The area selected for quantification was from the peri-infarct region of the ipsilateral hemisphere for all animals. The images were analyzed for percent of immunostaining per area with ImageJ software. These six values were then averaged for each brain.

Splenic images were taken with a Nikon 90i microscope using a 20x objective and NIS Elements BR 2.30 software at a high resolution. The images were processed and analyzed with Photoshop CS5 (Adobe Systems Inc., San Jose, CA). The intensity of the staining was measured in the histogram for the entire image and the amount of staining per image was analyzed. Six sections per spleen were analyzed for each rat.

Neuronal Cultures

Cortices from E18 rat embryos were dissociated with a solution of 0.25% trypsin/2.21mM EDTA for 10 min at 37°C. The solution was triturated to obtain a uniform single cell suspension. Then 40 ml of DMEM (Mediatech, Manassas, VA) was added and the solution was allowed to settle. The supernatant was transferred to a fresh conical tube and centrifuged at 1000 rpm for 10 min. The supernatant was aspirated off, the pellet was re-suspended in DMEM and the solution was allowed to settle. The debris from the bottom was removed with a pipette and the solution was centrifuged for 10 min at 1000 rpm. The supernatant was aspirated off and the cells were re-suspended in DMEM. Trypan blue exclusion was used to count viable cells and 3×10^5 cells in a final volume of 1 ml were plated in 24 well poly-L-lysine treated culture plates. Twenty-four hours later the media was changed to neurobasal complete (neurobasal media (Invitrogen), B-27 (Invitrogen), 0.05 mM L-glutamine (Mediatech)) for seven days. After a media change, the cells were used for oxygen glucose deprivation (OGD) experiments.

Mixed Glial Cultures

A 2.21mM EDTA solution containing 0.25% trypsin was used to dissociate cortices from P3 rat pups. The suspension was triturated and pelleted. The pellet was re-suspended in DMEM+, which consisted of DMEM (Mediatech), 2.5% fetal bovine serum, 10% horse serum, and 1% antibiotic/antimycotic (Mediatech). After Trypan Blue exclusion to assess cell viability, cells were seeded at a concentration of 1.5×10^7 cells in 75 cm² poly-L-lysine treated tissue culture flasks. The following day the media was changed to fresh DMEM+ and the cultures were incubated at 37°C for 8 days (Gottschall et al. 1995; Rowe et al. 2010).

Oligodendrocyte Purification

To separate the microglia cell fraction from the OL/astrocyte monolayer, flasks were mechanically shaken for 1 h and the media was discarded. Fresh DMEM+ was added to the flasks and incubated for 2 days at 37°C. Following the 2 day incubation, the flasks were mechanically shaken for 18 h to remove the astrocytes from the OLs and microglia. The media was collected and cells were pelleted and re-suspended in fresh DMEM+. Trypan Blue exclusion was used to count the viable cells. The media containing OLs and microglia was added to 10 cm plastic tissue culture dishes at a concentration of 10^7 cells/dish and incubated for 15 min at 37°C. This was repeated three times to assure microglial adherence to the plastic. Following the final incubation the dishes were gently agitated and the media was removed. The cells were pelleted, re-suspended in DMEM+, and plated on poly-L-lysine treated glass coverslips at a concentration of 3×10^5 cells/coverslip (McCarthy and de Vellis 1980). After 24 h the media was changed to neurobasal complete with 10 ng/ml of platelet derived growth factor-AA (PDGF-AA) (Barres et al. 1993; Yang et al. 2005) and the OLs were allowed to proliferate for 7 days. Afterwards, the PDGF-AA was withdrawn for 5 days allowing the OLs to progress to the mature phenotype (Yang et al. 2005). All experiments were conducted on cultures following the 5 day PDGF-AA withdrawal and all cultures used for experiments were 95% pure OLs (Hall et al. 2009b; Rowe et al. 2010).

Oxygen Glucose Deprivation and rIFN γ Administration

Mature OLs that were seeded on glass coverslips in 6 well culture plates were subjected to 24 h of OGD. Neurons that were seeded in 24 well culture plates were subjected to either OGD or normoxia for 24 h. OGD conditions were induced using DMEM without glucose and placing the cultures in an air tight chamber that was flushed with hypoxic

gas (95% N₂, 4% CO₂, and 1% O₂; Airgas, Tampa, Fl) for 15 min and sealed for 24 h at 37°C. Cultures exposed to normoxia were incubated in DMEM with glucose in a standard tissue culture incubator for 24 h at 37°C. The two groups were further divided into cultures that received 20 ng/ml of rIFN_γ or vehicle just prior to the 24 h OGD or normoxic conditions. The concentration of 20 ng/ml of rIFN_γ was previously shown to kill immature oligodendrocytes but not mature OLs (Horiuchi et al. 2006).

Lactate Dehydrogenase Assay

The amount of neuronal and OL cell death was determined using a lactate dehydrogenase (LDH) assay (Takara Bio, Inc, Madison, WI). Following 24 h of OGD or normoxia, the culture media was removed and centrifuged. Then 100 μl of media was added to 100 μl of LDH reagent and incubated in a 96 well plate for 30 min at room temperature protected from light. The plate was then read at 548 nm on the μQuant platereader (Bio-tek, Winooski, VT).

Statistical Analysis

All data are expressed as group mean ± SEM. Significance of the data was determined by ANOVA with a Dunnet's post hoc test for IFN_γ immuno-staining in the brain and neuronal culture survival. Following ANOVA, a Bonferroni's post hoc test was used for the rIFN_γ splenectomy treatment groups. A Tukey's post-hoc test was used following ANOVA to determine significance for the splenic IFN_γ protein levels. A two tailed t-test was used to for OL survival in culture. A value of p<0.05 was considered significant. All sections were blinded prior being analyzed by an investigator.

Results

IFN γ Levels are Increased in the Brain following MCAO

To determine if IFN γ is present in the brain following MCAO, its expression in the infarct was characterized over time. To quantify IFN γ levels, immunohistochemistry for IFN γ was performed on brain sections from sham operated animals and from animals euthanized at 3, 24, 48, 72, and 96 h following MCAO. IFN γ protein levels were significantly increased at 72 h ($p < 0.01$) and remained elevated at 96 h ($p < 0.05$) compared to sham operated rats 96 h after surgery (Fig. 1). Immunohistochemistry was also performed on brain sections from rats that received splenectomy two weeks prior to MCAO and were euthanized at 72 and 96 h post-MCAO. Splenectomy decreased IFN γ protein levels down to those not significantly different from sham MCAO at both 72 and 96 h post-MCAO. Additionally splenectomy reduces IFN γ protein levels significantly at 72 h compared to 72 h post-MCAO only and as well as at 96 h when compared to 96 h post-MCAO only.

IFN γ Protein Levels in the Spleen are Elevated at 24 h following MCAO

Splenic production of IFN γ was measured by immunohistochemical analysis of the spleen. IFN γ protein levels were significantly elevated at 24 h post-MCAO compared to 48, 72, and 96 h post-MCAO, and also elevated compared to the sham-operated rats at 48 and 96 h after surgery ($p < 0.0002$). Naïve spleens showed very low levels of IFN γ protein expression (Fig. 2).

IFN γ Expression by T Cells, NK Cells, and B Cells in and around the Infarct

Immunostaining for IFN γ was abundant in the infarct of rat brains at 96 h post-MCAO (Fig. 3A). Double staining with antibodies against immune cell markers and IFN γ showed

co-localization of CD3 (T cells) (Fig. 3B), CD161 (NK cells) (Fig. 3C), and CD45R (B cells) (Fig. 3D) with IFN γ . These results indicate T cells, NK cells and B cells were producing IFN γ in and around the infarct. CD11b positive cells did not co-localize with IFN γ staining cells, indicating that microglia/macrophages were not producing IFN γ (Fig. 3E).

T cells, B cells, NK cells, and Microglia/Macrophages are Present in the Ipsilateral Hemisphere following MCAO

Antibodies directed against immune cell surface markers showed that T cells (CD3), NK cells (CD161), B cells (CD45R), and microglia/macrophages (CD11b) are localized in the infarcted area of the ipsilateral hemisphere 96 h following MCAO (Fig. 4A-D). In splenectomized rats, there was a decrease in the immunostaining for T cells, NK cells, and B cells in the injured hemisphere (Fig. 4E-G). Microglia/macrophages in the infarct declined in the ipsilateral hemisphere of splenectomized rats (Fig. 4H). In splenectomized rats the predominant form of microglia/macrophages appear with an amoeboid morphology but these cells still display evident ramifications in the damaged area. Only microglia in the resting, ramified morphology were present in the contralateral hemispheres (Fig. 4I-L).

Administration of rIFN γ following MCAO Abolishes the Protective Effect of Splenectomy

IFN γ production originating from the spleen could contribute to delayed neural death and explain why splenectomy prior to MCAO is neuroprotective. To test this, rats underwent splenectomy or sham-splenectomy two weeks prior to MCAO. Animals were then administered rIFN γ (20 μ g/rat i.v.) or ddH $_2$ O at 48 and 72 h post-MCAO. Infarct volumes,

as measured by Fluoro-Jade staining, at 96 h post-MCAO showed splenectomized rats that received systemic rIFN γ had infarcts that were significantly greater than splenectomized vehicle rats ($p < 0.0001$). The splenectomized rIFN γ rats had infarcts that were not significantly different from either of the sham-splenectomized rat groups (Fig. 5E). There was an average of 5% infarct in the splenectomy vehicle brain sections (Fig. 5C), compared to the average infarcts of (50-70%) for all other treatment groups (Figs. 5A, 5B, and 5D).

Recombinant IFN γ Increases IFN γ Expression in the Infarct of Splenectomized Rats

Immunohistochemical analysis for IFN γ in the brain was performed to determine the effect of rIFN γ administration on levels of this cytokine in the infarct. IFN γ expression was significantly decreased in the infarct of splenectomized-vehicle rats (Fig. 6C) ($p < 0.02$) compared to all other groups (Fig. 6E). The addition of rIFN γ to splenectomized rats (Fig. 6D) increased IFN γ protein levels in the infarct to levels found in rats which underwent sham-splenectomy prior to MCAO (Fig. 6A and B).

Recombinant IFN γ is Not Cytotoxic to Cultured Primary Neurons or OLs

To determine if rIFN γ is directly toxic to neural cells, cultured neurons and OLs were treated with rIFN γ prior to OGD. Cell death as measured with LDH assays show that treatment with rIFN γ does not directly enhance death of neurons (Fig. 7A) in culture under normoxic or OGD conditions. Recombinant IFN γ does not increase the death of cultured OLs (Fig. 7B) exposed OGD conditions. Representative images of primary neuronal (Fig. 7C) and primary OL cultures (Fig. 7D) prior to experimentation are provided.

Discussion

The spleen is a key component in the immune response to ischemic injury of the brain and other organs (Okuaki et al. 1996; Savas et al. 2003; Jiang et al. 2007). Splenectomy is protective in models of ischemic (Ajmo et al. 2008), hemorrhagic (Lee et al. 2008), and severe traumatic brain injury (Li et al. 2011). Together, these studies suggest there is a splenic response that exacerbates neural injury by initiating a delayed inflammatory response.

Notably, $IFN\gamma$ perpetuates the pro-inflammatory response by promoting Th_1 cell differentiation while inhibiting Th_2 cell differentiation. Additionally, it is known to activate numerous immune cell types including microglia/macrophages, NK cells, B cells, and T cells, as well as vascular endothelial cells. Furthermore, this pro-inflammatory cytokine also influences antibody isotype production, up regulates both major histocompatibility complexes (MHC I and MHC II), induces changes in vascular endothelial cell adhesion, and increases the production of reactive oxygen species (Boehm et al. 1997). These actions are detrimental to the survival of compromised neural cells. In particular the enhanced Th_1 response seen with $IFN\gamma$ has been found to be detrimental in ischemic brain injuries. A Th_1 response to brain antigens has been shown in animal studies to result in a more severe injury (Becker et al. 2005) and is a poor prognostic factor regardless of stroke severity in people (Becker et al. 2011).

Experimental data suggest that $IFN\gamma$ plays an important role in exacerbating neural injury, as $IFN\gamma$ knockout mice show reduced infarct volume following transient MCAO (Yilmaz et al. 2006). In contrast, a different study demonstrated that there was no

difference in infarct volume between IFN γ knockout and wild type mice following MCAO (Lambertsen et al. 2004). However, this latter study used a different model of MCAO from the one used in the Yilmaz study. Increased serum levels of IFN γ have been detected in mice 24 h following MCAO (Liesz et al. 2009a).

In stroke patients IFN γ production was reduced 6 h following symptom onset. However, IFN γ expression returns to levels not significantly different than healthy controls 72 h following symptom onset. In these patients, IFN γ was being produced by the innate cells of the immune system, specifically $\gamma\delta$ T cells, NK cells, and natural killer T (NKT) cells (Peterfalvi et al. 2009). Both animal and human studies provide strong support for IFN γ and the innate immune system response in the progression of tissue damage in ischemic brain injury.

Splenic IFN γ protein levels were elevated at 24 h post-MCAO and decrease by 48 h. The spike of IFN γ found in the spleen of the rats suggests it is being produced by innate immune cells, in particular NK cells as they are a major source innate IFN γ (Boehm et al. 1997). This transient spike in IFN γ protein expression in the spleen at 24 h is consistent with rises in IFN γ mRNA in the spleens of mice 22 h following transient MCAO (Hurn et al. 2007).

Our results indicated an increase in IFN γ protein expression in the injured brain at 72 h post-MCAO, with expression remaining elevated at 96 h. These results are consistent with studies examining IFN γ mRNA levels in the brain following MCAO. For example, Li *et al.* (2001) demonstrated that IFN γ mRNA increased in the infarct at 2 days post-MCAO and remained elevated 6 days following MCAO (Li et al. 2001). Other reports

have shown that IFN γ mRNA levels were decreased in the mouse brain at 22 h following MCAO (Offner et al. 2006). An experiment by Liesz *et al.* (2011) in which mice were administered an antibody directed against CD49d (VLA-4) 24 h prior to transient MCAO provides further support for delayed IFN γ production in the brain. Trafficking of T and NK cells into the brain was decreased in the CD49d antibody treated mice compared to control mice. In the same experiment there was also a decrease in the amount of IFN γ mRNA at 72 h post-MCAO in the antibody treated mice compared to the control mice (Liesz et al. 2011). This experiment suggests that T cells and NK cells are a source of IFN γ in the brain at later time points following MCAO which is also consistent with our findings.

The increase in IFN γ expression in the brain at 72 h post-MCAO coincides with the time point at which microglia/macrophages become maximally activated in the brain (Leonardo et al. 2010). As IFN γ is a potent activator of microglia/macrophages, the delay in the activation of these cells in the brain suggests that splenic IFN γ is acting through other immune cells to elicit this delayed effect to the infarct. A direct systemic IFN γ response from the spleen would be expected to cause a more immediate response. Therefore, it is more likely that IFN γ production in the spleen acts on target cells within the spleen and these cells then migrate to other immune organs to interact with other cell types. These cells could then infiltrate the brain stimulating the microglia/macrophages to degrade the infarcted area in the brain. As T cells and NK cells have been found in the peri-infarct region producing IFN γ 96 h following MCAO, the likely sequence of events starts with an initial increase in IFN γ in the spleen leading to delayed neural injury.

Our results suggest the neuroprotection resulting from splenectomy is caused by the loss of IFN γ . Systemic administration of rIFN γ to splenectomized rats resulted in infarct volumes that were not different from sham-splenectomized rats, suggesting that spleen derived IFN γ is responsible for the delayed expansion of the penumbra. Interestingly, sham-splenectomized rats that received rIFN γ did not have larger infarcts than sham-splenectomized rat that received vehicle. This finding suggests the endogenous IFN γ response from the spleen is enough to cause maximal delayed neural damage following a stroke.

Splenectomy reduced the amount of IFN γ protein in the brain following MCAO and administration of rIFN γ restores IFN γ production in the brains of splenectomized animals to levels seen in sham-splenectomized rats. Additionally IFN γ expression was not significantly higher in the brains of sham-splenectomy rats that received rIFN γ compared to rats that received vehicle treatment. This observation provides evidence that the IFN γ from the spleen has a relationship to the IFN γ produced in the brain following MCAO. Whether this is a direct (systemic) or indirect (cellular) relationship is yet to be determined.

As previously reported, splenectomy reduced the number of Isolectin IB4 and myeloperoxidase (MPO) positive cells, activated microglia/macrophages and neutrophils respectively, in the infarcted hemisphere 96 h post-MCAO (Ajmo et al. 2008). Splenectomy reduces the number of peripheral immune cells, specifically T cells, B cells, and NK cells, in the ipsilateral hemisphere and alters the morphology of microglia/macrophages responding to the injury at 96 h following MCAO. A majority of the microglia in the splenectomized rats appear in transitional state with amoeboid-like

cell body with ramifications, not the completely amoeboid morphology observed in MCAO only rats at 96 h. As $\text{IFN}\gamma$ activates microglia/macrophages, the lack of this cytokine would maintain these cells towards a resting state. Therefore, blocking splenic $\text{IFN}\gamma$ could prove to be a therapeutic option in modulating the immune response following ischemic stroke.

Experiments with r $\text{IFN}\gamma$ were performed on cell cultures to ensure that the increase in infarct volume in the splenectomy-r $\text{IFN}\gamma$ group was due to activation of the immune system and not the result of the r $\text{IFN}\gamma$ being directly cytotoxic to neural cells. A previous study demonstrated that $\text{IFN}\gamma$ is not cytotoxic to primary mature OLs at 20 ng/ml (Horiuchi et al. 2006). This concentration was used to treat primary neural cell cultures under normoxic and OGD conditions. Recombinant $\text{IFN}\gamma$ is not directly cytotoxic to cultured neurons or OLs demonstrating that other cells through activation by $\text{IFN}\gamma$, like microglia, are eliciting their cytotoxic effect. This contention is further supported by (Bal-Price and Brown 2001) who showed that $\text{IFN}\gamma$ added to mixed brain cell cultures results in neuronal cell death. The neurotoxic effects of $\text{IFN}\gamma$ appear to be mediated through the activation of microglia/macrophages.

From these various experiments, blocking $\text{IFN}\gamma$ from facilitating a pro-inflammatory response to ischemic stroke is a potential way to reduce injury. Selectively blocking $\text{IFN}\gamma$ signaling will allow for targeting one facet of the immune response, leaving the anti-inflammatory or pro-regenerative facets able to respond to the injury.

