STEM CELL THERAPY AS A TREATMENT FOR ISCHEMIC STROKE

A THESIS

SUBMITTED TO THE FACULTY OF

UNIVERSITY OF MINNESOTA

BY

Laura Leigh Hocum Stone

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Walter C. Low PhD, Advisor

July, 2014

Acknowledgements

I would like to thank my advisor, Dr. Walt Low, for his support and guidance throughout the course of my time working in his lab. I would also like to thank my thesis committee, Dr. Alvin Beitz, Dr. LaDora Thompson, and Dr. Maxim Cheeran for their invaluable advice that helped to shape the body of work you see presented here.

Dedication

To my husband, Adam, and my family, whose never-ending support and encouragement helped me to achieve my goals.

Abstract

Despite the high prevalence and devastating outcome of ischemic stroke, there remain few options for treatment following stroke onset. The treatments that are currently available remain limited both in the time window following stroke in which they are effective, as well as their success in ameliorating stroke injury in the brain and limiting functional impairment. Current therapies for stroke, such as tissue plasminogen activator (tPA) are only effective up to 4.5 hours following stroke onset. We have tested a human umbilical cord blood-derived stem cell line that has shown both a significant reduction in stroke infarct volume as well as improved functional recovery following stroke in the rat when administered 48 hours following stroke onset. In the present study we have compared high vs. low passage non-hematopoietic umbilical cord blood stem cells (nh-UCBSCs) to determine whether highly expanded nh-UCBSCs are as therapeutically effective as low passaged cells, and their mechanisms of action. Using the middle cerebral arterial occlusion (MCAO) model of stroke in Sprague-Dawley rats, we administered nh-UCBSC by intra-venous administration two days following stroke induction. These human cells were injected into rats without any immune suppression, and no adverse reactions were detected. Both behavioral and histological analyses have shown that the administration of these cells reduces the infarct volume by 50% as well as improve the functional outcome of these rats following stroke for both high and low passaged nh-UCBSC. The brain transcriptome was compared between normal rats, and those with ischemic brain injury, and injury with nh-UCBSC treatment using next generation RNAseq analysis. Differences in the brain transcriptome revealed that ischemic brain injury was associated with significant increases in transcripts related to macrophage, T cell, and microglia function. Flow cytometry analysis of immune cells present in the brains of animals, in each of these three groups, confirmed infiltration of macrophages and T cells consequent to ischemia reduction to normal levels with nh-UCBSC treatment. Flow cytometry also revealed a restoration of normal levels of microglia in the brain following treatment. Overall, these data show a blunting of immune cell migration and activation that are typically activated in the brain following stroke, suggesting that nh-UCBSCs may act by inhibiting immune cell migration into the brain from the periphery, and possibly by inhibition of immune cell activation within the brain. Non-hematopoietic umbilical cord blood stem cells exhibit great potential to provide a novel therapy for stroke with no known ill-effects, and are effective at later time points following stroke than methods that are currently available in the clinic.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

tPA: tissue plasminogen activator

IV: intravenous

IA: intra-arterial

SVZ: subventricular zone

DG: dentate gyrus

NSC: neural stem cells

GCSF: granulocyte colony stimulating factor

MSC: marrow stromal cell

NSC: neural stem cell

ESC: embryonic stem cell

iPS: induced pluripotent stem cell

MAPC: multipotent adult progenitor cells

nh-UCBSC: non-hematopoietic umbilical cord blood stem cell

MCAO: middle cerebral artery occlusion

HUCB: human umbilical cord blood

NSS: neurological severity score

PFA: paraformaldehyde

PBS: phosphate buffered saline

Chapter 1

Neural Repair and Neuroprotection with Stem Cells in Ischemic Stroke

Laura L Stone[1,2,3], Andy Grande[2,3], Walter C Low[2,3]

[1] Graduate Program in Neuroscience, University of Minnesota, Minneapolis, MN 55455, USA
[2] Department of Neurosurgery, University of Minnesota, Minneapolis, MN 55455, USA
[3] Stem Cell Institute, University of Minnesota, Minneapolis, MN 55455 USA

Brain Sci. 2013, 3, 599-614; doi:10.3390/brainsci3020599

ABSTRACT

Stem cells have been touted as a potential source of cells for repair in regenerative medicine. When transplanted into the central nervous system stem cells have been shown to differentiate in to neurons and glia. Recent studies, however, have also revealed neuroprotective properties of stem cells. These studies suggest that various types of stem cells are able to protect against the loss of neurons in conditions of ischemic brain injury. In this article we discuss the use of stem cells for ischemic stroke and the parameters under which neuroprotection can occur in the translation of stem cell therapy to the clinical setting.