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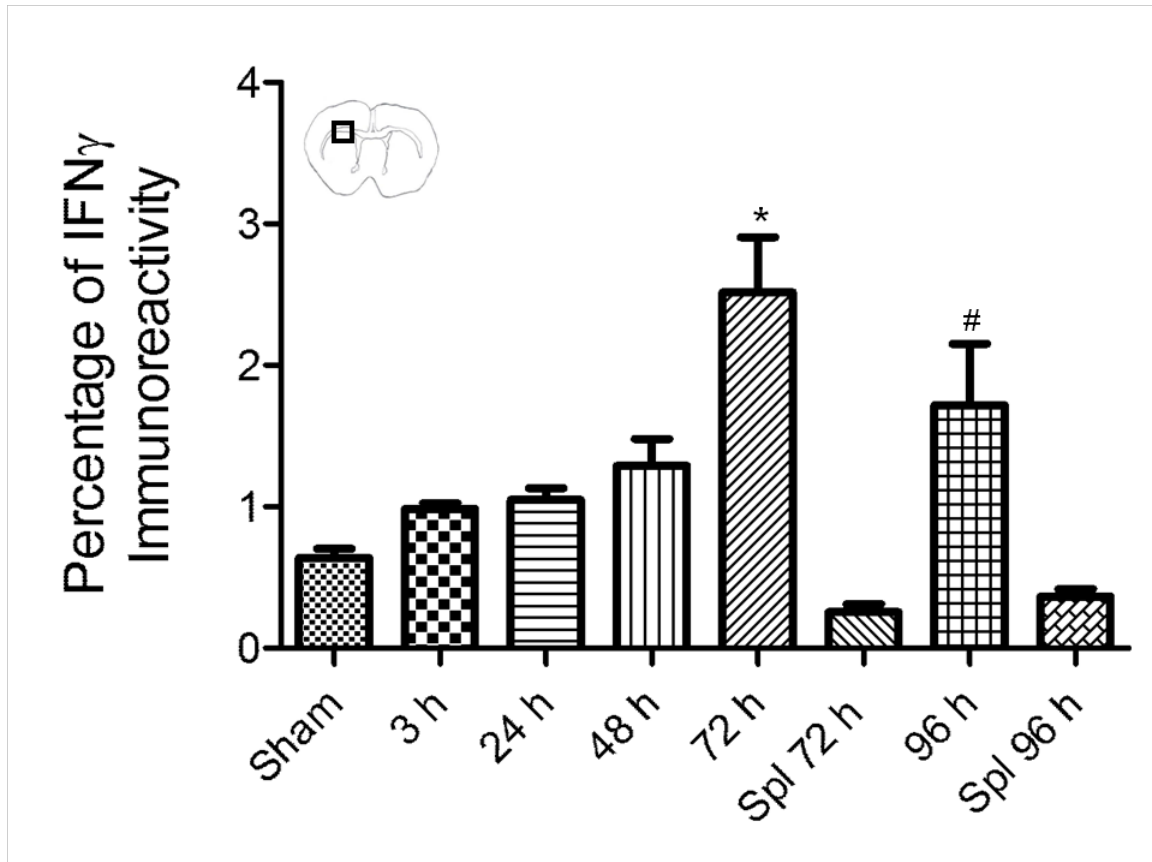


Figure 1: IFN γ levels increase in the injured brain post-MCAO. IFN γ immunohistochemistry of brain tissue from sham operated animals, animals that received splenectomies two weeks prior to MCAO and were euthanized at 72 and 96 h post-MCAO, and animals 3, 24, 48, 72, and 96 h post-MCAO. IFN γ protein levels were significantly higher at 72 and 96 h compared to sham operated animals and animals that received splenectomy prior to MCAO at 72 and 96 h post-MCAO (* $p < 0.01$; # $p < 0.05$). For each group $n \geq 3$. Box in brain graphic depicts area used for quantification of IFN γ levels. Sham denotes a sham MCAO and Spl denotes rats that underwent splenectomy prior to MCAO.

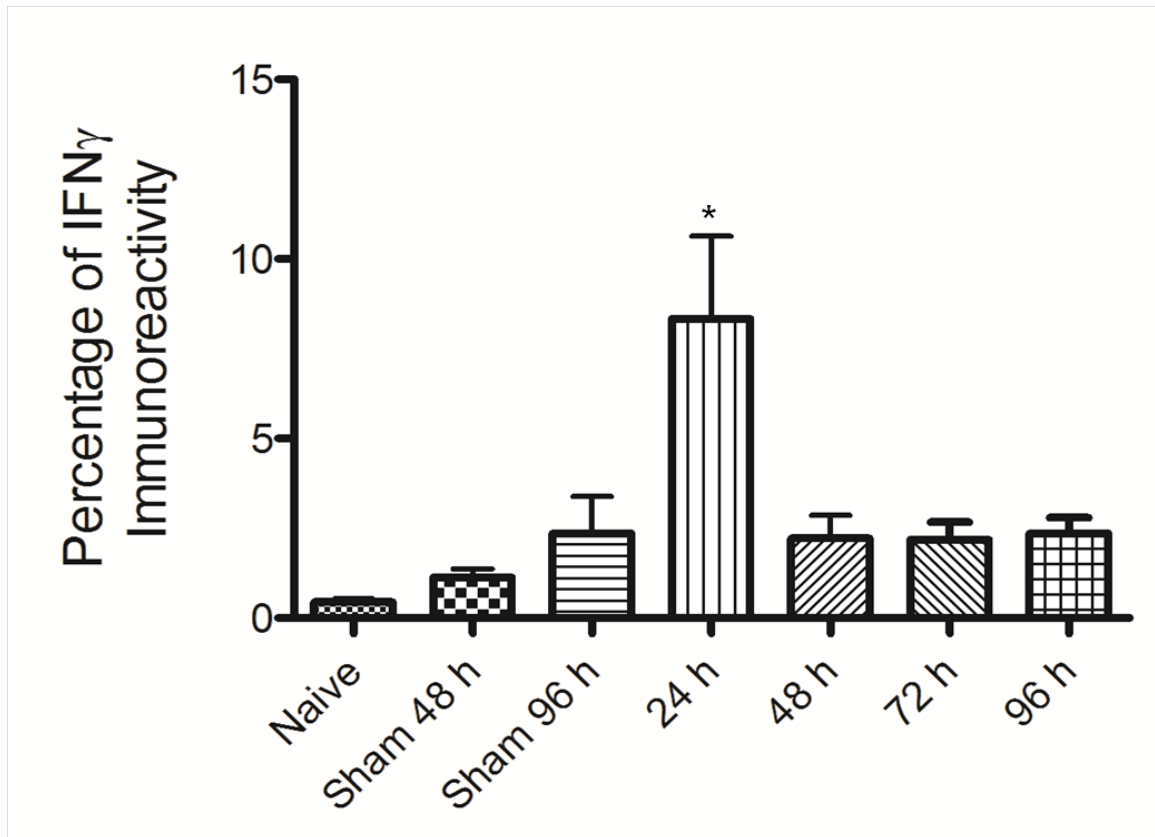


Figure 2: Splenic IFN γ production is elevated at 24 h post-MCAO. Spleens from animals 24, 48, 72, 96 h post-MCAO along with naïve, 48 and 96 h sham-MCAO were assayed using immunohistochemistry for IFN γ . IFN γ protein levels were found to be significantly elevated 24 h post-MCAO (* $p < 0.0002$) compared to the other groups.

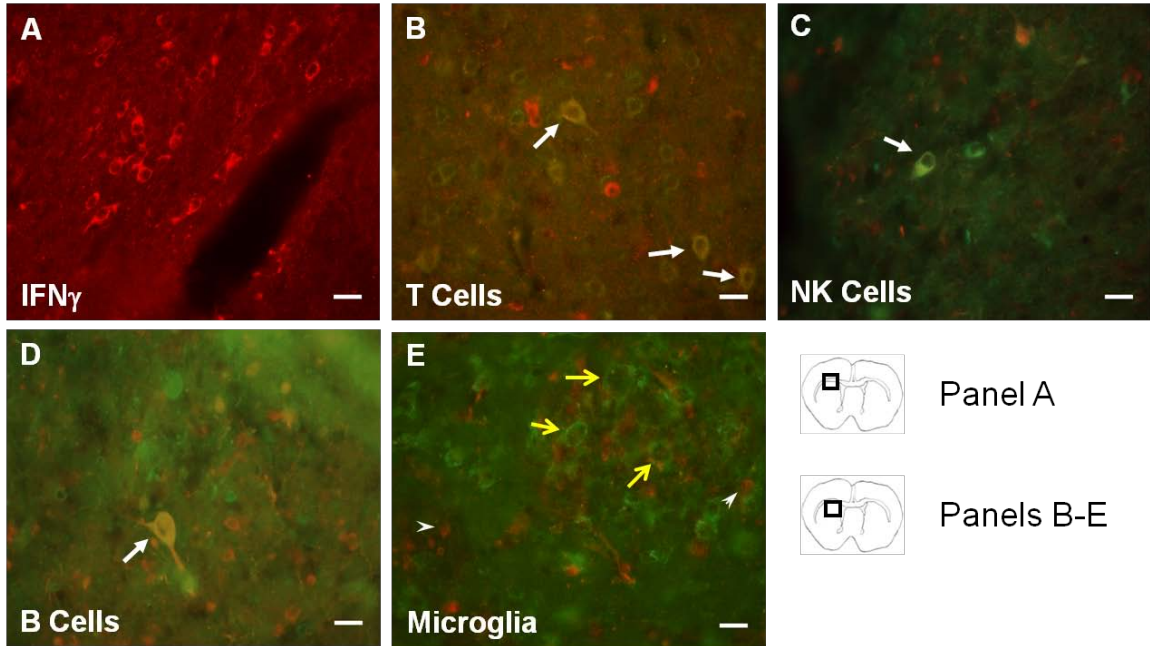


Figure 3: IFN γ expression in immune cells in the brain post MCAO. Representative brain sections from rats 96 h post-MCAO were stained with IFN γ and immune cell surface markers to identify what types of cells are expressing IFN γ in the infarct and peri-infarct. Micrographs show IFN γ (red) (A), and double staining merged images of IFN γ (red) with CD3 (green) for T cells (B), CD161 (green) for natural killer cells (C), and CD45R (green) for B cells (D); yellow cells with white arrows indicate areas of co-localization. A micrograph of staining with CD11b (green) for microglia/macrophages and IFN γ (red) (E) demonstrate a lack of co-localization of CD11b and IFN γ . In figure e, arrow heads indicate IFN γ positive cells and yellow arrows indicate CD11b positive cells. Scale bars = 20 μ m. Box in brain graphics depicts the regions where images were taken for a given micrograph.

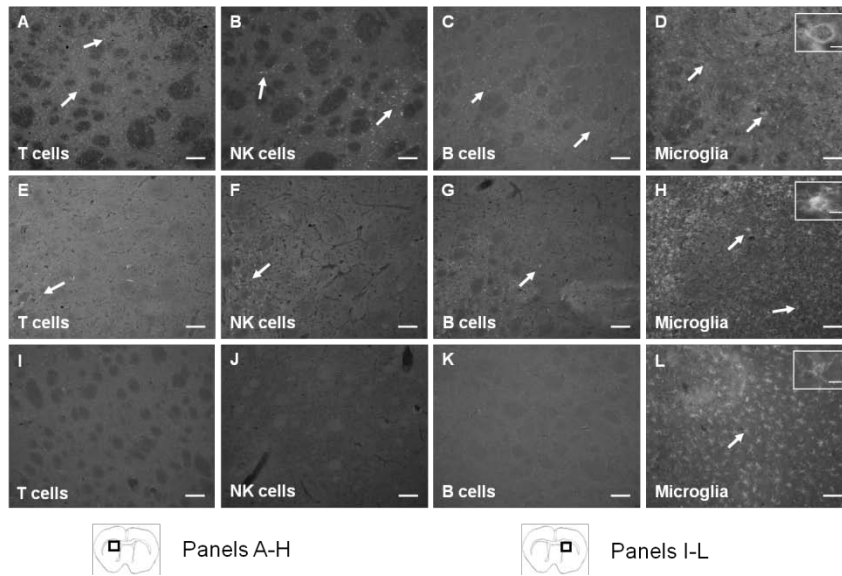


Figure 4: Differences in immune cell infiltrates in the brain following with splenectomy. T cells, B cells, NK cells, and microglia/macrophages are present in the ipsilateral hemisphere following MCAO. At 96 h post-MCAO immunohistochemistry for immune cell surface markers shows peripheral immune cells are present in the ipsilateral hemisphere. Micrographs show CD3 positive cells (T cells) (A), CD161 positive cells (NK cells) (B), CD45R positive cells (B cells) (C), and CD11b positive cells (microglia/macrophages) (D) in the infarcted hemisphere. Micrographs from splenectomized rats demonstrate a decrease in immunostaining for T cells (E), NK cells (F), B cells (G), and microglia/macrophages (H) in the ipsilateral hemisphere. However in the contralateral hemisphere there is an absence of staining for T cells (I), NK cells (J), and B cells (K). Only microglia/macrophages were detected in the contralateral hemispheres (L). Inserts provide representative images of the morphological states of the microglia/macrophages present in each group and show an amoeboid cell (D), an amoeboid cell with evident ramifications (H), and a ramified cell (L). Scale bars = 100 μ m. The scale bar of the inserts = 20 μ m. Box in brain graphics depicts the regions where images were taken for a given micrograph.

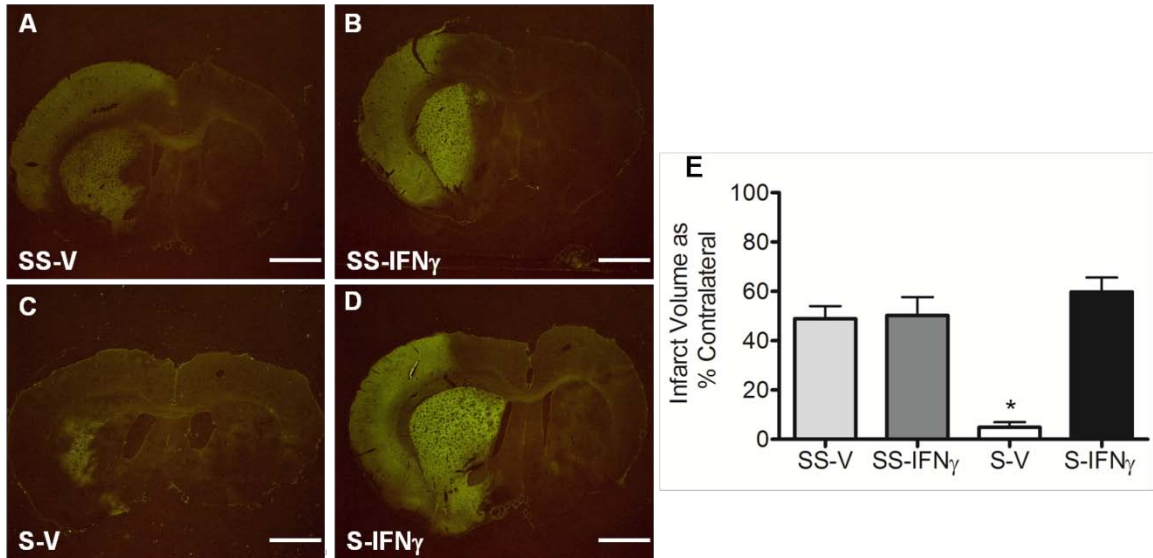


Figure 5: Recombinant IFN γ increases neural injury following MCAO in splenectomized rats. Recombinant IFN γ increases infarct volume in splenectomized rats at 96 h post-MCAO to levels not different from sham-splenectomized rats. Infarct volumes were measured as a percentage of the contralateral hemisphere with Fluoro-Jade staining. Graph depicts average infarct volumes for each group at 96 h post-MCAO (E). The splenectomy-vehicle treated rats had significantly lower infarcts than the other treatment groups (* $p < 0.0001$). The splenectomy-IFN γ treated rats had infarcts that were not significantly different from the sham-splenectomy groups. Representative images for each treatment group at 96 h post-MCAO: sham-splenectomy-vehicle (SS-V) $n=4$ (A), sham-splenectomy-rIFN γ (SS-IFN γ) $n=6$ (B), splenectomy-vehicle (S-V) $n=4$ (C), and splenectomy-rIFN γ (S-IFN γ) $n=6$ (D). Scale bars = 2 mm.

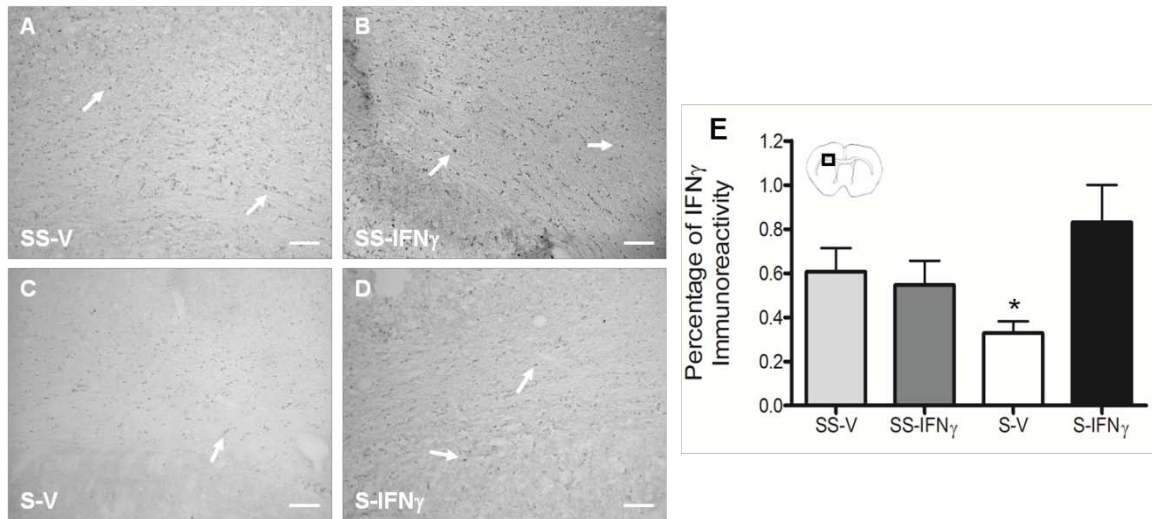


Figure 6: Recombinant IFN γ increases IFN γ expression in the infarct of splenectomized rats. The graph shows splenectomy results in a significant decrease in IFN γ protein expression at 96 h post-MCAO (* $p < 0.02$) (E). However rats that received splenectomy and rIFN γ had IFN γ protein levels not significantly different than the rats which underwent sham-splenectomy prior to MCAO. Representative images from each treatment group at 96 h following MCAO: sham-splenectomy-vehicle (SS-V) (A), sham-splenectomy-rIFN γ (SS-IFN γ) (B), splenectomy-vehicle (S-V) (C), and splenectomy-rIFN γ (S-IFN γ) (D). Box in brain graphics depicts the regions where images were taken for a given micrograph.

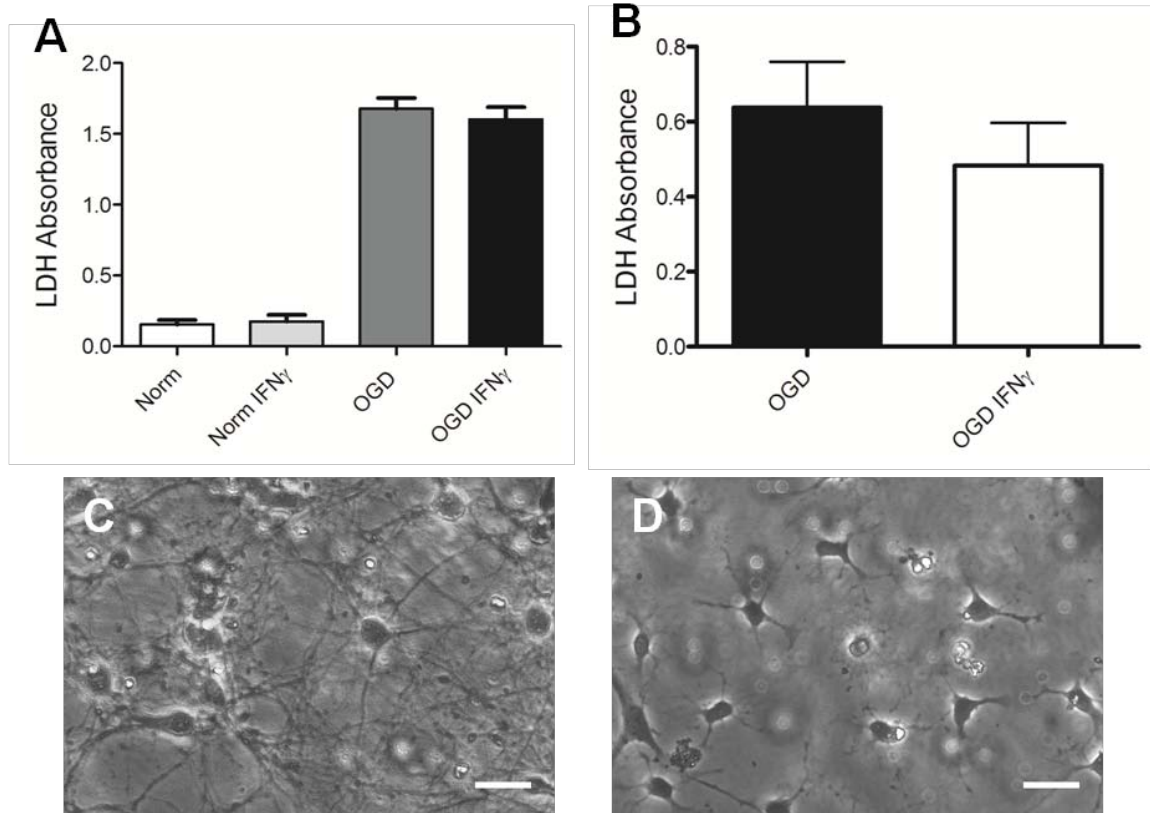


Figure 7: Recombinant IFN γ is not cytotoxic to cultured primary neurons or OLs.

Primary neuronal and OL cultures were treated with 20 ng/ml of rIFN γ under normoxic and OGD for 24 h. Recombinant IFN γ does not increase the amount of cell death, as measured by LDH, in neuronal cultures under normoxic or OGD conditions for 24 h (A). Oligodendrocytes subjected to 24 h of OGD and rIFN γ did not have significantly different survival rates (B). Representative images depict neuronal (C) and OL (D) cultures prior to treatment. Scale bars = 30 μ m.

Chapter 2:

A Transient Decrease in Spleen Size following Stroke Corresponds to Splenocyte Release into Systemic Circulation

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Note to Reader

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Abstract

The splenic response to stroke is a proinflammatory reaction to ischemic injury resulting in expanded neurodegeneration. Splenectomy reduces neural injury in rodent models of hemorrhagic and ischemic stroke, however the exact nature of this response has yet to be fully understood. This study examines the migration of splenocytes after brain ischemia utilizing carboxyfluorescein diacetate succinimidyl ester (CFSE) to label them *in vivo*. The spleen was found to significantly decrease in size from 24 to 48 h following

middle cerebral artery occlusion (MCAO) in rats compared to sham operated controls. By 96 h post-MCAO the spleen size returned to levels not different from sham operated rats. To track splenocyte migration following MCAO, spleens were injected with CFSE to label cells. CFSE positive cell numbers were significantly reduced in the 48 h MCAO group versus 48 h sham and CFSE labeled cells were equivalent in 96 h MCAO and sham groups. A significant increase of labeled lymphocyte, monocytes, and neutrophils was detected in the blood at 48 h post-MCAO when compared to the other groups. CFSE labeled cells migrated to the brain following MCAO but appear to remain within the vasculature. These cells were identified as natural killer cells (NK) and monocytes at 48 h and at 96 h post-MCAO NK cells, T cells and monocytes. At 96 h post-MCAO CFSE labeled cells are producing interferon gamma. Splenocytes are released from the spleen following ischemic brain injury, enter into systemic circulation and migrate to the brain exacerbating neural injury.

Introduction

Stroke is a complex neural injury that progresses through several phases following the initial insult. The peripheral immune system plays a delayed role in the progression of neural injury following stroke. The presence of peripheral immune cells in the brain following ischemic stroke has been well documented in animal studies (Schroeter et al. 1994; Stevens et al. 2002). The role that these cells play in the progression of neural injury following stroke is still being investigated, as the origin of these cells is not clearly understood. Recent evidence indicates that the spleen is at least one of the sources of these immune cells.

The spleen is a large reservoir of immune cells and red blood cells. Its removal reduces neurodegeneration in a variety of brain injury models (Ajmo et al. 2008; Lee et al. 2008; Li et al. 2011; Das et al. 2011). Moreover, splenectomy decreases cellular death following ischemic-reperfusion injury in other organs, including the liver (Okuaki et al. 1996), intestines (Savas et al. 2003), kidney (Jiang et al. 2007), and heart (Leuschner et al. 2010). Splenectomy also decreases the number of immune cells in the brain (Ajmo et al. 2008; Seifert et al. 2012) following middle cerebral artery occlusion (MCAO). Additional studies have shown that preventing peripheral immune cell infiltration is neuroprotective following MCAO. Antibodies generated against cellular adhesion molecules prevent immune cell extravasation into the brain and decrease infarct volume (Chopp et al. 1996; Kanemoto et al. 2002; Liesz et al. 2011). Cytokine production within the brain following MCAO is also decreased with splenectomy (Seifert et al. 2012). These studies illustrate that the spleen is a focal point for the immune response to tissue injury.

In addition to peripheral immune cells being present in the ischemic brain they produce and secrete proinflammatory cytokines while in the brain. Several cytokines and chemokines have up regulated mRNA expression in the brain following transient MCAO in mice. (Chang et al. 2011; Hurn et al. 2007; Offner et al. 2006a) Elevated levels of interferon gamma ($IFN\gamma$) protein have been found in the brain following permanent MCAO in rats (Seifert et al. 2012). Additionally, intracerebroventricular injection of antibodies directed against either tumor necrosis factor alpha ($TNF\alpha$) or $IFN\gamma$ following MCAO decreased infarct volume (Liesz et al. 2009). These data demonstrate that the presence of peripheral immune cells is detrimental following stroke, whether these cells are acting through a cellular cytotoxic mechanism or through the secretion of

proinflammatory cytokines and chemokines. Elucidating the source of these cells should provide some insight into the mechanisms by which these cells elicit a proinflammatory response following ischemic brain injury.

This study aims to determine temporal changes in splenic mass in rats following MCAO to determine if splenic contraction leads to an increase in circulating splenocytes. It has been well documented that there are peripheral immune cells present in the brain hours to days following MCAO (Stevens et al. 2002), however the origin of these cells remains to be elucidated. The spleen contributes to the proinflammatory response following MCAO and is a major reservoir of immune cells. Using carboxyfluorescein diacetate succinimidyl ester (CFSE), a fluorescent molecule that covalently binds to intracellular proteins, allowed splenocytes to be tracked in this study. CFSE is used in lymphocyte trafficking studies *in vivo* (Weston and Parish 1990) and can be detected for up to 8-10 cell divisions (Lyons 2000). It has also been shown to be detected out to 20 days in transplanted hepatocytes (Karrer et al. 1992). This suggests CFSE is a stable molecule that allows cells to be tracked using fluorescence. By labeling splenocytes *in vivo* with CFSE prior to MCAO, splenocyte migration was tracked following MCAO.

Materials and Methods

Animal Care

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Male Sprague-Dawley rats (300-350g) were used for the *in vivo* experiments. All rats were purchased from Harlan Labs (Indianapolis, IN), maintained on a 12 h light/dark cycle (6 am – 6 pm) and given access to food and water *ad libitum*.

Splenic CFSE Injections

Splenic CFSE injections were performed five days prior to MCAO by making a midline skin incision at the caudal terminus of the 13th rib on the anatomical left. The abdominal wall was opened along midline and the spleen was externalized through the incision with blunt forceps. The spleens were injected with 250µl of a 4mg/ml solution of CFSE (Molecular Probes, Eugene, OR) in dimethyl sulfoxide (DMSO). The injections were evenly spaced out in five sites of 50µl per site along the spleen. The spleen was then reinserted into the abdominal cavity. The incision was then closed with sutures, first closing the abdominal cavity and then the skin incision. To obtain baseline CFSE labeling, a group of CFSE only rats were euthanized five days following the splenic injections; at the same time point the other groups underwent MCAO or sham surgeries.

Laser Doppler Blood Flow Measurement

Laser Doppler was used to monitor blood perfusion (Moor Instruments Ltd, Devon, England). A hole was drilled 1 mm posterior and 4 mm lateral from Bregma, and a guide screw was placed. The laser doppler probe was inserted into the guide screw, and the tip of the probe was placed against the surface of the brain. Rats that did not show \geq 60% reduction in perfusion during MCAO were excluded from this study (Ajmo et al. 2006; Ajmo et al. 2008; Hall et al. 2009). Sham operated rats had the guide screw and laser doppler probe placed and blood flow was monitored to ensure that there was not a drop in cerebral blood flow during the sham procedure.

Permanent Middle Cerebral Artery Occlusion

MCAO surgery was performed using the intraluminal method originally described by Longa *et al.* (Longa et al. 1989) and previously reported (Ajmo et al. 2006; Ajmo et al.

2008; Hall et al. 2009). Briefly, rats were anesthetized. Then blunt dissection was performed to isolate the common carotid artery, the internal carotid artery (ICA), and the external carotid artery (ECA). A 40 mm monofilament was introduced into the ECA, fed distally into the ICA, and advanced to the origin of the MCA. The filament was tied off on the ECA to produce a permanent occlusion. The incision was then sutured closed and the rat was allowed to wake in a fresh cage. Sham operations were performed for the CFSE rats at 48 and 96 h post-MCAO to ensure there no confounding factors from the CFSE injections.

Tissue Extraction and Sectioning

The animals were euthanatized with ketamine/xylazine mix, 75 mg/kg and 7.5 mg/kg respectively, intraperitoneal (i.p.) at 3, 24, 48, 51, 72 and 96 h post-MCAO, and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB). The spleens and thymi were removed prior to perfusion. Spleens were weighed immediately following removal and were subsequently placed in RPMI complete (RPMI-1640 without Phenol Red media (Cellgro, Manassas, VA), antibiotic/antimycotic (Cellgro), 29.2 mg/ml L-glutamine, low IgG FBS (Cellgro), and 14.2M 2-mercaptoethanol) cell culture media. The thymi were placed in 4% paraformaldehyde in PB. Thymi were subsequently sliced into 16µm sections with a cryostat and thaw mounted on glass slides. The brains were harvested, post fixed in 4% paraformaldehyde, and immersed in 20% followed by 30% sucrose in phosphate buffered saline (PBS). Brains were frozen and sliced into 30 µm sections with a cryostat. Coronal sections were taken at six points from 1.7 to -3.3 mm from Bregma. Sections were either thaw mounted on glass slides or placed in Walter's Anti-freeze cryopreservative and stored at -20°C. All tissue processing was done under limited lighting to protect the CFSE dye.

Fluoro-Jade Staining

Brain sections mounted on glass slides were stained with Fluoro-Jade, which labels degenerating neurons. This method was adapted from that originally developed by Schmued *et al.* (Schmued et al. 1997) and has been described by Duckworth *et al.* (Duckworth et al. 2005). Slides were dried, placed in 100% ethanol for 3 min, 70% ethanol for 1 min, and then ddH₂O for 1 min. Slides were oxidized using a 0.06% KMnO₄ solution for 15 min followed by three 1 min rinses with ddH₂O. Slides were stained in a 0.001% solution of Fluoro-Jade (Histochem, Jefferson, AR) in 0.1% acetic acid in the dark for 30 min. Slides were rinsed 4 times with ddH₂O for 3 min each time, allowed to dry at 45°C for 20 min, cleared with xylene and then cover slipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Infarct Quantification

Fluoro-Jade stained tissue was digitally photographed with Zeiss Axioskop2 (Carl Zeiss Inc, Thornwood, NY) microscope controlled by Openlab software (Improvision, Waltham, MA) at a magnification of 1x. Area of neurodegeneration was measured using the NIH ImageJ software. To compensate for possible edema in the ipsilateral hemisphere, the area of the contralateral hemisphere was also measured. Infarct volumes were then calculated by the total area of ipsilateral staining divided by the total contralateral area for a given animal. Infarct quantification was done for all animals.

Immunohistochemistry

The slides were dried at 45°C for 1 h then rinsed with PBS pH 7.4. Endogenous peroxidase activity was extinguished by incubating the slides for 20 min in 3% hydrogen peroxide. Slides were placed in permeabilization buffer containing 10% serum, 3% 1M

lysine, and 0.3% Triton X-100 in PBS for 1 h at room temperature. Next, sections were incubated overnight at 4°C in a primary antibody solution (PBS with 2% serum and 0.3% Triton X-100) in a humidified chamber. Slides were subsequently washed with PBS and incubated with a secondary antibody solution (PBS, 2% serum, 0.3% Triton X-100) for 1 h. For staining with metal-enhanced 3, 3'-diaminobenzidine (DAB) visualization sections were washed in PBS (3 x 5 min) following secondary antibody solution and incubated in an avidin/biotin/horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were washed in PBS, and DAB (Pierce, Rockford, IL) was used for color development. Slides were washed thoroughly with PBS and dried for 1 h at 45°C then dehydrated, rinsed with xylene and cover slipped using DPX.

For fluorescence staining, the same procedure was followed up to the incubation with the fluorescently labeled secondary antibody, though sections were not incubated in hydrogen peroxide. Slides were washed with PBS after secondary incubation, dried, rinsed in xylene, and then cover slipped using DPX. Slides were protected from light during these steps. Double-labeled immunohistochemistry, for CFSE and immune cell surface markers or IFN γ was achieved by incubating the slides with primary antibodies, followed by incubation with secondary antibodies conjugated to 594nm fluorophores as CFSE fluoresces at 488nm.

The following primary antibodies were used: goat anti-rat IFN γ (1:200; R&D Systems, Minneapolis, MN), mouse anti-rat CD3 for T cells (1:2,000; BD Biosciences, San Jose, CA), mouse anti-rat CD161 for NK cells (1:1,000; Serotec, Raleigh, NC), and mouse anti-rat CD11b for microglia/macrophages (1:3,000; Serotec). Anti-fluorescein antibodies

were used to detect the presence of any CFSE molecules: Alexa-Fluor® 488 goat-anti fluorescein (1:1,000; Invitrogen, Carlsbad, CA) and biotinylated rabbit anti-fluorescein (1:3,000; Invitrogen). Alexa-Fluor® 594 rabbit anti-mouse (1:300; Invitrogen) secondary was used in conjunction the immune cell surface markers. Alexa-Fluor® 594 goat anti-rabbit (1:300; Invitrogen) were used as secondary antibodies for IFN γ .

Image Capture

Tissue sections that were double labeled were viewed on the Leica SP2 confocal microscope (Leica Microsystems, Buffalo Grove, IL). Images were taken at a magnification of 63x. Each fluorophore was scanned sequentially and then the two images were merged.

Splenic Cell Counts

Fresh spleens from the CFSE injected rats were removed prior to perfusion, weighed, and placed in RPMI complete in stomacher bags. The spleens were disassociated and strained with a 70 μ m filter into a 50 ml conical. The volume of the conical was brought up to 40 ml. The cells were pelleted by centrifuging at 1500rpm for 5 min. The supernatant was discarded and the cells were resuspended in 5 ml of ACK lysis buffer (0.15M NH $_4$ Cl, 1M KHCO $_3$, 0.1M Na $_2$ EDTA in water pH equaled 7.4) for 5 min. The volume was adjusted to 40 ml with RPMI complete and then centrifuged at 1500rpm for 5 min. The supernatant was discarded and the cells were resuspended in 30 ml RPMI complete. Cells were counted using Trypan Blue exclusion of dead cells. Additionally, 40 μ l of the solution was placed on a slide and cover slipped with a 22mm circular cover slip (380mm 2), three slides were used per spleen to obtain CFSE cell counts for each spleen. Using a reticle with a 10x10 grid (1mm 2 area with 10x objective using Zeiss AxioSkop2 microscope, Carl Zeiss Inc) cells were counted in three different areas on

each cover slip. A total number of CFSE positive cells and total number of cells for each area was recorded and then the nine total counts for each rat were averaged to give total number of CFSE positive cells and the percentage stained with CFSE for each time point.

Giemsa Staining and Analysis

Giemsa staining was used to identify the immune cell types within circulation following MCAO. Blood samples were obtained using cardiac puncture at the time of euthanasia. Blood smears were made on by placing a small drop of blood on a non-charged slide. All slides were air dried then fixed with methanol for 5 min. For Giemsa staining slides were washed with distilled water three times for 2 min. The slides were then submerged in Giemsa (Sigma-Aldrich, St. Louis, MO) stain for 4 min. The reaction was stopped by adding distilled water. To remove excess staining an additional three washes with distilled water were performed for 3 min each. Slides were then dried overnight and cover slipped with DPX mounting media. These slides were then used to count the total number of CFSE positive cells on the slide and identify these cells, as well as get a leukocyte count for each animal.

The total number of CFSE cells was determined by counting the number of cells on the total slide, two slides per animal. The cells were also identified using the Giemsa staining. Additionally leukocytes were counted by identifying 100 cells per slide, two slides per animal.

Statistical Analysis

All data are expressed as group mean \pm SEM. Significance of the data was determined by ANOVA with a Dunnet's post hoc test for the spleen weights and the CFSE cells in

the blood. A Tukey's post hoc test was used for the CFSE cells in the spleen. A value of $p < 0.05$ was considered significant. All sections were blinded prior being analyzed by an investigator.

Results

The Spleen Transiently Decreases in Size following MCAO in Rats

Spleens were weighed at different time points following MCAO. Spleen weights were measured at 3, 24, 48, 51, 72, and 96 h post-MCAO and sham procedure. Previously we have demonstrated 96 h post-MCAO as the time point at which the infarct is stable in this permanent model of MCAO (Newcomb et al. 2006). The spleen was found to significantly decrease in size at 24, 48, and 51 h following MCAO compared to sham operated animals ($p < 0.05$). However, spleen weights were not significantly different from sham controls at 3, 72 and 96 h post-MCAO (Fig 8).

CFSE is a Safe and Effective Method to Label and Track Splenocytes in vivo

To track splenocyte migration following MCAO, rat spleens were injected with CFSE to label splenocytes *in vivo*. None of the animals injected appeared ill or died (data not shown). To determine if the presence of CFSE adversely affected splenic cells, Trypan blue exclusion was utilized to assess cell viability. There was no difference in the number of live splenocytes at any of the time points tested (Fig 9A). Additionally CFSE labeled approximately 20 percent of splenocytes by five days (Fig 9B). The CFSE injections did not alter infarct volume (data not shown).

Changes in the Number of CFSE Positive Cells within the Spleen following MCAO

The CFSE only group received splenic injections of CFSE and was euthanized five days later, the time point at which the remaining groups received sham or MCAO surgery. The total number of CFSE positive splenocytes was decreased in the 48 h MCAO group compared to the 48 h sham and the CFSE only groups ($p < 0.0001$; Fig 10). No differences were detected between the 96 h MCAO and sham groups.