INTRODUCTION

Stroke is one of the most common causes of death in the United States, with over 750,000 cases per year in the US (Roger et al. 2012). Moreover, those who

suffer stroke and survive do so at great cost to society, with direct costs estimated at $18.8 billion per year (Roger et al. 2012). Despite the high prevalence of stroke, there remain only limited options for therapy in the clinic, none of which are effective for restoring lost neurological function. In addition, available treatments are only effective at the acute phase of stroke. The lack of effective treatments for such a pervasive disease have made novel approaches to stroke therapy a focus of both preclinical and clinical research in recent years. One of the most prominent approaches to stroke therapy that is emerging from this body of research is the use of exogenous stem cells as a therapy for ameliorating stroke deficits and restoring neurological function.

TREATMENT

Currently available treatments for ischemic stroke focus on revascularization – removal of the clot in the brain to restore normal blood flow, while restoring normal cranial pressure to reduce damage due to swelling. The only currently proven therapy for stroke is tissue plasminogen activator (tPA) which disrupts the clot, but is only effective if administered via IV within 4.5 hours of the stroke occurrence, or delivered via IA administration up to 6 hours following stroke (Donnan et al. 2003).

In addition, there are surgical methods of mechanically removing the clot, but these methods are very invasive and carry their own risks. Devices have been developed to mechanically disrupt the clot, such as the penumbra system, which

operates by aspirating and extracting the thrombus (Bose et al. 2008), and the Merci Clot Retrieval Device, which also mechanically removes the embolism and restores blood flow (Kim et al. 2006). The Solitaire Revascularization Device is a stent-based device that allows immediate restoration of blood flow with the placement of a stent, followed by clot removal (Jahan 2010). These surgical interventional techniques have shown benefit, but there are no double-blinded, randomized, controlled studies showing this benefit, and any studies conducted have only involved small patient numbers. While the methods for restoring blood flow to areas of ischemia are improving, we have yet to improve stroke outcome over what was first demonstrated in the NINDS trial in 1995 with IV tPA at <3 hours after stroke onset (Group 1995).

The most critical factor affecting stroke therapy is that currently only 5% of patients are treated for stroke (Roger et al. 2012). There are many factors which contribute to this fact, including a lack of public awareness of stroke symptoms, and lack of ability to reach appropriate care in time for current treatments to be effective. Due to these facts, there is a great need to develop additional therapies for patients at later time points, and for those who have a completed infarct. Stem cell based therapies are proving to be very promising candidates for treatments that are not only effective at later time points following stroke onset, but addressing the complex pathophysiology of stroke and providing neurological repair. This review will outline current preclinical and clinical research in the emerging field of stem cell therapeutics in stroke research.

STEM CELL THERAPIES

Historically, the dogma put forth by Ramon Y Cajal that the total number of cells in the brain were fixed and that there was no cellular regeneration or presence of endogenous stem cells in the brain was widely accepted (Colucci-D'Amato, Bonavita and di Porzio 2006). This dogma was accepted well into the 20th century, until studies by Altman and Das (1965) provided evidence for the existence of stem cells in the brain. These early studies on neural stem cells as the basis for neurogenesis were subsequently confirmed by the Nottebohm group (Nottebohm 1989). The discovery of neurogenesis in the brain lead to the discovery of neurogenic niches in the subventricular zone (SVZ) and the dentate gyrus (DG) (Doetsch 2003). Research in the field also revealed enhanced neurogenesis after injury in both neurogenic niches as well as non-neurogenic regions such as the cortex and striatum (Parent 2003). These discoveries have opened the door for investigating the potential use of endogenous neurogenesis as a treatment of stroke.

A. Neuroregeneration

One of the potential mechanisms by which stem cells can provide therapy for stroke victims is through neuroregeneration. In order for stem cells to produce successful neuroregeneration, several conditions must be met: they must be able to produce multiple types of neurons and glia; the new cells must be able to migrate to the site of injury; and they must be able to integrate with existing circuitry by initializing and maintaining appropriate functional connections with

neighboring cells. The adult brain is populated by endogenous stem cells known as neural stem cells (NSCs) which are capable of differentiating into different types of neurons and glia (Weiss et al. 1996).