MCAO Induces Changes in Circulating Leucocytes and CFSE Positive Cells

Circulating CFSE positive cells were identified by using blood smears and the cell types were determined using Giemsa staining. When blood smears from all the different groups were compared, an overall increase in CFSE positive cells was observed in circulation 48 h following MCAO ($p < 0.0007$). This increase resulted in an increase in circulating lymphocytes ($p < 0.005$), neutrophils ($p < 0.0005$), and monocytes ($p < 0.02$). Giemsa staining revealed a significant increase in lymphocytes at 48 h post-MCAO and in the 96 h sham and MCAO groups ($p < 0.001$). Neutrophils were significantly increased in the 48 h MCAO and 96 h sham groups ($p < 0.001$). In contrast, monocytes were significantly decreased in all groups compared to the CFSE only ($p < 0.0001$) (Table 1).

CFSE Cells Migrate to the Brain post-MCAO

CFSE labeled cells were present in the brains in the ipsilateral hemisphere of animals that underwent the MCAO procedure only and not in the sham operated animals (Fig 11A and D). The cells were mainly located in the striatum of the injured brain at 48 and 96 h post-MCAO (Fig 11B and E). Upon higher magnification cells remained within the cerebral vascular, not extravasating into the brain parenchyma (Fig 11C and F).

Identification of CFSE Positive Cells in the Brain following MCAO

To identify the types of CFSE labeled cells in the brain following MCAO, double labeling with staining for immune cell surface markers was employed. The double labeling demonstrated the presence of CFSE positive monocytes (CD11b) and NK cells (CD161) at 48 h post-MCAO (Fig 12A and B). At 96 h following MCAO monocytes, NK cells and T cells (CD3) were present (Fig 12C-E). There were not any CFSE positive T cells in the brains of animals 48 h post-MCAO (data not shown).

IFN γ Production by CFSE Labeled Cells in the Brain

Since IFN γ is a potent inflammatory cytokine, IFN γ production by CFSE cells was assessed. Double labeling for IFN γ was performed on brain sections to determine if any of the CFSE positive cells were expressing this cytokine. IFN γ staining co-localized with CFSE at 96 h post-MCAO (Fig 13) but not at 48 h following MCAO (data not shown).

Discussion

The spleen is a highly proinflammatory organ following ischemic brain injuries (Ajmo et al. 2008; Lee et al. 2008; Li et al. 2011; Das et al. 2011) as well as ischemic liver (Okuaki et al. 1996), intestine (Savas et al. 2003), kidney (Jiang et al. 2007), and heart (Leuschner et al. 2010) injury. In animal studies splenectomy reduces injury in all of these organs. The spleen uses both a cellular and humoral response to tissue injuries. The humoral response has been characterized following stroke since there are many reports citing the splenic production of inflammatory cytokines and chemokines after this type of brain injury. These cytokines include increased IFN γ protein following MCAO in rats (Seifert et al. 2012). Increased cytokine mRNA for interleukin 1 beta (IL-1 β)

(Schwartz et al. 2008; Lee et al. 2008), TNF α (Lee et al. 2008; Schwartz et al. 2008; Hurn et al. 2007), IFN γ (Hurn et al. 2007), and IL-6 (Hurn et al. 2007; Lee et al. 2008) have been found in mice or rats following ischemic or hemorrhagic stroke. Chemokine mRNA for CXCL2 and CXCL10 have been found to be elevated following MCAO in mice (Offner et al. 2006a; Hurn et al. 2007). The cellular response has not been characterized as well as the humoral response following stroke. There are some data in a mouse model of transient MCAO (Offner et al. 2006b). However, cellular tracking has not been utilized following stroke.

Other investigators have found that the spleen decreases in size following transient MCAO in mice and that this decrease in spleen size is due to apoptosis of the cells and a loss of functional centers within the spleen (Offner et al. 2006b). In rats, permanent MCAO results in a transient decrease in spleen size from 24-72 h post-MCAO. This effect is the result of a catecholamine surge following MCAO (Meyer et al. 2004) which activates α_1 -adrenergic receptors on the splenic capsule causing a contraction of the smooth muscles in the capsule. This leads to the transient decrease in splenic size observed in rats. Blocking the α_1 -adrenergic receptors with prazosin or carvedilol prevents the decrease in spleen size seen at 48 h following MCAO (Ajmo et al. 2009) while carvedilol is also neuroprotective (Savitz et al. 2000; Ajmo et al. 2009). Contraction of the splenic smooth muscle is associated with an increase in circulating white blood cells and erythrocytes (Bakovic et al. 2005). The splenocytes which are released into circulation following MCAO could account for the peripheral immune cells found in the brain after stroke. While there are differences between Offner *et al.* (2006) and the current findings this could be due the different species used in the studies, mice versus rats, or the type of stroke surgery performed in each, transient MCAO versus permanent

MCAO. Further investigations into how the spleen reacts to ischemic strokes in patients will help elucidate the role the spleen plays in stroke pathogenesis.

The spleen responds to MCAO induced injury differently than injury associated with a sham surgery. Sham operated rats at 48 h post-surgery have CFSE positive cell populations within the spleen not different than CFSE only rats. MCAO rats in both the 48 h and 96 h groups and those in the 96 h sham group all have significantly decreased CFSE positive cell populations within the spleen compared to CFSE only and 48 h sham groups. This indicates that the brain injury induces a different splenic response, as CFSE labeled cells are decreased in the spleen at 48 h post-MCAO compared to the 48 h sham operated group. The significant increase in circulating cells seen at 48 h post-MCAO demonstrates the injury to the brain induces splenocyte migration. Another study also found a decrease in splenocyte counts at 48 h post-MCAO compared to sham operated rats (Gendron et al. 2002). However, the sham operation induces a delayed splenic response as there is not a significant difference in the number of CFSE positive cells within the spleen at 96 h in the sham or MCAO treated groups. This indicates that the spleen does have a delayed response to a general insult like a sham operation. The splenic response to the sham operation at 96 h is also supported by the similar increases in circulating lymphocytes in both the 96 h MCAO and the 96 h sham groups.

Interestingly, while the spleen is decreased in size 48 h following MCAO, there is a significant increase in the number of circulating CFSE positive cells, regardless of immune cell type. The total number of circulating monocytes is decreased in all treatment groups compared to the CFSE only group, which was used as a CFSE injection control group. While the total number of circulating monocytes is decreased at 48 h there was a significant increase in CFSE positive monocytes demonstrating most of

the monocytes in circulation following MCAO at 48 h are from the spleen. This is also consistent with evidence that there is a population of undifferentiated monocytes in the spleen that are released into circulation and travel to the heart following myocardial infarction in mice (Swirski et al. 2009). In a transient mouse MCAO model an increase in circulating monocytes was observed at 96 h following MCAO (Offner et al. 2006b), which is inconsistent with the data presented in this study. The differences again could be due to the different models of MCAO and species used for each study. Additionally, few CFSE positive cells were found in the thymus (data not shown).

Circulating CFSE positive cells were found only in the brains of MCAO treated rats at 48 and 96 h following MCAO. There were no CFSE positive cells in the brains of sham-operated rats at any time point. These CFSE positive cells were identified as NK cells and monocytes at 48 h following MCAO, while at 96 h post-MCAO T cells were also present. The cells appear to be localized to the blood vessels. While there is clear documentation of the presence of immune cells within the infarct following MCAO (Stevens et al. 2002), it does not appear that the majority of these cells are of splenic origin. The types of immune cells found in the brain are consistent with the time course for different immune cell populations to appear within the brain following stroke (Stevens et al. 2002). Following the temporal pattern of immune cell infiltration 48 h post-MCAO, there was the presence of monocytes and a lack of CFSE positive T cells. Additionally, the presence of both T cells and monocytes 96 h post-MCAO is consistent with the infiltration of these cells, as T cells are found later as part of the delayed adaptive immune response (Stevens et al. 2002). The presence of these cells is known to exacerbate delayed neural injury following stroke and removal of the spleen decreases the number of peripheral immune cells that enter the brain (Ajmo et al. 2008; Seifert et al. 2012). Therefore, if splenocytes are not entering the brain parenchyma, but

enhancing the pro-inflammatory response to the injured neural tissue, then the cells might be secreting inflammatory mediators. These inflammatory cytokines or chemokines could be the method by which splenocytes are detrimental following stroke versus being directly cytotoxic to neural cells. Secretion of proinflammatory cytokines which activate microglia/macrophages at the site of injury would be detrimental to the survival of neural cells following stroke.

One highly proinflammatory cytokine that activates microglia/macrophages, $IFN\gamma$, is elevated in brains of rats post-MCAO (Seifert et al. 2012). Additionally, there is an increase in $IFN\gamma$ mRNA days following stroke in mice (Li et al. 2001), and blocking $IFN\gamma$ using neutralizing antibodies injected directly into the brain at 72 h post-MCAO is neuroprotective (Liesz et al. 2009). At 96 h post-MCAO, CFSE positive cells were producing $IFN\gamma$, which is consistent with the cell types that have been documented in the brain 96 h following MCAO (Stevens et al. 2002). This production of $IFN\gamma$ could subsequently activate microglia/macrophages in a proinflammatory manner contributing to increased neural injury. These data are consistent with the time when microglia/macrophages become maximally activated following MCAO (Leonardo et al. 2010). While $IFN\gamma$ producing cells were not found at 48 h following MCAO, there are several other cytokines that could be produced by the cell types present early following a stroke. Possible early proinflammatory mediators include IL-12, IL-8, IL-18, and CXCL2. IL-12 and IL-18 are strong inducers of $IFN\gamma$ synthesis by T cells and NK cells (Schroder et al. 2004). While IL-8 and CXCL2 are highly chemotactic and both are up regulated following MCAO (Newman et al. 2005; Hurn et al. 2007; Offner et al. 2006a). Expression of any or all of these molecules would increase neural injury following stroke.

This set of experiments has identified the cellular splenic response following permanent MCAO in rats using CFSE as a way to track immune cell migration following MCAO. There is a transient decrease in splenic size, likely mediated by catecholaminergic activity following stroke. This decrease in spleen size is associated with an increase in circulating splenocytes which migrate to the brain and secrete the proinflammatory cytokine $IFN\gamma$. This production of $IFN\gamma$ coincides with the time when microglia/macrophages are becoming maximally activated and have the ability to create delayed neural injury. These data provide some insight into the mechanism by which the spleen is detrimental in ischemic brain injuries and why splenectomy is neuroprotective in these types of injuries. Further investigation into the splenic reaction in stroke patients would provide insight into how the peripheral immune system can be modulated following stroke to improve neurological outcomes.

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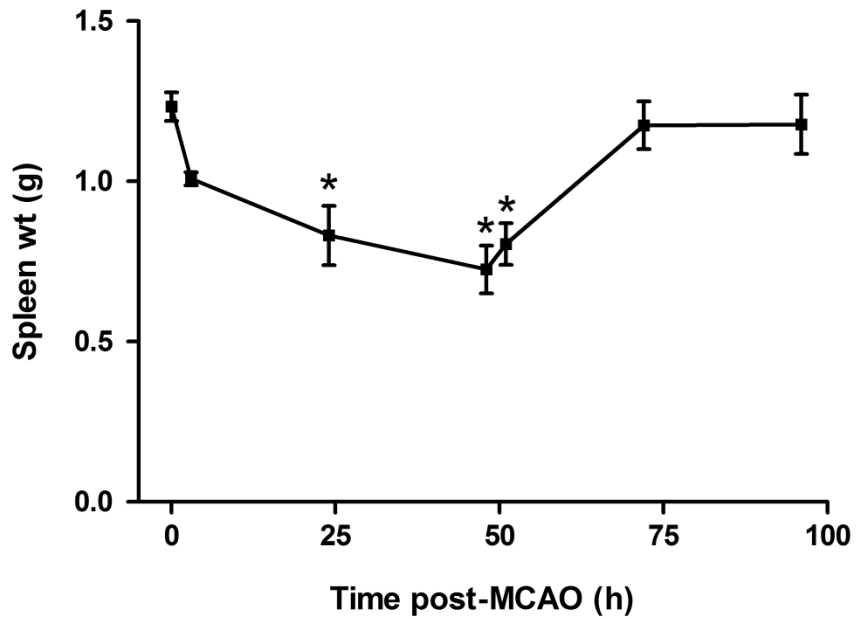


Figure 8: The spleen transiently decreases in size following MCAO in rats. Mean spleen weights plotted over time following MCAO. Spleen weights were significantly decreased by the 24, 48, and 51 h time points compared to sham operated controls (* $p < 0.05$). Spleen weights were not significantly different from sham operated controls at the 3, 72, and 96 h time points.

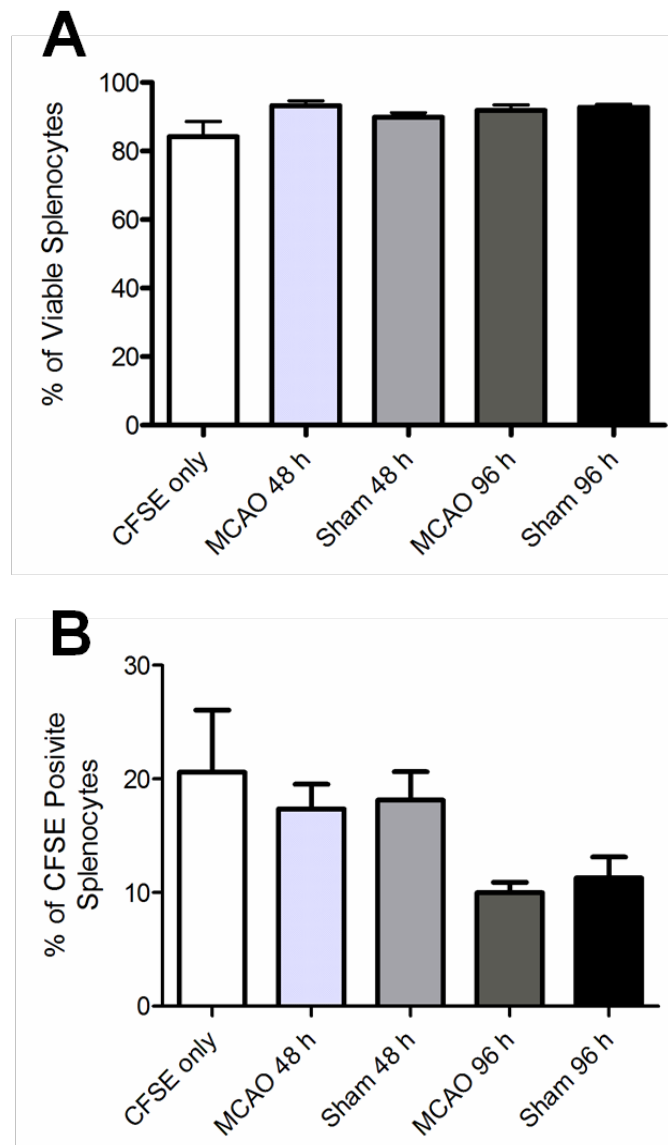


Figure 9: CFSE is a safe and effective method to label splenocytes *in vivo*. When CFSE is injected directly into the spleen, it is not cytotoxic. CFSE was found to be safe when used out to nine days following the initial injections (96 h post-MCAO) (A). Five days following splenic injection of CFSE an average of 20% of splenocytes were CFSE positive (B). The CFSE only group received splenic CFSE injections and was euthanized 5 days post injection, the same time point at which the other groups underwent MCAO or sham surgery.

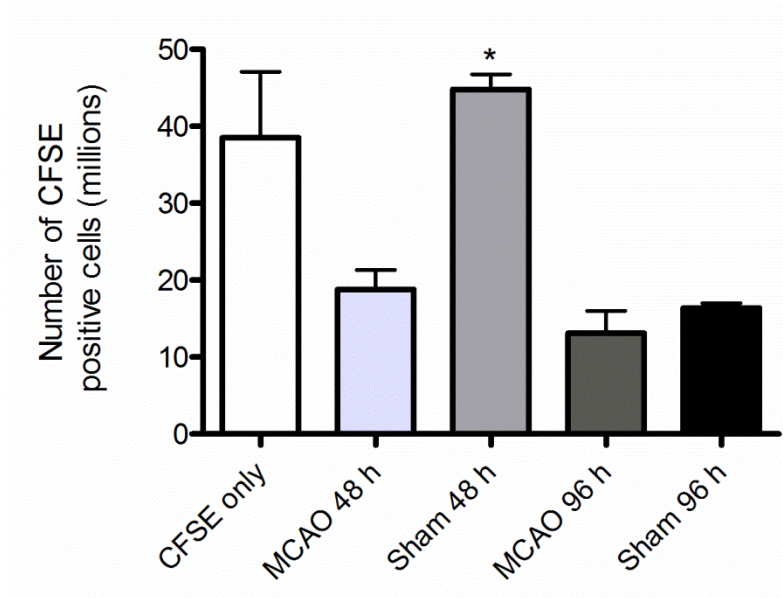


Figure 10: The number of CFSE positive cells within the spleen decreases following MCAO. The total number of CFSE positive splenocytes was increased in the 48 h sham MCAO group compared to all the other groups except the CFSE only group (* $p < 0.0001$).

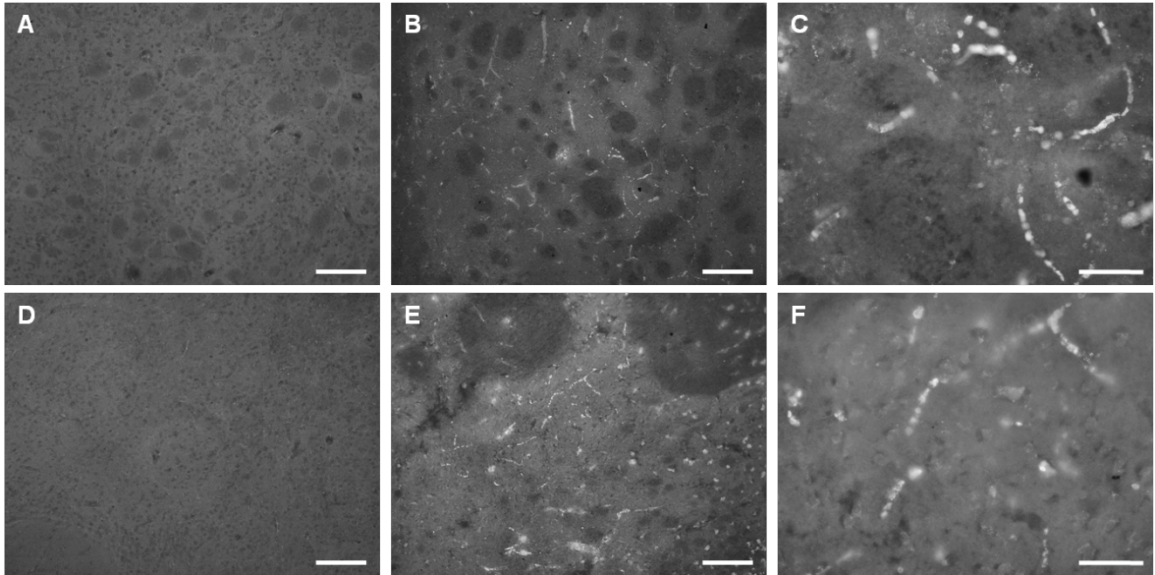


Figure 11: CFSE cells migrate to the brain post-MCAO. Micrographs A, B and C are from 48 h following surgery showing representative sections from sham (10X), MCAO (10X) and MCAO (40X), respectively. Micrographs D, E and F are from 96 h following surgery showing representative sections from sham (10X), MCAO (10X) and MCAO (40X), respectively. CFSE labeled splenocytes are present in brain sections from the rats that underwent MCAO and reside primarily in blood vessels. Scale bars for A, B, D, and E = 100 μ m. Scale bars in C and F = 50 μ m.

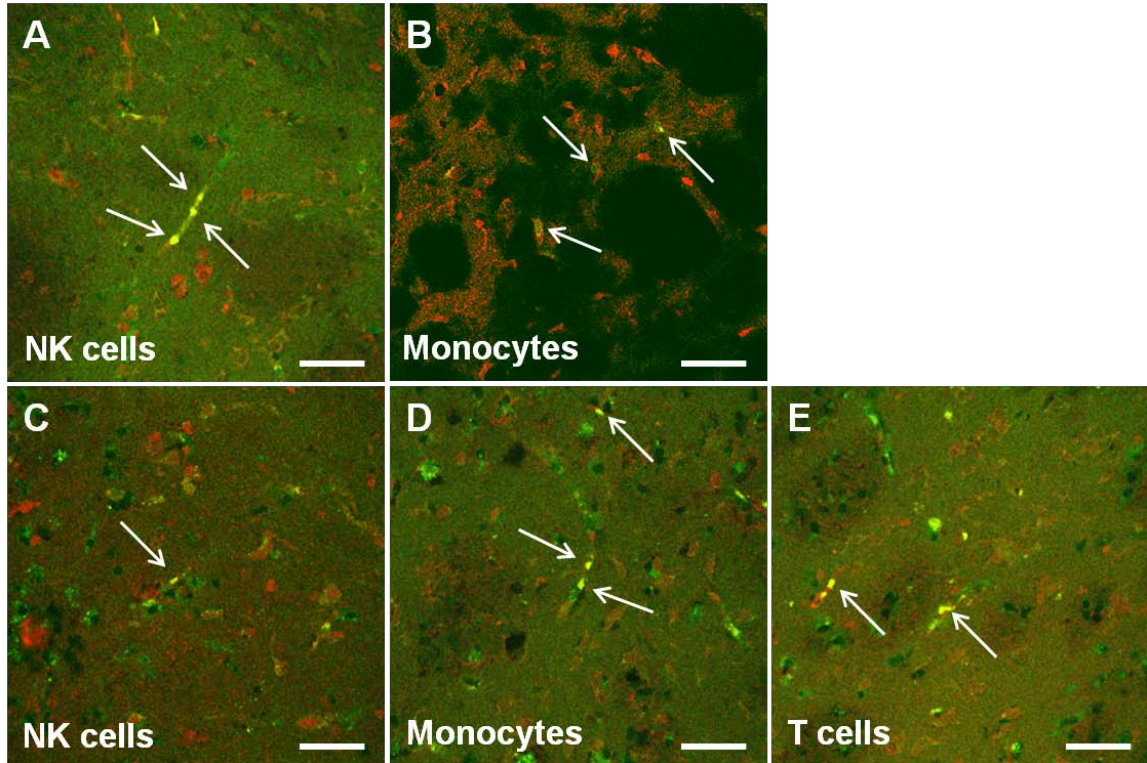


Figure 12: Identification of CFSE positive cells in the brain following MCAO. Brain sections from CFSE treated rats at 48 and 96 h post-MCAO were immunostained with antibodies that recognize CD161 (NK cell), CD11b (monocytes), and CD3 (T cell). CD161 expression co-localized with CFSE labeled cells at 48 h (A) and 96 h (C). CD11b co-localized with CFSE labeled cells at 48 h (B) and 96 h (D). CD3 immunoreactivity was only detected with CFSE at 96 h (E). Arrows indicate double labeled cells. Scale bars = 50µm.

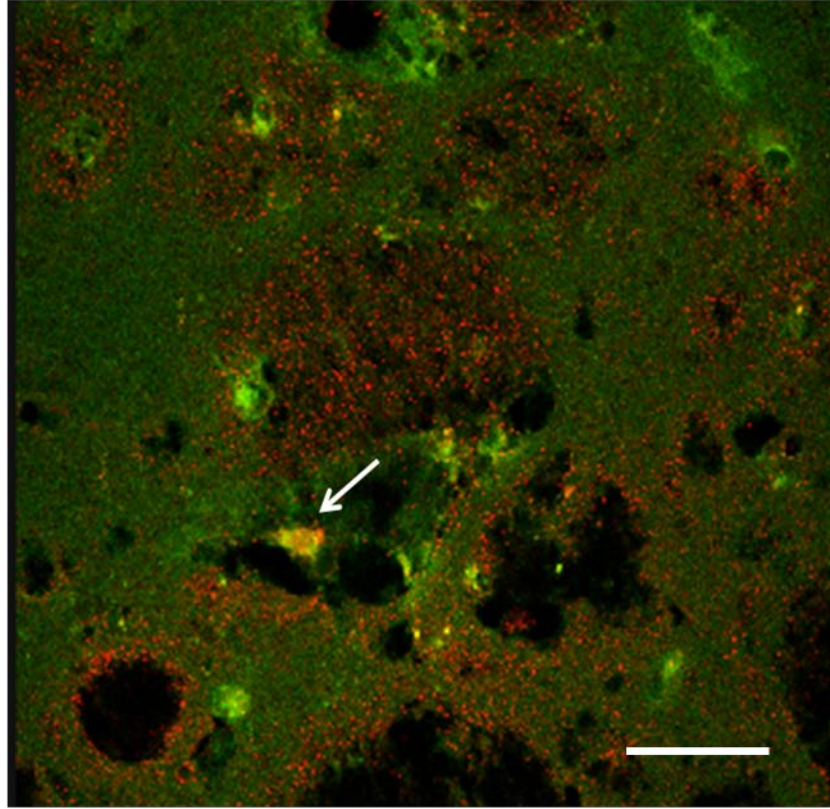


Figure 13: IFN γ production by CFSE positive cells in the brain. Brain sections from a CFSE injected rat at 96 h post-MCAO were immunostained with antibodies that recognize IFN γ . Antibodies directed against IFN γ co-localized with CFSE labeled cells at 96 h. Arrow indicates area of co-localization of labeling. Scale bars = 50 μ m.

Table 1: CFSE positive cells significantly increase in the blood at 48 h in MCAO operated rats.

	CFSE only ^a	48 h MCAO	48 h Sham	96 h MCAO	96 h Sham
Blood Smears - CFSE Labeled Cells (mean count of entire smear) *p<0.05 vs. all other groups					
Total CFSE labeled cells	0.25	9.50*	3.00	3.00	1.25
Lymphocytes	0.25	3.25*	1.00	0.83	0.25
Neutrophils	0.00	2.50*	1.25	1.00	0.75
Monocytes	0.00	3.00*	0.75	0.83	0.75
Basophils	0.00	0.75	0.00	0.33	0.00
Blood Smears - Giemsa Staining (mean count out of 100 leukocytes) *p<0.05 vs. CFSE only					
Lymphocytes	25.50	39.00*	31.25	65.17*	58.50*
Neutrophils	13.25	38.38*	23.25	21.00	27.88*
Monocytes	59.25	20.00*	43.00*	10.42*	10.50*
Basophils	1.13	1.63	1.50	2.50	1.00

In 48 h post MCAO operated rats, CFSE positive cells were significantly increased in the blood compared to all other groups (* p<0.0007). With Giemsa staining there was a significant decrease in monocytes in the blood in all treatment groups compared to CFSE only. There was an increase in lymphocytes in all surgery groups but was only significant in the 48 h MCAO, 96 h sham and MCAO groups. Additionally there was an increase in neutrophils in all groups that was only significant in the 48 h MCAO and 96 h sham groups (* p ≤ 0.02).

^a CFSE only rats were euthanized at 5 days post injection, this is the same time point all other groups underwent MCAO or sham surgery.

Chapter 3:

Interferon-inducible Protein 10 Levels Increase following Stroke and Inhibition of Interferon Gamma Signaling reverses this Increase

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Abstract

The delayed immune response to stroke is responsible for the increased neural injury that continues to occur after the initial ischemic event. This delayed immune response has been linked to the spleen, as splenectomy prior to middle cerebral artery occlusion (MCAO) is neuroprotective. Previous studies have shown that the inflammatory cytokine interferon gamma (IFN γ) is linked to the splenic response enhancing neural injury following MCAO. Presence of IFN γ activates the expression of the inflammatory interferon-inducible protein 10 (IP-10). This study was designed to determine if IP-10 via IFN γ signaling is up regulated in the brain and spleen following MCAO. Expression of IP-10 was found to increase in the brain and the spleen following MCAO. In an attempt to

block IFN γ signaling and increases in IP-10 antibodies recognizing IFN γ were administered. IFN γ neutralizing antibody administration reduced the levels of IP-10 in the brain but not in the spleen. T cell infiltration was reduced in the MCAO-damaged brains of the IFN γ antibody treated animals relative to ones receiving isotype antibodies. Neutralizing IFN γ interferes with the pro-inflammatory cascade that involves IP-10 and the recruitment of T cells to the brain following ischemic stroke.

Introduction

The spleen has been shown to be largely responsible for the peripheral immune response which increases neural injury after ischemic stroke. The spleen is a large reservoir for immune cells and splenectomy prior to middle cerebral artery occlusion (MCAO) is neuroprotective in rats (Ajmo et al. 2008) and mice (Jin et al. 2013). Interferon gamma (IFN γ) is a pro-inflammatory cytokine that relays the splenic response to MCAO. IFN γ levels increase in the brain 72 h post MCAO in rats (Seifert et al. 2012b) and mice (Jin et al. 2013). Splenectomy decreases the elevated levels of IFN γ in brain following MCAO (Seifert et al. 2012b; Jin et al. 2013). Additionally, splenocytes have been tracked *in vivo* following MCAO and these cells migrate to the injured brain (Seifert et al. 2012a). Splenic IFN γ levels spike 24 h post MCAO (Seifert et al. 2012b) suggesting the spleen plays a detrimental role following stroke through IFN γ .

It has been well documented that IFN γ increases following experimental stroke (Offner et al. 2006; Hurn et al. 2007; Seifert et al. 2012b; Jin et al. 2013) and that blocking it is neuroprotective (Liesz et al. 2009; Liesz et al. 2011; Yilmaz et al. 2006). Therefore, interfering with IFN γ signaling should result in decreased infarct volume and reduced

inflammation following stroke. This delayed increase in IFN γ protein in the brain following MCAO indicates that this inflammatory cytokine is a relevant therapeutic target. Downstream signaling of IFN γ induces the expression of the chemokine interferon-inducible protein 10 (IP-10), also known as CXCL10. IP-10 is a pro-inflammatory chemokine that selectively drives the propagation of the Th₁ response by interacting with CXCR3 receptor (Loetscher et al. 2001). Microglia/macrophages produce IP-10 in response to IFN γ stimulation (Luster 2002) and IP-10 cause the chemotaxis of Th₁ cells to the site of injury. In addition, IP-10 can prevent the activation of Th₂ cells by competitive antagonism of the CCR3 receptor (Loetscher et al. 2001). This interaction of IP-10 with CXCR3 and CCR3 creates a pro-inflammatory feed forward mechanism recruiting more IFN γ producing cells to the site of injury, leading to more IFN γ production which in turn leads to more IP-10 production.

Studies on IP-10 in experimental stroke show that IP-10 mRNA is up regulated early in the brain at 6 and 22 h following MCAO in mice. In the spleen IP-10 mRNA levels are increased at 22 h post MCAO in mice (Offner et al. 2006; Hurn et al. 2007). Protein levels of IP-10 have not been quantified in the brain or spleen following MCAO or at longer time points after MCAO. Further investigation into the role of IP-10 following brain ischemia will elucidate the IFN γ /T cell response in stroke.

This study was designed to determine if IP-10 protein levels are significantly elevated in the brain and the spleen following MCAO. Previous studies have demonstrated increased levels of IFN γ , the main inducer of IP-10 synthesis, in the brain (Seifert et al. 2012b; Jin et al. 2013) and the spleen post MCAO (Seifert et al. 2012b). A neutralizing antibody directed against IFN γ was administered starting 24 h following MCAO to

determine if delayed systemic blocking of IFN γ would result in reduced neural injury. IP-10 levels were also measured in the spleen and the brain. T cell recruitment to the brain was also investigated as IP-10 is a chemoattractant for Th₁ cells. Blocking IFN γ will provide insight into the entire IFN γ , IP-10, T cell recruitment inflammatory loop following stroke.

Methods and Materials

Animal Care

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Male Sprague-Dawley rats (300-350g) were used for the following experiments. All rats were purchased from Harlan Labs (Indianapolis, IN), maintained on a 12 h light/dark cycle (6 am – 6 pm) and given access to food and water *ad libitum*.

Laser Doppler Blood Flow Measurement

Laser Doppler was used to monitor blood perfusion (Moor Instruments Ltd, Devon, England). A hole was drilled 1 mm posterior and 4 mm lateral from Bregma, and a guide screw was placed. The laser doppler probe was inserted into the guide screw, and the tip of the probe was placed against the surface of the brain. Rats that did not show \geq 60% reduction in perfusion during MCAO were excluded from this study (Ajmo et al. 2006; Ajmo et al. 2008; Hall et al. 2009). Sham operated rats had the guide screw and laser doppler probe placed and blood flow was monitored to ensure that there was not a drop in cerebral blood flow during the sham procedure.

Permanent Middle Cerebral Artery Occlusion

MCAO surgery was performed using the intraluminal method originally described by Longa *et al.* (Longa *et al.* 1989) and previously reported (Ajmo *et al.* 2006; Ajmo *et al.* 2008; Hall *et al.* 2009). Briefly, rats were anesthetized. Blunt dissection was performed to isolate the common carotid artery, the internal carotid artery (ICA), and the external carotid artery (ECA). The ECA was ligated and cut. Then a 40 mm monofilament was introduced into the ECA, fed distally into the ICA, and advanced to the origin of the MCA. The filament was tied off on the ECA to produce a permanent occlusion. The incision was then sutured closed and the rat was allowed to wake in a fresh cage.

Treatment Injections

A goat anti-rat polyclonal IFN γ neutralizing antibody (R&D Systems, Minneapolis, MN) and a goat IgG isotype antibody (R&D Systems) were reconstituted with phosphate buffered saline (PBS) to a concentration of 100 μ g/ml. Animals in the antibody treatment study were randomly assigned to one of three treatment groups: IFN γ neutralizing antibody, IgG isotype control or the PBS control. Beginning at 24 h post MCAO animals were administered either 5 μ g (0.05 ml) of a goat anti-rat IFN γ neutralizing antibody, a goat IgG isotype control, or an equivalent amount of PBS via an intraperitoneal (i.p.) injection. Treatment was administered at 24, 48, and 72 h post MCAO.

Tissue Extraction and Sectioning

The animals were euthanatized with a ketamine/xylazine mix, 75 mg/kg and 7.5 mg/kg respectively, i.p. at 24, 48, 72 or 96 h post-MCAO for the time course experiment and at 96 h post-MCAO for the antibody treatment experiment. Anesthetized animals were then

perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB). The spleen and thymus were removed prior to perfusion. Spleens were weighed immediately following removal and were subsequently snap frozen and stored in the -80°C freezer with the thymi. The brains were harvested, post fixed in 4% paraformaldehyde, and immersed in 20% followed by 30% sucrose in PBS. Brains were frozen and sliced into 30 µm sections using a cryostat. Coronal sections were taken at six points from 1.7 to -3.3 mm from Bregma. Sections were either thaw mounted on glass slides or placed in Walter's Anti-freeze cryopreservative and stored at -20°C.

Fluoro-Jade Staining

Brain sections mounted on glass slides were stained with Fluoro-Jade, which labels degenerating neurons. This method was adapted from that originally developed by Schmued *et al.* (Schmued et al. 1997) and has been described by Duckworth *et al.* (Duckworth et al. 2005). Slides were dried at room temperature overnight, placed in 100% ethanol for 3 min, 70% ethanol for 1 min, and then ddH₂O for 1 min. Slides were oxidized using a 0.06% KMnO₄ solution for 15 min followed by three 1 min rinses with ddH₂O. Slides were stained in a 0.001% solution of Fluoro-Jade (Histochem, Jefferson, AR) in 0.1% acetic acid in the dark for 30 min. Slides then were rinsed 4 times with ddH₂O for 3 min each time, allowed to dry at 45°C for 20 min, cleared twice with xylene and then cover slipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Infarct Quantification

Fluoro-Jade stained tissue was digitally photographed with Zeiss Axioskop2 (Carl Zeiss Inc, Thornwood, NY) microscope controlled by Openlab software (Improvision, Waltham,

MA) at a magnification of 1x. Area of neurodegeneration was measured using the NIH ImageJ software. The area of the contralateral hemisphere was also measured and used to compensate for possible edema in the ipsilateral hemisphere. Infarct volumes were then calculated by the total area of ipsilateral staining divided by the total contralateral area for a given animal. Infarct quantification was done for all animals.

Immunohistochemistry in the Brain

The slides were dried at 45°C for 1 h then rinsed with PBS pH 7.4. Endogenous peroxidase activity was extinguished by incubating the slides for 20 min in 3% hydrogen peroxide. Slides were placed in permeabilization buffer containing 10% serum, 3% 1M lysine, and 0.3% Triton X-100 in PBS for 1 h at room temperature. Next, sections were incubated overnight at 4°C in a primary antibody solution (PBS with 2% serum and 0.3% Triton X-100) in a humidified chamber. Slides were subsequently rinsed with PBS and incubated with a secondary antibody solution (PBS, 2% serum, 0.3% Triton X-100) for 1 h. For staining with metal-enhanced 3, 3'-diaminobenzidine (DAB) visualization sections were rinsed with PBS following secondary antibody solution and incubated in an avidin/biotin/horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were washed in PBS, and DAB (Pierce, Rockford, IL) was used for color development. Slides were washed thoroughly with PBS and dried for 1 h at 45°C then dehydrated, rinsed twice with xylene and cover slipped using DPX.

For fluorescence staining, the same procedure was followed up to the incubation with the fluorescently labeled secondary antibody, though sections were not incubated in hydrogen peroxide. Slides were rinsed with PBS after secondary incubation, dried,

rinsed twice in xylene, and then cover slipped using DPX. Slides were protected from light during these steps. Double-labeled immunohistochemistry, for IP-10 and immune cell surface markers was achieved by incubating the slides with primary antibodies, followed by incubation with secondary antibodies conjugated to 594nm or 488nm fluorophores.

The following primary antibodies were used: rabbit anti-rat IP-10 (1:5,000; abcam; Cambridge, MA), mouse anti-rat CD3 for T cells (1:2,000; BD Biosciences, San Jose, CA), and mouse anti-rat CD11b for microglia/macrophages (1:3,000; Serotec). Alexa-Fluor® 488 goat anti-rabbit (1:300; Invitrogen) secondary was used for all IP-10 staining. Alexa-Fluor® 594 goat anti-mouse (1:300; Invitrogen) secondary was used in conjunction the immune cell surface markers for double staining with IP-10. Alexa-Fluor® 488 rabbit anti-mouse (1:300; Invitrogen) secondary was used when only staining for CD3 T cell.