Many *in vivo* studies have demonstrated the production of both immature and mature neurons that migrate towards the striatum following a stroke. Ischemia-induced neurogenesis was first documented in the hippocampus 1998 (Liu et al. 1998). This study showed an amplification of endogenous neurogenesis following global ischemia, but did not show any replacement of the CA1 pyramidal cells that are lost in ischemia. Similar amplifications of neurogenesis in neurogenic regions, such as the dentate gyrus and sub-ventricular zone, have been shown following focal ischemia (Arvidsson et al. 2002, Parent et al. 2002). One of the drawbacks of endogenous neurogenesis as a therapy for stroke is that the new cells have limited capabilities to migrate to the site of injury. Granulocyte colony stimulating factor (G-CSF) has arisen as a potential therapy to allow for the migration of endogenous stem cells to the site of ischemic injury (Borlongan and Hess 2006).

Despite studies showing the ability to recruit endogenous new neurons to the site of injury, there are very few studies that have been able to show new neurons extending axons to appropriate targets, and there has been no evidence of existing neurons extending axons to new neurons *in vivo*. Given these results, we are still a long way from being able to use endogenous stem cells as a viable treatment for stroke injury.

B. Neuroprotection

Developments in the field of stem cell research have identified an application of exogenous stem cells as a vehicle for neuroprotective effects. One of the major benefits to these studies is that this approach to stroke therapy has shown to provide benefits far past the 3 hour time window of current therapeutic approaches for stroke.

Several labs (Cui et al. 2012, Rowe et al. 2012b, Xiao et al. 2005), have tested various exogenous stromal stem cells intravenously in rodents after an induced stroke. These studies have revealed the potential for these types of treatment to provide multiple benefits, including reduced stroke volumes, improved functional outcomes, and an extended time window for treatment – up to 48 hours following stroke onset. These studies in rodent models of stroke show great promise for future stroke therapies, due to their ability to improve stroke outcome and recovery, and most importantly, do so at a time point later than any currently available treatments.

Recent studies have focused on more specific cell types or stem cell populations that have been administered. Various mechanisms of delivery including intravenous (IV), intra-arterial (IA), and direct injection into the brain have been attempted. IV delivery of exogenous stem cells is desirable because it is the least invasive of all administration techniques. One drawback to this method is that many of the injected cells end up being caught in peripheral organs such as the liver, spleen and lungs. In phase I and II clinical trials, IV

administration of autologous mesenchymal stromal cells were shown to be safe in both the short and long term (Bang et al. 2005a, Lee et al. 2010). IA delivery is more invasive, infusing cells directly into the artery that is perfusing the ischemic tissue. The benefit of this method is that it allows the cells to bypass any peripheral organs and go directly to the site of injury. Some concerns with this method of delivery are the potential for the injected cells to form microvascular occlusions, thus worsening the ischemia and resulting in higher mortality rates (Li et al. 2010b, Walczak et al. 2008). Finally, direct intra-cerebral injection is highly invasive and carries many risks, such as initial human studies showing adverse side effects such as seizures, subdural hematoma and worsening of motor function(Kondziolka et al. 2005a, Savitz et al. 2005).

Despite the wealth of research in the field of therapeutic applications of stem cells, there is no unified theory for the mechanism of action exhibited by these cells. One of the longest held views on the mechanism of action of exogenous stem cells as a treatment for stroke is that administered cells migrate to the site of injury and are able to replace diseased and dead endogenous cells by engraftment and differentiation into functional neural and glial cells. Support for this theory comes from a number of studies in animal models, such as one by Hayase et al., (2009) in which stem cells were injected into the cortex three days after stroke onset, and were found in the brain 100 days later, with evidence of projections and expressing positive markers for glutamatergic, dopaminergic, and

GABAergic neurons. The animals that were injected with these cells also showed significant functional recovery.