IP-10 Immunohistochemistry in the Spleen

Spleens were fixed in 4% paraformaldehyde overnight. The spleens were then placed in a solution of 20% glycerol and 2% dimethyl sulfoxide (DMSO) and embedded in a gelatin matrix using MultiBrain Technology© (NeuroScience Associates, Knoxville, TN). The block of spleens was rapidly frozen in isopentane with crushed dry ice (-70°C). Using a microtome the block was sliced into 25 µm sections. Six consecutive sections were taken and collected in Antigen Preservation solution (50% ethylene glycol, 49% PBS pH 7.0, 1% polyvinyl pyrrolidone). The spleen sections were stained free floating in Tris-buffered saline (TBS) solutions. Endogenous peroxide activity was extinguished by treatment with 3% hydrogen peroxide for 15 min. After washing with TBS sections were incubated for 30 min in permeabilization buffer (TBS with 0.3% TritonX-100 and 10%

rabbit serum). Following permeabilization, slides were incubated overnight at room temperature with primary antibody in TBS with 2% rabbit serum. The sections were rinsed with TBS and incubated in secondary biotinylated antibody in TBS with 2% rabbit serum for 1 h. After being rinsed with TBS, sections were incubated with an avidin/biotin/horseradish peroxidase complex (Vectastain Elite ABC kit) for 1 h. Staining was visualized with DAB (Sigma-Aldrich, St. Louis, MO). The sections were then mounted on gelatinized slides, dried, dehydrated, cleared with xylene, and cover slipped with Permount (Fischer Scientific, Pittsburg, PA). The primary antibody used was rabbit anti-rat IP-10 (abcam) and the secondary antibody was biotinylated goat anti-rabbit (Vector Laboratories,).

IP-10 Immunohistochemistry Quantification

IP-10 stained tissue sections were digitally photographed with Zeiss Axioskop2 microscope controlled by Openlab software at a 10x magnification. Three images from each of 4 Bregma points (1.7 mm to -1.3 mm) were taken for a total of twelve images per brain. The area selected for quantification was the striatum of the ipsilateral hemisphere for all animals. The images were analyzed for percent of immunostaining per area with ImageJ software. These twelve values were then averaged for each brain.

Splenic images were taken with a Nikon 90i microscope using a 20x objective and NIS Elements BR 2.30 software at a high resolution. The images were processed and analyzed with Photoshop CS5 (Adobe Systems Inc., San Jose, CA). The intensity of the staining was measured in the histogram for the entire image and the amount of staining per image was analyzed. Six sections per spleen were analyzed for each rat.

Confocal Image Capture

Tissue sections that were double labeled were viewed on the Leica SP2 confocal microscope (Leica Microsystems, Buffalo Grove, IL). Images were taken at a magnification of 63x. Each fluorophore was scanned sequentially and then the two images were merged.

Statistical Analysis

All data are expressed as group mean \pm SEM. Significance of the data was determined by ANOVA with Fischer's Least Significant Difference post hoc test for all analysis. A value of $p < 0.05$ was considered significant. All sections were blinded prior being analyzed by an investigator.

Results

IP-10 Levels are Elevated in the Brain following MCAO

To determine if IP-10 protein is expressed in the brain following MCAO, its protein expression in the striatum of the ipsilateral hemisphere was characterized over time. To quantify IP-10 levels, immunohistochemistry for IP-10 was performed on brain sections from animals euthanized at 48, 72, and 96 h following MCAO and naïve or sham operated rats. IP-10 protein levels were significantly increased at 72 h and remained elevated at 96 h ($p < 0.01$) compared to sham operated rats 96 h after surgery (Fig 14A). Micrographs of representative sections from the striatum are depicted for naïve (Fig 14B), 48 h (Fig 14C), 72 h (Fig 14D), and 96 h post MCAO (Fig 14E).

Splenic IP-10 Levels Increase after MCAO and Remain Elevated

In the spleen IP-10 protein levels were quantified using immunohistochemistry. Spleens from animals euthanized 24, 48, 72, and 96 h post MCAO and sham or naïve rats were used to perform immunohistochemistry to determine IP-10 protein expression. IP-10 levels were significantly elevated at 24 h and remained elevated out to 96 h following MCAO compared to naïve spleens ($p < 0.0007$). The sham operated animals had increased levels of IP-10 but this did not reach statistical significance (Fig 15A). Micrographs of representative sections from the spleen are depicted for naïve (Fig 15B), sham (Fig 15C), 24 h (Fig 15D), 48 h (Fig 15E), 72 h (Fig 15F), and 96 h post MCAO (Fig 15G).

IP-10 Producing Cells in the Brain following MCAO

IP-10 expression is induced by $IFN\gamma$ in cells of the monocytic origin. Double staining with IP-10 and CD11b, a marker for monocytes, was performed on the brains from animals 96 h post MCAO. IP-10 and CD11b co-localized in the striatum of the ipsilateral hemisphere (Fig 16). This co-localization was determined using confocal microscopy.

$IFN\gamma$ Neutralizing Antibody Administration Decreases Infarct following MCAO

To determine if the pro-inflammatory $IFN\gamma$ signaling pathway contributes to increased neural injury following MCAO, an $IFN\gamma$ neutralizing antibody was administered 24, 48, and 72 h post MCAO. Infarct volumes, as measured by Fluoro-Jade staining, were significantly decreased in the $IFN\gamma$ neutralizing antibody group compared to the vehicle control ($p < 0.007$). When compared to the IgG control the $IFN\gamma$ antibody group was approaching significance ($p = 0.0588$) (Fig 17).

IFN γ Neutralizing Antibody Decreased IP-10 in the Brain

IP-10 protein expression was quantified in the striatum of the ipsilateral hemisphere of the IFN γ antibody, vehicle, and IgG treated rats. The IP-10 immunoreactivity was significantly decreased in the IFN γ antibody treatment group compared to the IgG isotype and PBS controls (Fig 18D). Representative micrographs from the striatum demonstrate IP-10 staining was elevated in the PBS (Fig 18A) and IgG isotype control (Fig 18B) groups compared to IP-10 staining in the IFN γ neutralizing antibody treated group (Fig 18C).

IP-10 Levels in the Spleen Increase with Antibody Administration

IP-10 levels were measured in the spleen of animals treated with PBS, IgG isotype, or IFN γ neutralizing antibody. Splenic IP-10 immunoreactivity was increased in the both the antibody treated groups compared to the PBS treated group (Fig 19D). Representative micrographs show reduced IP-10 staining in the spleens of PBS treated animals compared to rats receiving an antibody (Fig 19A). IP-10 staining was increased equally in the groups of animals that received an IgG (Fig 19B) or IFN γ antibody (Fig 19C).

The Amount of CD3 Immunoreactivity Appears to Decrease in the Brains of IFN γ Antibody Treated Animals

Brain sections from animals that underwent MCAO and then administered an IFN γ neutralizing antibody, an IgG isotype antibody, or PBS were stained using an anti CD3 antibody to visualize the presence of T cells. At 96 h post MCAO the amount of CD3

immunostaining in the striatum appeared to be decreased in the IFN γ antibody treated group (Fig 20C) compared to the IgG (Fig 20B) and PBS (Fig 20A) control groups.

Discussion

The spleen reacts to bodily injuries by eliciting an inflammatory response that further exacerbates the cellular damage. Splenectomy is protective in a variety of ischemic injuries in other organs including the liver (Okuaki et al. 1996), kidney (Jiang et al. 2007), intestines (Savas et al. 2003), and heart (Leuschner et al. 2010). Additionally, removal of the spleen is neuroprotective in several types of brain injuries including ischemic stroke (Ajmo et al. 2008; Jin et al. 2013), intracerebral hemorrhage (Lee et al. 2008), and traumatic brain injury (Li et al. 2011; Das et al. 2011; Walker et al. 2010). Radiation of the spleen following MCAO also reduces infarct volume (Zhang 2013). The removal of splenocytes either physically with splenectomy or with radiation results in protection from ischemic injuries. Splenocytes mediate the expression of inflammatory cytokines and are universally detrimental to ischemic injuries in mouse and rat injury models.

In the spleen the levels of IFN γ spike at 24 h post MCAO (Seifert et al. 2012b). This very transient increase in IFN γ leads to a prolonged increase in the expression of IP-10 that begins at 24 h and remains elevated at least out to 96 h post MCAO. This demonstrates the long lasting effects IFN γ can have even when it is present for only a brief period of time. IP-10 levels are increased in the sham operated animals but the increase is not as high as the animals that underwent MCAO, suggesting the splenic response to a brain injury could cause the production of higher levels of IP-10. The elevated levels of IP-10 at 24 h are consistent with data that found significantly higher mRNA levels of IP-10 in the spleen 22 h following stroke in mice (Offner et al. 2006; Hurn et al. 2007).

IP-10 levels in the brain parallel the expression pattern of IFN γ where expression does not become elevated until 72 h and remain elevated at 96 h post MCAO. These results are consistent with a delayed response and up regulation of protein compared to mRNA levels. Studies looking at mRNA levels of IP-10 in the brain following MCAO found increased mRNA as early as 6 h post MCAO (Offner et al. 2006). As expected, IP-10 within the infarct is expressed in cells of the monocytic lineage (CD11b⁺), which probably are microglia. IFN γ is known to activate monocytes to produce IP-10 (Boehm et al. 1997). Studies that inhibited or neutralized IFN γ activity resulted in decreased infarcts. The problem with these studies is that none of the methods used to inhibit IFN γ are viable treatment options. IFN γ ^{-/-} mice had decreased infarct volumes compared to wild type mice (Yilmaz et al. 2006). Inhibiting IFN γ with a neutralizing antibody injected directly into the brain three days following MCAO decreased infarct volume (Liesz et al. 2009). Additionally, using an antibody directed at CD49d (very late antigen 4, VLA4) prevented immune cells from entering the brain following MCAO leading to decreased infarct volume. This treatment precluded the cells producing IFN γ from entering the injured brain. Animals in this study were pretreated with antibody or administration was started 3 h post MCAO, not extending the therapeutic window (Liesz et al. 2011). A relevant approach to target IFN γ signaling would be to systemically administer an agent directed against IFN γ activity after MCAO.

Systemic administration of an IFN γ neutralizing antibody significantly decreases infarct volume when compared to vehicle controls. These results are consistent with previous observations where IFN γ was blocked (Yilmaz et al. 2006; Liesz et al. 2009; Liesz et al.

2011). Additionally, striatal IP-10 levels in the brain are significantly decreased with IFN γ neutralization. The amount of CD3 immunoreactivity appears to be reduced in the striatum of these rats as well. This is expected as IFN γ is known to induce IP-10 production (Boehm et al. 1997) and IP-10 is a strong chemoattractant for pro-inflammatory IFN γ producing T cells (Groom and Luster 2011). The infarct volumes for the IFN γ neutralizing antibody and the IgG control were approaching significance demonstrating that an increase in the sample size is likely required to attain a statistically significant level. Moreover, a potentially more effective approach would be to use these antibodies in conjunction with pharmaceuticals that block the IFN γ or its downstream intracellular signal transduction.

Administration of a goat antibody that is perceived by the immune system as foreign resulted in a localized inflammatory response in the spleen. Both groups of rats that received a goat antibody had elevated levels of IP-10 compared to the PBS control group. IP-10 production can be induced in the presence of high levels of IFN β or IFN α . However, IFN γ is a more potent inducer of IP-10 (Groom and Luster 2011). The reaction in the spleen is further evidence that a goat neutralizing antibody may not be the most optimal method to inhibit IFN γ or its signaling. As mentioned above, agents exist that block IFN γ receptor activation or its signaling. Its signaling is obstructed by Jak $_{1/2}$ or STAT1 inhibitors. There are several Jak $_{1/2}$ inhibitors currently in clinical trials for rheumatoid arthritis (RA) and cancer. One Jak $_{1/2}$ inhibitor, INCB028050, is in clinical trials for RA and in preclinical trials, using rats, 10mg/kg/d was found to decrease IFN γ signaling by 65 percent in the animal model of RA (Fridman et al. 2010).

IFN γ appears to be a strong target for stroke therapeutics. The reduction in IP-10 and T cell recruitment are two positive effects of inhibiting IFN γ signaling. The next goal would be to identify an agent that selectively targets IFN γ without causing an inflammatory response itself. Targeting only the pro-inflammatory IFN γ response after stroke without shutting down the whole immune system is a potential therapeutic approach worthy of further investigation.

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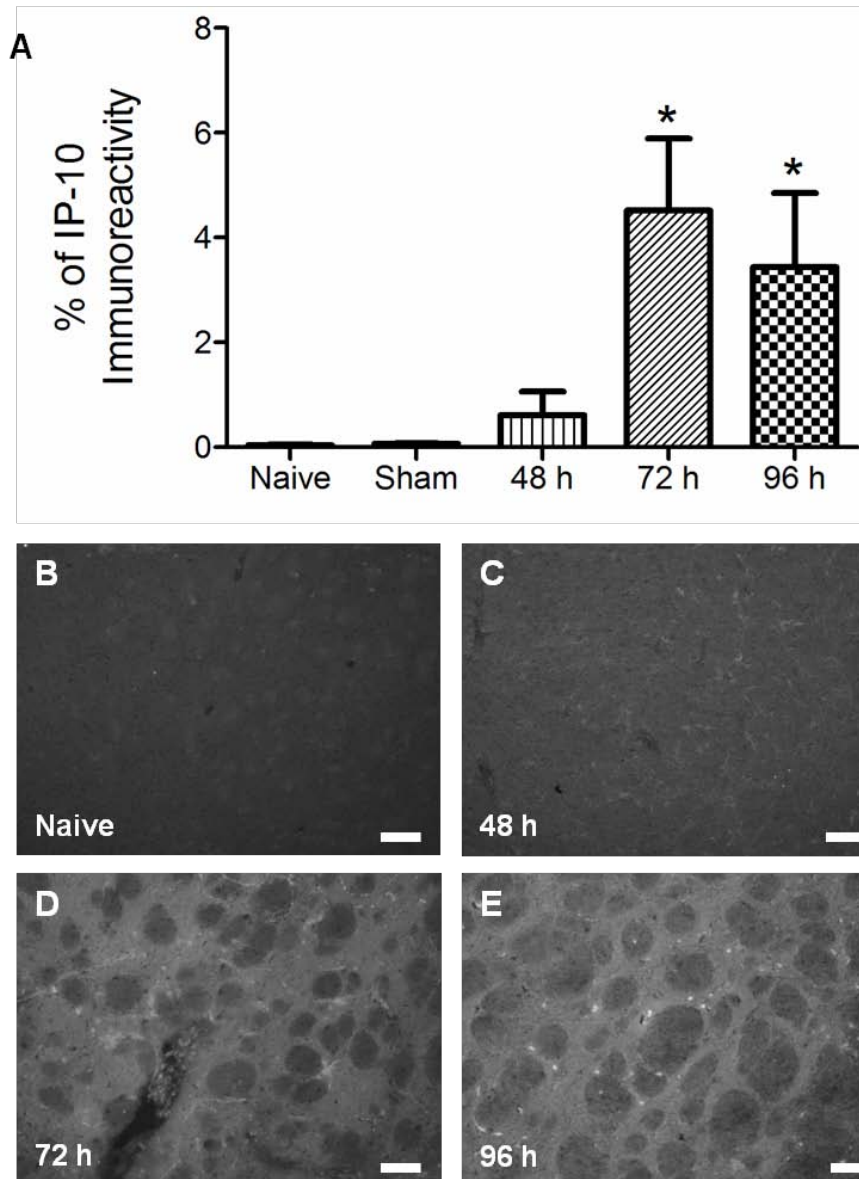


Figure 14: Quantification of IP-10 levels in the brain post MCAO. Immunohistochemical quantification of striatal IP-10 protein levels in the brains of naïve, sham, 48, 72, and 96 h post MCAO demonstrate IP-10 levels are significantly elevated at 72 and 96 h post MCAO compared to naïve brains ($*p < 0.01$) (A). Representative micrographs of IP-10 stained brains from naïve (B), 48 h (C), 72 h (D), and 96 h (E) post MCAO. Scale bar = 100 μ m.

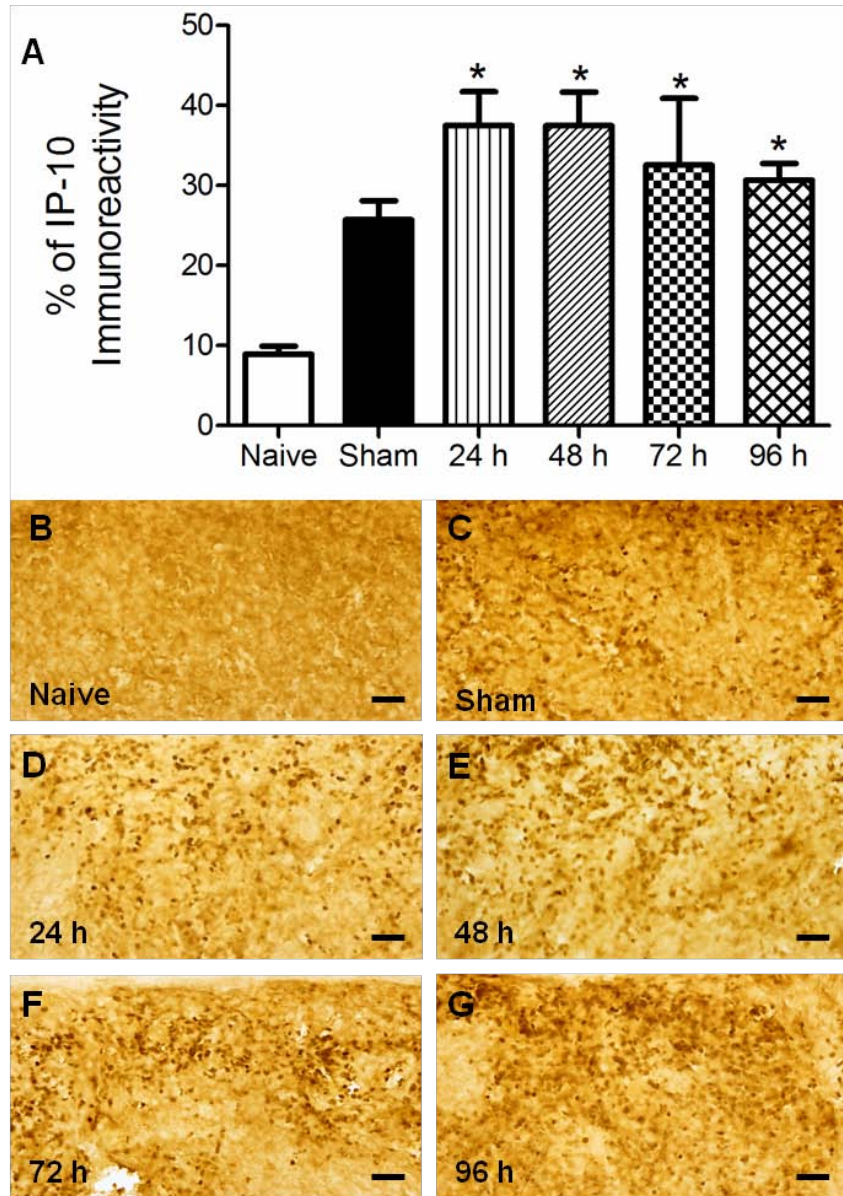


Figure 15: Quantification of IP-10 levels in the spleen post MCAO.

Immunohistochemical quantification of IP-10 protein levels in the spleens of naïve, sham, 24, 48, 72, and 96 h post MCAO demonstrate IP-10 levels are significantly elevated at 24 h and remain elevated out to 96 h post MCAO compared to naïve spleens (* $p < 0.0007$) (A). Representative micrographs of IP-10 stained spleens from naïve (B), sham (C), 24 h (D), 48 h (E), 72 h (F), and 96 h (G) post MCAO. Scale bar = 120 μ m.

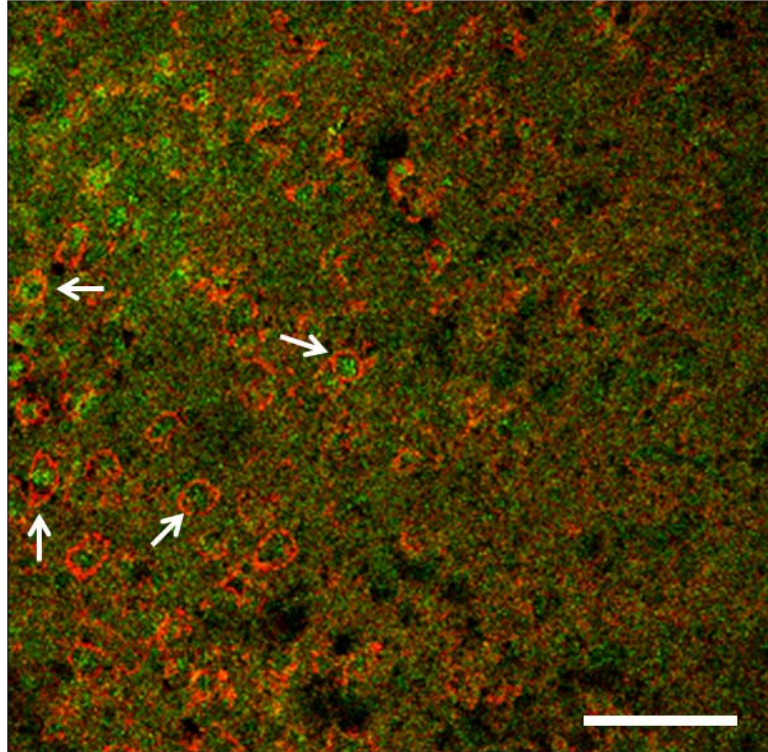


Figure 16: IP-10 producing monocytes in the infarct following MCAO. Confocal micrograph shows amoeboid CD11b positive cells (red) with intracellular IP-10 (green) in the striatum of the ipsilateral hemisphere 96 h following MCAO. Scale bar equals 75µm. Arrows indicate areas of co-localization.

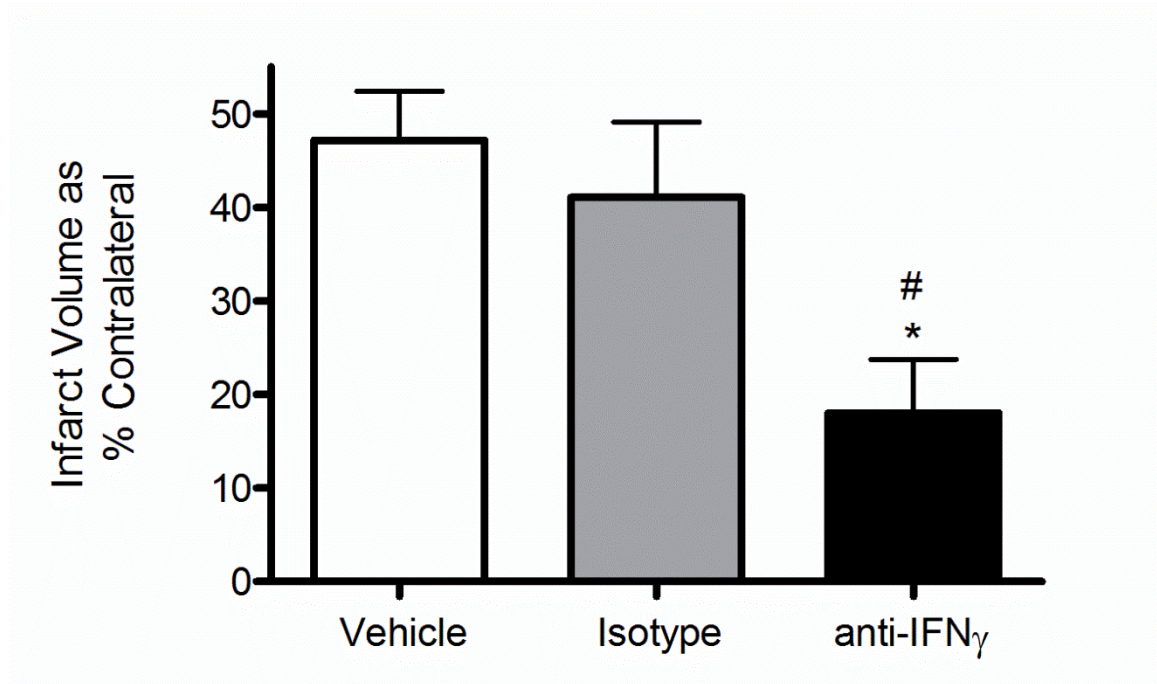


Figure 17: IFN γ neutralizing antibody administration following MCAO decreases infarct volume. Administration of an IFN γ at 24, 48, and 72 h post MCAO significantly decreased infarct volume at 96 h when compared to the vehicle group (* $p < 0.007$). Infarct volumes are decreased compared to isotype controls. However this did not reach significance (# $p = 0.0588$).

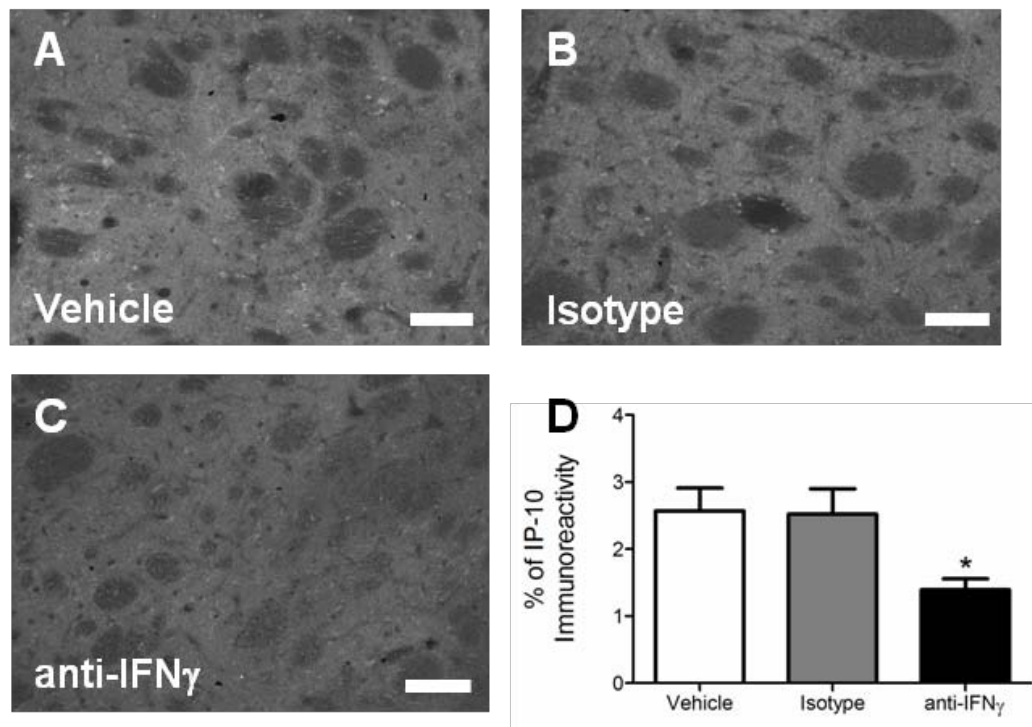


Figure 18: Quantification of IP-10 levels in the brain post MCAO with administration of an IFN γ neutralizing antibody. Immunohistochemical quantification of striatal IP-10 protein levels in the brains of vehicle, IgG isotype, and IFN γ antibody 96 h post MCAO demonstrate IP-10 levels are significantly decreased in the IFN γ antibody treated group compared to the vehicle treated group (* $p < 0.009$) (D). Representative micrographs of IP-10 stained brains from vehicle (A), IgG isotype (B), and IFN γ antibody (C) treatment groups 96 h post MCAO. Scale bars = 100 μ m.

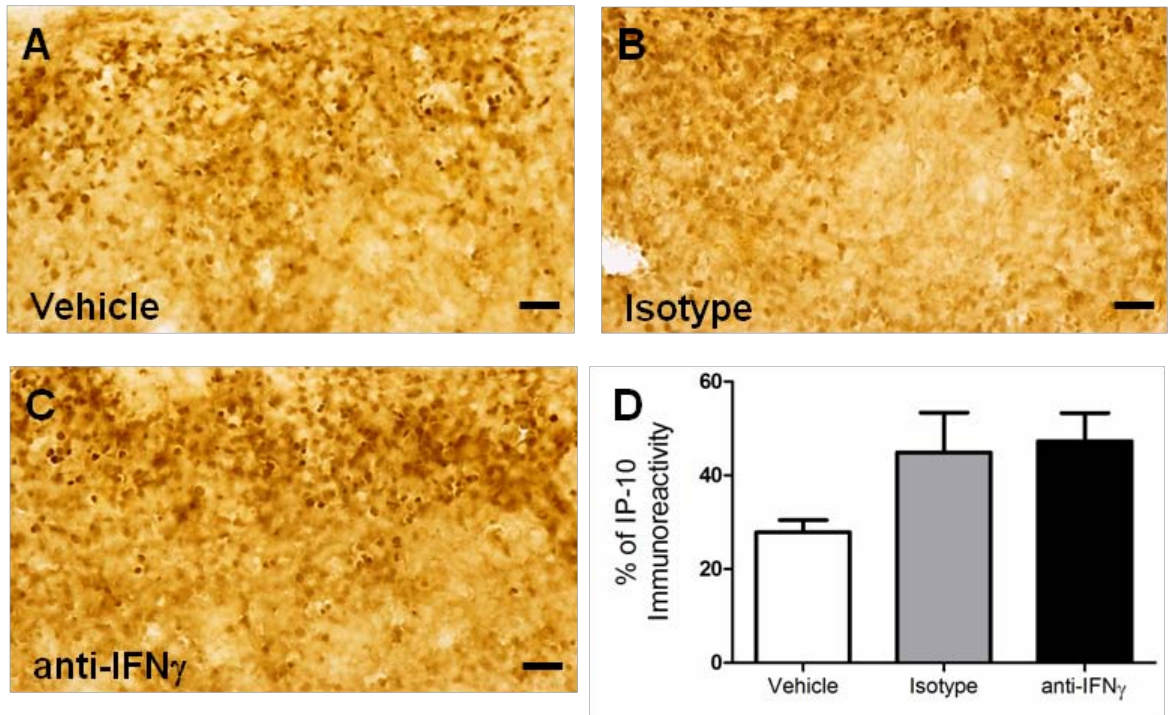


Figure 19: Quantification of IP-10 levels in the spleen post MCAO with administration of an IFN γ neutralizing antibody. Immunohistochemical quantification of IP-10 protein levels in the spleens of vehicle, IgG isotype, and IFN γ antibody 96 h post MCAO demonstrate IP-10 levels are elevated in the IFN γ antibody and the IgG groups compared to the vehicle treated group (D). Representative micrographs of IP-10 stained spleens from vehicle (A), IgG isotype (B), and IFN γ antibody (C) treatment groups 96 h post MCAO. Scale bars = 120 μ m.

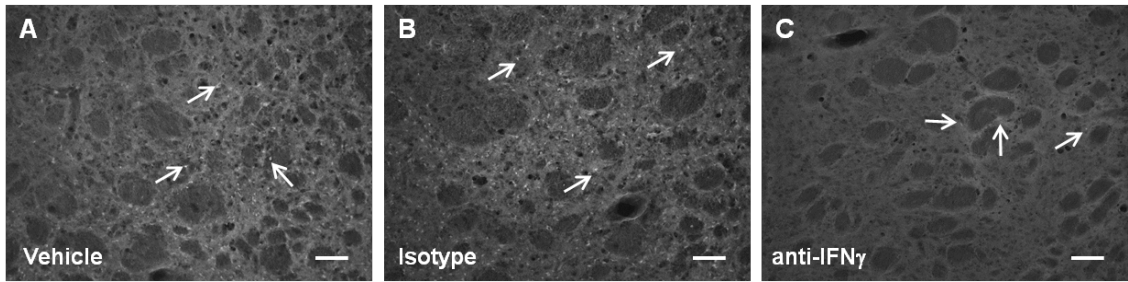


Figure 20: CD3 immunoreactivity appears to be decreased in IFN γ antibody treated brains. The amount of T cells in the brain following MCAO appears to be decreased. Images are from the striatum of the ipsilateral hemisphere. There appears to be decreased CD3 (T cell) immunoreactivity in the brains of IFN γ antibody treated animals (C) compared to the amount of staining in the two control groups vehicle (A) and isotype (B). Scale bars equal 100 μ m. Arrows indicate areas of staining.

Conclusion

Stroke is a complex neurologic disorder that occurs 795,000 times per year. It is the leading cause of disability and the fourth leading cause of death. The current therapeutic treatment for ischemic stroke is rTPA. Unfortunately only 3-5 percent of stroke patients receive this treatment. Investigations into understanding the underlying mechanisms of neural cell death have provided some insight into developing potential new therapies. A majority of the initial work focused on the very early events that lead to neural injury, including glutamate excitotoxicity (Ikonomidou and Turski 2002) and calcium dysregulation (Cheng et al. 2004). Then the early immune response was recognized as a contributing factor to neural injury and therapies were developed to target the immune system with antibodies against intracellular adhesion molecule (ICAM) (Enlimomab Acute Stroke Trial Investigators 2001). All of these therapies failed in clinical trials. There have been many different speculations as to why these different agents failed. The preliminary studies were done in rodent models of stroke but the rodent brain is different from the human brain with regards to ratio of white to gray matter and the development of the cortex. In other preliminary studies therapies were administered prior to experimental stroke or at a clinically irrelevant time point. Additionally, most of the preliminary studies were completed in healthy young male animals, which do not accurately represent the human population that is most commonly affected by stroke. Further research into the mechanisms of neural injury has lead to the discovery of the role the immune system plays in enhancing delayed neural injury. Studies have identified the immune cell types that are present in the brain at various time points

following MCAO and have reported lymphocytes, T cells in particular, are increased in the infarct days following stroke. Unlike monocytes and other innate immune cells, which are present in the brain hours following stroke, T cells begin to significantly increase in number around 72 h following MCAO (Stevens et al. 2002).

Other studies began to examine the adaptive immune response following MCAO and found that lymphocytes play a detrimental role following stroke. SCID mice, which lack the ability to produce lymphocytes, had decreased infarcts compared to WT mice at 96 h following tMCAO (Hurn et al. 2007). Rag^{-/-} mice, which are not capable of producing lymphocytes, also had reduced infarcts compared to WT mice. When looking at specific lymphocyte subtypes T cells both, CD4^{-/-} (Th cells) and CD8^{-/-} (cytotoxic T cells) mice, had reduced infarcts compared to WT mice. However, B cell^{-/-} mice did not have significantly different infarct volumes when compared to WT mice, suggesting B cells do not play a detrimental role following stroke (Yilmaz et al. 2006). These studies implicate the peripheral immune system in the exacerbation of neural cell death following stroke.

The spleen is a reservoir of peripheral immune cells. Splenectomy prior to pMCAO decreases infarct volume by 80% out to four days following pMCAO (Ajmo et al. 2008); the infarct has stabilized by four days in our pMCAO model (Newcomb et al. 2006). In addition to decreasing infarct size splenectomy also decreases the number of neutrophils, (Ajmo et al. 2008) T cells, NK cells, and decreases microglia/macrophage activation (Seifert et al. 2012). Removing the spleen decreases infarct volume after tMCAO in mice (Jin et al. 2013) and reduces brain edema following ICH (Lee et al.

2008). The mechanism behind the splenic response is poorly understood but the spleen as a plausible therapeutic target for stroke.

Removing splenocytes has a beneficial effect on stroke outcomes. The exact role that these cells play in exacerbating neural injury after stroke is unclear. These cells could be migrating to the brain following stroke and increasing injury by cell mediated effects. Splenocytes could also remain in the spleen and contribute to neural injury by releasing inflammatory mediators like cytokines. It is also possible that both of these scenarios are contributing to immune mediated cell death following stroke. While immune cells are present in the brain after stroke, these could originate from other organs besides the spleen. However, our data shows that splenocytes of monocytic and T cell origin migrate to the injured brain but appear to remain in the blood vessels and release inflammatory cytokines, such as $IFN\gamma$.

Cytokines have also been extensively studied in experimental models of stroke and in stroke patients. These studies have lead to contradicting evidence regarding the role certain cytokines play following stroke. Some cytokines have demonstrated neuroprotective effects and other inflammatory cytokines are elevated too early to be a therapeutic target. $IL-1\beta$ is elevated in the brain within hours following stroke. $IL-10$ is neuroprotective following experimental stroke and higher levels of plasma $IL-10$ are associated with better outcomes in patients. $TNF\alpha$ has contradictory effects following experimental stroke and its effects are dependent on its concentration. $IL-6$ has both inflammatory and protective effects depending on the timing of its expression following stroke. The majority of cytokines studied following experimental stroke are elevated early

in the brain and produced by cells of the innate immune system. These contradictory results at early time points after stroke have not lead to the development of any therapeutics targeting any of the above mentioned cytokines, despite the fact that there are FDA approved therapies which target $TNF\alpha$ and IL-6.

The adaptive immune system initiates a response days after stroke and targeting it would extend the therapeutic window. $IFN\gamma$ is a pro-inflammatory cytokine that is primarily produced by the adaptive immune system. One of the primary mechanisms by which $IFN\gamma$ modulates the immune response is to activate cells of monocytic origin, including microglia and macrophages. In response to a pathogen T cells and NK cells produce $IFN\gamma$ to activate and increase the cytotoxic properties of macrophages. The primary goal of the Th_1 response is to increase the killing of intracellular pathogens. $IFN\gamma$ is considered to be the signature cytokine associated with Th_1 responses. This response is the body's major defense against viral and bacterial pathogens. However, elevated levels of $IFN\gamma$ following stroke as shown in our studies leads to increased cell death through over activation of microglia/macrophage in the injured brain.

Previous work in our laboratory has shown microglia/macrophages become maximally activated at 72 h post pMCAO (Leonardo et al. 2010). These data coincide with the findings in aim 1 that $IFN\gamma$ levels are elevated in the brain at 72 h after pMCAO. The spleen is the mediator of the inflammatory response following stroke as demonstrating that its removal reduces injury. Additional data from aim 1 indicate the spleen is a major source of $IFN\gamma$, as levels spike at 24 h in the spleen and splenectomy prior to pMCAO

reduces brain levels of IFN γ . Adding IFN γ systemically to splenectomized animals reverses the protective effects of splenectomy and increases infarct volume to levels not statistically different from sham splenectomized animals and brain IFN γ levels return to levels seen in intact animals. Moreover, the effects of IFN γ are not directly toxic to neural cells. Co-incubation with IFN γ during normoxia and OGD did not result in significantly increased death to cultured neurons or oligodendrocytes. However, neuronal cultures that contain IFN γ activated microglia did have significantly more cell death (Bal-Price and Brown 2001). This suggests that IFN γ alone is not directly toxic to neurons or OLs but if microglia are present then IFN γ activates these cells in a pro-inflammatory nature that is detrimental to neural cells.