The evidence for engraftment and transdifferentiation, however, has been heavily scrutinized, and many studies have provided evidence against this theory of stem cells as the sole mechanism of action. It has been shown that adult hematopoietic stem cells cannot differentiate into functional neurons. One of the pitfalls many studies face is that they look for neuronal markers for evidence of transdifferentiation, but do not test the engrafted cells for functionality. A study by (Roybon et al. 2006) showed that when hematopoietic stem cells were grown in culture, they were able to differentiate into neuron-like cells, but these cells were not capable of producing an action potential. When these same cells were transplanted into the brain, there was no evidence of a neuronal phenotype. Instead, the injected cells differentiated into microglia, or died shortly after transplantation. This study, and many others like it (Xiao et al. 2005), have shown that while there are often significant improvements in stroke outcome following stem cell administration, and engraftment and transdifferentiation may not be the mechanism by which these cells are acting.

An emerging theory as to the mechanism of action of therapeutic stem cells has arisen from a large number of studies that have shown a beneficial effect of the administration of stem cells following stroke, but have found little evidence of extensive engraftment or cell replacement in the brain. It has been suggested that the mechanism by which exogenous stem cells act is by altering the local

immune response at the site of injury and modulating chronic inflammation. The tissue surrounding any type of brain injury has been shown to be highly pro-inflammatory, with increases in pro-inflammatory cytokines such as IL-1a, IL-1b, IL-6, and TNF-α upregulated at 48 hours following injury (Harting et al. 2008).

Several studies have shown that administration of exogenous stem cells into a stroke animal have resulted in a reduction in inflammatory cytokines, as well as an upregulation in anti-inflammatory cytokines. (Liu et al. 2009) showed that MSC that were injected into the cortex following stroke in a rat model not only decreased the infarct size, but that IL-10 was up regulated and TNF-α was down regulated following MSC administration, suggesting an anti-inflammatory effect of the MSCs.

An *in vitro* study of MSCs grown in contact culture with NSCs showed an increase in IL-6 production as well as a decrease in apoptosis. These results suggest that the direct implantation of MSCs that come into contact with endogenous NSCs stimulates the local immune response through NFkB activity (Walker et al. 2010). This result was not replicated in *in vitro* studies without cell-cell contact.

When looking to apply cell therapies in the clinic, opting for less invasive therapies is preferable. IV and IA administration of stem cells have been studied in many animal models of stroke and brain injury. These studies generally show little to no cell engraftment in the brain, but do show decreases in infarct volume as well as improvements in functional outcome measures. One common

observation is that this type of administration results in what is known as the 'pulmonary first pass effect' (Fischer et al. 2009). IV administration results in the majority of injected cells becoming caught in the lungs, spleen, kidney, and liver. Yet significant infarct reduction and improvement in functional recovery has been repeated in numerous studies.

One suggested mechanism of action in these instances is modulation of the systemic immune response which stimulates anti-inflammatory and pro-survival responses that ameliorate stroke injury. There is evidence that systemically administered stem cells interact with immune cells in multiple organ systems. For example, stem cells that become caught in the lungs have been shown to interact with pulmonary macrophages and modulate the systemic inflammatory response (Nemeth et al. 2009). As previously discussed, modulation of the inflammatory response is key in improving stroke outcome. It has also been shown that IV administration of MSCs results in a decrease in the pro-inflammatory cytokines TNF-α and IL-6 in the serum, as well as an increase in the anti-inflammatory cytokine IL-10 (Nemeth et al. 2009). Systemically administered stem cells can also interact with splenocytes to have an effect on the overall immune response following stroke. A study by (Lee et al. 2008), systemically administered NSCs in ischemic rats, resulting in improved functional outcomes and reduced infarct size, though very few transplanted cells were found in the cortical tissue. Cytokine analysis showed a decrease in the pro-inflammatory cytokines TNF-α and IL-6 in both the brain and the spleen, and

histology showed a large number of NSCs present in the splenic tissue. Stroke animals receiving NSCs that had splenectomies did not show any improvement following ischemic injury, providing a strong case for the necessity of NSC interaction with splenocytes for improved stroke recovery.

Alterations in the pro- and anti-inflammatory cytokine profiles of stroke animals as a result of stem cell therapy may be crucial to ameliorating stroke deficits. In addition to affecting the inflammatory profile, stem cells can secrete cytokines that promote angiogenesis and neovascularization (Neuhoff et al. 2007). It is, perhaps, by altering the local and systemic immune system that provides the benefit that is seen following stem cell administration, even when no engraftment occurs.