Splenectomy provides further evidence that the splenic response is involved in generating the inflammatory response in the brain following stroke. As splenectomy reduces the amount of peripheral immune cells in the brain compared to sham splenectomized animals. Cells from both the innate and adaptive immune systems are significantly decreased in the brain with the absence of the spleen during stroke. NK and T cells are the major source of IFN γ in the brain so the absence of these cells will result in a reduced activation of microglia/macrophages. These data implicate the spleen in the IFN γ inflammatory response and the peripheral immune cell response that increase neural injury following MCAO.

These current studies on the role of IFN γ in stroke are supported by previously published literature. IFN γ mRNA is increased in the brain beginning 48 h post MCAO and remained

elevated out to six days (Li et al. 2001). Direct injection of an IFN γ neutralizing antibody into the brain was only effective at reducing infarct if administered at 72 h following tMCAO in mice and was not effective if administered 15 min post tMCAO (Liesz et al. 2009). Additionally, IFN γ ^{-/-} mice had reduced infarcts compared to WT mice following tMCAO (Yilmaz et al. 2006). Microglia/macrophages become maximally activated at 72 h following pMCAO (Leonardo et al. 2010) showing the presence of IFN γ is necessary for the activation of these cells. IFN γ mRNA is increased in the spleen at 22 h following tMCAO in mice (Hurn et al. 2007) supporting the finding that IFN γ levels spike within the spleen at 24 h post pMCAO. Our findings with splenectomy and IFN γ have recently been replicated in a mouse model of tMCAO (Jin et al. 2013) showing an identical response in a different species and model of stroke. These experiments demonstrate there is a connection between the spleen, IFN γ , and post stroke immune mediated neural injury.

The delay in increased IFN γ levels expressed in the injured brain suggests that the splenocytes are migrating to the brain following ischemic stroke. Previous studies have used knockout mice and irradiated WT mice injected with green fluorescent protein (GFP) mouse bone marrow to study the reaction of the immune system to stroke. These studies have shed important light on what types of cells are found in the brain following stroke and identifying cells that are detrimental to neural cell survival after a stroke. However, none of these studies have addressed the specific role splenocytes play in the post stroke inflammatory reaction in the brain. The second aim labeled splenocytes with CFSE prior to MCAO and tracked the migration of spleen cells following MCAO. This will allow the use of an intact animal and still be able to track cells *in vivo* following MCAO without using adoptive transfer of labeled cells.

The spleen transiently decreases in size after pMCAO in rats from 24-72 h post pMCAO. Increased circulating levels of CAs mediate this effect by activation of α_1 adrenergic receptors that are expressed on the splenic smooth muscle capsule. Administration of prazosin, an α_1 adrenergic blocker, prevents the decrease in spleen size observed at 48 h post pMCAO (Ajmo et al. 2008). Stress from a stroke induces increased circulating levels of CAs in people and animals. Splenic contraction has been associated with physically stressful situations, like repeated breath holds that result in repeated apneas. The physical stress caused splenic contraction and the release of red and white blood cells into circulation. This increase in circulating red and white blood cells does not occur in splenectomized individuals (Bakovic et al. 2005). The changes in spleen size after pMCAO determined the time points that were chosen for the study presented in aim 2, when the spleen is decreased in size at 48 h and at 96 h post pMCAO when the spleen has returned to size. CFSE was found to be nontoxic out to nine days post injection and labels 20 percent of splenocytes at five days post injection. At 48 h there is a significant decrease in splenic CFSE labeled cells and a concurrent significant increase in CFSE positive cells in circulation following pMCAO which is not observed in sham MCAO or 96 h post MCAO. This increase in circulating splenocytes corresponds to the decrease in spleen size, demonstrating that contraction of the spleen leads to the release of splenocytes into circulation following pMCAO. This is consistent with data on splenic contraction leading to increased circulating levels of red and white blood cells (Bakovic et al. 2005).

While there was an overall increase in circulating splenocytes there is an overall decrease in circulating monocytes indicating the majority of these cells in circulation are likely to be of splenic origin. This is consistent with findings that the spleen is a large reservoir of undifferentiated non-tissue specific monocytes (Swirski et al. 2009). The decrease in spleen size is consistent with the decrease in CFSE labeled splenocytes in the spleen, and the increase in circulating CFSE labeled cells at 48 h in the pMCAO group and not any of the other groups. The lymphocyte response also appears to be pMCAO specific as they were significantly elevated in circulation in the 48 and 96 h pMCAO groups and the 96 h sham MCAO. At 48 h following pMCAO the most likely lymphocyte in the circulation are NK cells, as these cells are involved in the innate immune response. T cells and B cells could be contributing to the increase seen at 48 h, but these cells generally take longer to become activated. Further evidence of this finding is that there are more lymphocytes in circulation at 96 h regardless of treatment. This suggests the adaptive response is more robust at 96 h than at 48 h. With data demonstrating splenocytes enter into circulation following pMCAO the injured brain was examined for CFSE label splenocytes.

CFSE positive cells are found in the brains of rats at 48 and 96 h after pMCAO only and not in the sham MCAO treated groups. Additionally, CFSE positive cells were only found in the ipsilateral hemisphere. It has been previously published that immune cells are present in the brain following MCAO (Stevens et al. 2002); however the tissue origin of these cells was unknown. These data demonstrate that some of the immune cells found in the brain after MCAO are directly from the spleen. Additionally double staining with immune cell surface markers identified monocytes and NK cells at 48 h and monocytes, NK cells, and T cells at 96 h post pMCAO. No CFSE positive T cells were found at 48 h

following pMCAO. These data are consistent with previous data on the timing of the migration of different immune cell populations into the brain following MCAO. Monocytes have been found in the brain as early as 18 h post MCAO and are seen in the brain out to 96 h following MCAO (Stevens et al. 2002). This is consistent with observing splenic monocytes in the brain at 48 and 96 h post pMCAO. Additionally, T cells are not observed in significant numbers in the brain until 72 h following MCAO (Stevens et al. 2002). This would explain why splenic T cells are present at 96 h and not at 48 h post pMCAO.

Using confocal microscopy it appears as though the CFSE positive cells in the brain are remaining in the vasculature and not extravasating into the infarct. Previous studies have demonstrated the detrimental role splenocytes, or the spleen, have on neural injuries, as splenectomy is neuroprotective prior to MCAO (Seifert et al. 2012; Jin et al. 2013; Ajmo et al. 2008). If the splenocytes in the brain appear to be remaining in the vasculature this makes it unlikely they are having directly cytotoxic effects on neural cells. Secretion of cytokines is another method of how these cells could be negatively influencing cellular survival within the infarct. Double staining indicated CFSE positive cells are producing $IFN\gamma$ in the brain at 96 h but not at 48 h following pMCAO. Other studies have demonstrated that $IFN\gamma$ protein levels are elevated at later time points in the brain (Seifert et al. 2012; Jin et al. 2013), and that peripheral immune cells are the source of $IFN\gamma$ (Seifert et al. 2012). CFSE positive cells at 48 h could be producing any number or combination of different cytokines or chemokines. For example, monocytes secrete $TNF\alpha$, IL-6, IL-12, IL-8, and the MCP family of chemokines (Boehm et al. 1997) all of which could be detrimental to neural tissue within the infarct.

IFN γ producing CFSE positive splenocytes were found in the ipsilateral hemisphere of the brain 96 h following pMCAO. These data provide a connection between the spleen, IFN γ , and the post stroke immune response. This connection suggests blocking IFN γ signaling could be neuroprotective following pMCAO. One way to block IFN γ signaling would be to neutralize circulating IFN γ with an antibody directed against it. In order to demonstrate the efficacy of any treatment neutralizing IFN γ activity, expression of IFN γ -dependent IP-10 was analyzed. IP-10 is pro-inflammatory chemokine and a potent T cell chemoattractant. The ability of IP-10 to modulate the Th₁ response and the fact that IFN γ induces a robust expression of IP-10 in activated cells makes this molecule an ideal indicator of IFN γ activation. Aim 3 was designed to identify a molecular footprint of IFN γ induced activation by investigating the expression profile of IP-10 following pMCAO and determine the effects of neutralizing IFN γ with an antibody after pMCAO on IP-10 expression in the brain and spleen.

IP-10 expression in the brain is significantly increased at 72 h and remains elevated at 96 h post pMCAO. This expression profile mimics the expression of IFN γ in the brain after pMCAO (Seifert et al. 2012). Splenic IP-10 expression is increased at 24 h and remains elevated out to 96 h post pMCAO. However, unlike the brain, IP-10 expression in the spleen does not mirror the response of IFN γ in the spleen that spikes at 24 h post pMCAO and returns to sham operated levels (Seifert et al. 2012). The data from the brain and the spleen are consistent with findings in mice following tMCAO where IP-10 mRNA is up regulated in the brain beginning at 6 h and remains elevated at 22 h. In the spleen IP-10 mRNA is increased at 22 h post tMCAO (Hurn et al. 2007; Offner et al.

2006). Microglia/macrophages are the main target cells of IFN γ and following pMCAO monocytes (CD11b⁺ cells) are the primary source of IP-10 in the brain. This is consistent with other reports that cells of monocytic origin produce IP-10 (Boehm et al. 1997).

To inhibit IFN γ signaling, a neutralizing antibody directed against IFN γ was administered at 24, 48, and 72 h post pMCAO. The IFN γ neutralizing antibody significantly reduced infarct volume at 96 h following pMCAO compared to the vehicle control group. However, infarct volume was reduced but not significantly when the IFN γ antibody group was compared to the IgG isotype control group ($p=0.058$). In addition to reducing infarct volume, administration of an IFN γ neutralizing antibody significantly reduced the levels of IP-10 protein in the brain at 96 h following pMCAO. However, administration of a goat antibody, directed against IFN γ or the IgG isotype, resulted in increased levels of IP-10 in the spleen compared to vehicle treated rats 96 h post pMCAO. The decreased levels of IP-10 in the brain demonstrate that IFN γ signaling is being inhibited by the neutralizing antibody. The spleen reacted in a pro-inflammatory manner to the presence of a perceived threat of a foreign protein, the goat antibody. Both antibody treated groups had increased splenic IP-10 levels compared to the vehicle group. While IFN γ is a potent inducer of IP-10 it is not the only cytokine that can induce its production. TNF α , IFN α , and IFN β can all induce IP-10 production, especially when they are expressed in combination (Groom and Luster 2011). Even activation of TLR4 can induce low levels of IP-10 production (Luster 2002).

In addition to reducing IP-10 levels in the brain, the IFN γ neutralizing antibody also appears to reduce the amount of T cells in the ipsilateral hemisphere 96 h following pMCAO. IP-10 is a potent chemoattractant for Th₁ cells and has the unique ability to act as an antagonist on Th₂ cells. IP-10 is a member of the CXC family of chemokines and binds the CXCR3 receptor on Th₁ cells which attracts more Th₁ cells to the injured area, in this case the brain. The recruitment of more Th₁ cells increases the amount of IFN γ and other pro-inflammatory mediators in the brain. IP-10 has the ability to simultaneously inhibit the activation of Th₂ cells by acting as an antagonist at the CCR3 receptor. Studies have shown IP-10 binding to CCR3 prevents any of the CCR3 ligands from binding the receptor and activating the cell (Loetscher et al. 2001). The ability of IP-10 to recruit more pro-inflammatory Th₁ cells and block the Th₂ response creates a highly inflammatory environment.

Blocking IFN γ activity after stroke appears to be an ideal approach to reducing neural injury. The administration of a less immunogenic agent to interfere with IFN γ signaling could prove to be a promising therapeutic. There are other points in the IFN γ signaling pathway that could be targeted to blunt this response. IFN γ signals through Jak_{1/2}, which activate STAT1. Both points in the pathway could be targeted. There are currently several Jak_{1/2} inhibitors in clinical trials for rheumatoid arthritis (RA) and cancer. One inhibitor, INCB028050, is in clinical trials for RA and in preclinical studies using a rat model of RA a dose of 10 mg/kg/d decreased IFN γ production by 65 percent (Fridman et al. 2010).

Selectively interfering with $IFN\gamma$ and the Th_1 response does not inhibit the other aspects of the immune system. Different facets of the immune system are involved with tissue repair and regeneration. Therefore, agents that suppress the immune system as a whole are detrimental because they block the beneficial immune responses as well as the responses that protect against infectious agents.

The Spleen, $IFN\gamma$, and IP-10: The Pro-Inflammatory Loop in Response to Stroke

Strokes involving occlusion of the MCA, which is the most commonly occluded vessel in ischemic stroke patients, damage a number of frontal brain areas including the insular cortex. The insular cortex is mainly perfused by the MCA. Insular cortical damage results in sympathetic dysregulation and increased levels of circulating CAs in patients and animals (Meyer et al. 2004; Cechetto et al. 1989). This increase in CAs has several implications following stroke. Studies in rats suggest activation of α adrenergic receptors on the splenic capsule causes splenic contraction and the release of splenocytes into systemic circulation. Additionally CAs affect most immune cells, in particular Th cells. Th_1 cells express the β_2 adrenergic receptor and when their activation increases the intracellular levels of cyclic adenosine monophosphate (cAMP) activating protein kinase A (PKA). This cascade inhibits Th_1 cells from producing IL-2, $IFN\gamma$, and decreases proliferation. However, Th_2 cells do not express β_2 receptors and are unaffected by CAs. This would suggest that the immune system cannot generate a Th_1 inflammatory response following strokes that involve the MCA and could be happening in stroke patients. However, naïve Th cells also express β_2 receptors and when these receptors are activated by the presence of CAs then naïve Th cells differentiate in response to an antigen into Th_1 cells. These cells have also been shown to produce two to four fold the amount of $IFN\gamma$ than cells not activated in the presence of CAs (Swanson et al. 2001).

Moreover, the opening of the BBB by MMPs and other proteases along with the neural cell death causes neural antigens to leak into systemic circulation (Herrmann et al. 2000; Wunderlich et al. 1999). These antigens are seen as novel antigens to the immune system as the BBB under normal conditions shields the brain from the immune system. The spleen is a major site of blood filtration so it is possible then that after a stroke naïve Th cells in the spleen are becoming activated against neural antigens in circulation. With increased circulating levels of CAs, a pool of Th₁ cells is created that are very reactive to neural antigens. These cells could then lead to a pro-inflammatory IFN γ driven immune response to the brain following stroke. This would result in two different immune reactions occurring in the body at the same time, a blunted Th₁ response to pathogens and a strong Th₁ response to the brain. Stroke patients that develop an infection within 15 days of stroke onset have an increased Th₁ response to neural antigens and a poorer outcome at 90 days compared to patients that did not develop an infection. This poor outcome occurred regardless of initial stroke severity (Becker et al. 2011).

This new pool of Th₁ cells which are primed to respond to neural antigens with highly elevated levels of IFN γ could explain the early spike in IFN γ in the spleen, which would lead to the delayed increase in the brain. IFN γ levels in the brain become elevated at the same time that microglia/macrophages are becoming maximally activated (Leonardo et al. 2010). These cells in response to IFN γ produce ROS and increase recruitment of more immune cells that results in more neural injury. Additionally microglia/macrophages increase the expression of IP-10, which attracts more Th₁ cells and blunts the Th₂ response. Now a positive feedback loop has been set up where more Th₁ cells produce

more IFN γ that activate more microglia/macrophages to produce more IP-10 to attract more Th $_1$ cells.

Removal of any piece of this loop: the spleen, T cells, or IFN γ , results in decreased neural injury. The spleen appears to be the initial launch point for this response to stroke. Splenectomy prior to MCAO reduces infarct volume, IFN γ levels in the brain, and immune cell infiltration into the brain. Knockout mice have shown that knocking out all lymphocytes, T cells, or IFN γ reduces infarct volume compared to WT mice. Selectively blocking IFN γ also reduces infarct, IP-10 levels, and T cell recruitment. All of these experiments selectively target an aspect of the inflammatory loop following stroke and successfully decreases neural injury. This suggests the pro-inflammatory loop established after a stroke is a potential therapeutic target. As previously mentioned selectively targeting IFN γ only interferes with one facet of the immune response. This leaves the other parts of the immune system that are critical to tissue repair, regeneration and responding to infectious agents intact. An ideal stroke treatment will extend the therapeutic window, decrease inflammation, and promote tissue repair. Inhibiting IFN γ meets two of the three criteria by extending the treatment window from 4.5 h to 24 h and decreases inflammation. Blocking IFN γ signaling has the possibility to indirectly promote tissue repair if the immune response is shifted from an inflammatory tone to a more regenerative/repairing tone. Inhibiting IFN γ could also be combined with a treatment that selectively targets tissue repair.

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Proposed Treatments Targeting the Splenic Response Following Stroke to Decrease Neural Injury

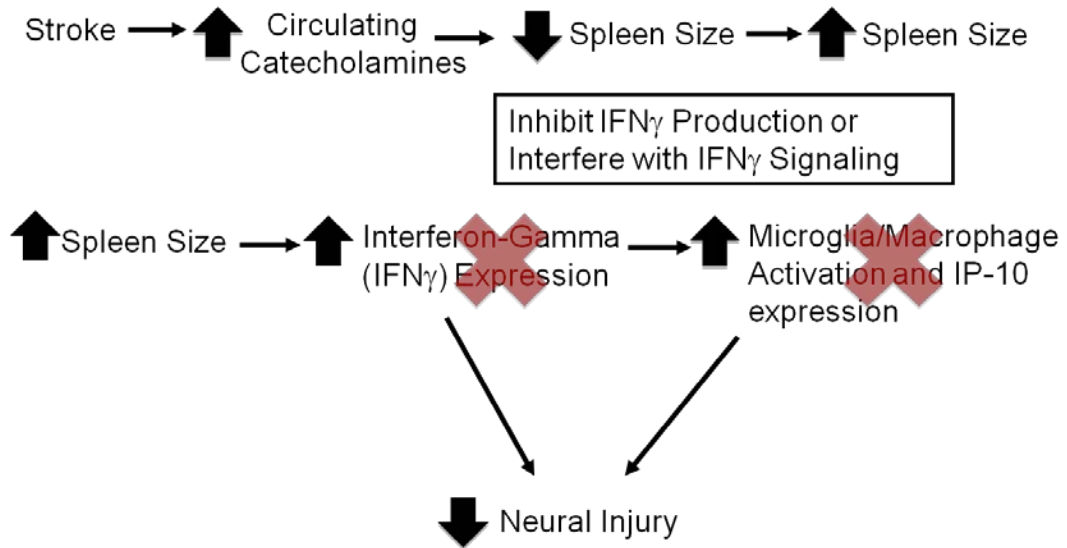


Figure 21: Proposed treatments targeting the splenic response following stroke to decrease neural injury. The splenic response to ischemic stroke can be detrimental. Blocking IFN γ signaling following ischemic stroke leads to decreased neural injury by decreasing interferon-inducible protein 10 (IP-10) and T cell recruitment to the injured brain. This suggests inhibiting IFN γ signaling could be a stroke therapeutic.

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