STEM CELL TRANSPLANTATION FOR TREATMENT OF STROKE

A. Goals for stem cell transplantation

In order for cell transplantation to successfully provide therapy, cells must either cross the blood brain barrier and influence the local stroke milieu, influence the systemic immune response, or replace cells lost to ischemia, resulting in improved outcome and reduced injury. If used to generate new neurons, these new neurons must mature, form synaptic connections and not die. If used for neuroprotection, then it will be important to understand if it is needed for them to cross the blood brain barrier, as well as understanding the effect of first pass through the lungs and the liver. It will be important to know the drawbacks and advantages of each type of cell administration: IV, IA, and direct injection. It must

also be determined whether or not it is better to use autologous cells which likely will require time before given to a stroke patient or to have banked cells that can be given immediately.

B. Stem cell types

Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) have long been considered the gold standard for pluripotency, and are considered ideal for therapy due to their ability to differentiate into any cell type including neurons and glia. One of the major drawbacks of using this cell type is that they can create teratomas if they are injected in an undifferentiated state (Seminatore et al. 2010). The post-ischemic environment has been indicated as a cause of teratoma formation from undifferentiated cells, yet hyperproliferation has also been documented in cells that were injected at a later stage of differentiation, and this effect is independent of the surrounding environment (Seminatore et al. 2010). It is very difficult to create a homogenous culture of ESCs that are completely without undifferentiated cells that may cause teratoma formation. It is critical that any graft used for therapy in humans be devoid of undifferentiated cells. These cells also are surrounded by political and ethical concerns that have limited research and would make them more difficult to apply to the clinical setting.

Several studies have shown ESCs transplanted into the ischemic brain that exhibit neuronal markers and synaptic connectivity. (Buhnemann et al. 2006) injected ESC derived neural precursors into mouse cortex and found the

transplanted cells survived up to 12 weeks, although the number of surviving cells was much lower than the number that were injected. The surviving transplanted cells differentiated into glial cells and neurons of several different neurotransmitter producing types. Similar evidence of the functionality of transplanted ESCs has been shown by Takagi et al. (2005), in which NPCs derived from monkey ESCs were injected into MPTP monkeys – a primate model of Parkinson's Disease. They found that the transplanted cells were able to function as dopaminergic neurons. In addition, animals receiving the cell transplant exhibited improved behavioral recovery. A recent study showed that transplantation of ES-NPCs into healthy murine cerebral cortex results in manifestation of dendritic and axonal connections. In addition, these transplanted cells did not form inappropriate connections (Ideguchi et al. 2010).

Inducible Pluripotent Stem Cells (iPSCs)

The reprogramming of adult fibroblast cells to exhibit properties of embryonic stem cells, has led to the generation of so-called inducible pluripotent stem cells (iPS cells). Until recently, issues surrounding immune rejection, potential for the formation of tumors, and ethical concerns decreased the attraction of pursuing stem cell transplantation as a therapy for stroke. However, landmark studies by Yamanaka identifying iPS, or inducible pluripotent stem cells, provided a promising new direction for stem cell therapy. iPS cells are derived by genetically reprogramming adult fibroblasts into an embryonic stem cell-like state by expressing a set of genetic factors that allow the cells to exhibit pluripotency

(Takahashi et al. 2007, Yu et al. 2007). These cells are being developed in several therapeutic settings due to their ability to differentiate into cells that are characteristic of all three embryonic germ layers. An additional benefit of these reprogrammed cells as cell therapies is that fibroblasts from patients can be taken and reprogrammed, thus eliminating the need for immune suppression.

Since the initial study by Yamanaka and his colleagues, many studies have emerged on the production of iPS cells using various combinations of factors and generating iPS cells from various tissue sources. Due to the limited availability of these cells, very few stroke-specific studies have been conducted using iPS cells as therapy. Kawai et al., (2010) studied the administration of iPS cells in a rodent model of stroke, and found severe teratoma formation in the brains of both the stroke and sham operated animals.

These results show similar caveats to the use of ESCs for therapy – i.e., the administration of undifferentiated iPS cells may result in teratoma formation, making them unsuitable for therapy in their undifferentiated state. A recent study has demonstrated that the epigenetic changes induced in the process of generating iPS cells can induce immune rejection (Pera 2011). Whether this will occur in clinical practice, however, remains unknown. While iPS cells can be expanded and can be used to produce various cell types, it remains difficult to generate highly purified cell populations and even when directed to differentiate into specific neuronal populations there is still a significant potential to form teratomas in vivo (Kawai et al. 2010).

Neural Stem Cells (NSCs)

Another type of cell that has been investigated for its therapeutic properties is the neural stem cell (NSC). These cells can be isolated from several regions (the sub-ventricular zone and the sub-granular zone) in the central nervous system of embryos as well as in adults and can be grown and expanded in culture as neurospheres (Arvidsson et al. 2002). Unlike ESCs, NSCs are not pluripotent, but they can differentiate into many, but not all, types of CNS cells. They have been shown to differentiate into neurons, astrocytes, and oligodendrocytes. While some studies have used grafts of exogenous NSCs, others have attempted to recruit endogenous NSCs to the site of injury as a potential therapy. A study by Li et al., (2010a) demonstrated that the endogenous NSCs in the adult brain that are activated by ischemia are necessary for neuroprotection of the ischemic brain. By inhibiting cell proliferation in the brain following ischemia, they found a large increase in infarct size and functional deficit, indicating a crucial role for endogenous NSCs in stroke recovery. Another study has shown that the intra-ventricular administration of NSCs results in improved functional recovery at 28 days following stroke, and also found that NSCs expressing HIF-1a further improved recovery (Wu et al. 2010). While many studies have shown the potential therapeutic benefit of both endogenous and transplanted NSCs, one of the limiting factors of this type of treatment is the poor survival rate of transplanted cells. It has been reported that as little as 1-3% of the transplanted

cells survive, and an even smaller percentage have been shown to differentiate into neurons (Toda 2001). Despite the low survival rate, studies of NSCs have shown consistent therapeutic benefits in animal models of ischemic stroke.

Adult tissue-derived stem cells

One of the most popular sources of cells for cell-based therapy is adult tissue-derived stem cells. These include, but are not limited to, bone marrow derived stem cells, umbilical cord blood derived stem cells, and teratocarcinoma cells. Bone marrow derived cells are among the most commonly researched adult stem cell types, and were identified as potential therapeutic targets due to the endogenous behavior of bone marrow cells.

Marrow Stromal Cells (MSCs) are a type of bone marrow derived stem cell that have been widely studied for their therapeutic benefits. It has been demonstrated that these cells migrate towards tissue injury signals *in vitro* (Menon et al. 2007), however, *in vivo* models have shown a very poor survival rate of transplanted MSCs (Coyne et al. 2006). Despite the low rate of cell survival following transplantation, many studies have shown improved outcome following ischemic injury after the transplantation of MSCs (Willing et al. 2003). A second type of bone marrow derived stem cell is multipotent adult progenitor cells (MAPC). These cells were originally thought to be superior to MSCs due to their ability to generate functional cell types from all three embryonic germ layers, including neurons with mature electrophysiological properties (Serafini et al. 2007). However, studies of transplantation of MAPCs resulted in improved

recovery from ischemia (Zhao et al. 2002), but did not show robust evidence of engraftment (Burns et al. 2006).

Another type of adult-derived stem cells are teratocarcinoma cells, which are a variant of embryonic stem cells that have been derived from an immortalized cell line of germ cell tumors. These cells are able to differentiate into a pure population of neurons when exposed to retinoic acid (Damjanov 1990). These cells have been tested in clinical trials (Kondziolka et al. 2000) as a treatment for stroke and shown to be safe as well as exhibit engraftment as postmitotic neuronal cells. The patients in this study showed a small amount of functional improvement, but this improvement was not found to be statistically significant (Kondziolka et al. 2005b). Although these cells avoid any ethical concerns such as those that arise from ESC use, questions of safety will always be a concern with this cell line, due to the tumorigenic nature of the donor cells.

A promising source of therapeutic adult-derived stem cells is umbilical cord blood, which has been shown to be rich in stem cells, varying fractions of which have been tested in animal models of ischemic stroke as a potential therapy. Administration of these cells has shown a dramatic decrease in infarct size as well as improved functional recovery (Xiao et al. 2005, Rowe et al. 2012a, Cui et al. 2012). A recent study combining the administration of Simvastatin in addition to human umbilical cord blood stem cells in a rat model of stroke showed an increase in injected cells migrating into the brain. As a result, they showed increased neural plasticity and improved neurological outcome (Cui, et al., 2012).

Many studies using umbilical stem cells show little to no evidence of surviving transplanted cells in the brain following systemic delivery, suggesting that cell replacement is not the mechanism of action for the beneficial effects of these cells.

An extension of the concept of reprogramming somatic cells to become iPSCs is the method of direct reprogramming to generate neurons directly from adult fibroblasts. Recent studies have demonstrated the production of several neuronal types through the approach of direct reprogramming (Meissner, Wernig and Jaenisch 2007). Since these cells do not re-enter the cell cycle, there is no potential to form tumors. One of the limitations of using these types of cells as a therapy for stroke is that it is difficult to generate a sufficient number of cells, generation of directly reprogrammed cells takes time, and therefore may not be produced in time for administration when needed.

As described previously, many types of adult derived stem cells exhibit an innate tropism towards a site of injury. Some researchers are harnessing the ability of these cells to migrate towards injury sites and reprogramming them to act as vehicles of gene therapy. Adult stem cells can be made to deliver therapeutic molecules that are anti-inflammatory, pro-angiogenic, pro-survival, or anti-apoptotic (Muller, Snyder and Loring 2006). MSCs have been modified to express PlGF (Placental Growth Factor), which is angiogenic to impaired non-neural tissue. Stroke animals that received MSCs expressing PlGF showed a decrease in infarct size, as well as an increase in functional recovery and

angiogenesis at the site of injury (Liu et al. 2006). Another study utilized MSCs expressing Ang-1, and found that the stroke animals that received the genetically modified cells showed an increase in angiogenesis and neovascularization, specifically at the border of the infarct area, as well as overall increased cerebral blood flow and a decrease in infarct size (Onda et al. 2008). These studies suggest that gene therapy using stem cells as vehicles may provide added benefit and improved outcome following ischemic stroke.

CLINICAL TRIALS

The promising results of preclinical research in this field have prompted a large number of preliminary clinical studies to be launched in recent years. One of the earlier clinical trials to investigate the efficacy of stem cells as a treatment for stroke tested the safety and efficacy of mesenchymal stem cells (MSCs) in a randomized and controlled phase I/II trial (Bang et al. 2005b). This study recruited patients that had acute cerebral infarction within the area of the middle cerebral artery, and aspirated bone marrow from these patients one week following stroke onset. MSCs were isolated from the marrow and administered intravenously between five and seven weeks following stroke. Short term follow up showed no adverse effects from the cell administration, and functional recovery improved in patients receiving MSC therapy according to the Barthel Index and the modified Rankin Score. A long term follow up study of the same patients confirmed that intravenous delivery of MSCs is safe, and that some

patients may derive a functional benefit, though they noted that functional improvement was associated with serum levels of stromal cell-derived factor-1, as well as the involvement of the subventricular region of the lateral ventricle (Lee et al. 2010).

A second clinical trial also tested the effects of bone marrow-derived mononuclear cells as a treatment for stroke (Moniche et al. 2012). In this study, patients were administered intra-arterially between 5 and 9 days following stroke. This study found no adverse effects related to the cell transplantation. However, there was also no significant neurological improvement at 180 days following the cell administration. There was a trend towards functional improvement in the Barthel Index correlating with the number of CD34+ cells that were injected.

Of the many ongoing clinical trials in this field, the majority focus on determining the safety and efficacy of bone marrow derived stem cells as a treatment for stroke. Other cell types being studied include: modified stem cells, umbilical cord blood derived stem cells, and adipose derived stem cells. The majority of the ongoing clinical trials are administering the therapeutic cells systemically either via IV or IA administration , as this method of delivery is the least likely to cause complications. The studies encompass therapies for both chronic and acute ischemic stroke. A summary of current ongoing stem cell trials for stroke can be found at the website ClinicalTrials.gov and are listed for convenience below in Table 1. As the present time the results of these current studies have yet to be reported and are anxiously awaited.

Table 1. Ongoing Clinical Trials of Stem Cell Therapies for Ischemic Stroke.

Stem Cell Type	Administration	Therapeutic Time Point Following Stroke	Clinical Trial ID
Autologous PBSC (CD34+)	Intercerebral Implantation	na	NCT00950521
Mesenchymal Stem Cells	IV	<6 weeks	NCT00875654
Autologous bone marrow stem cell	IV	7-30 days	NCT01501773
autologous bone marrow CD34+	Infusion into MCA	5-9 days	NCT00761982
Autologous BMSC	IV	5 weeks	NCT01468064
Autologous Bone Marrow CD34+	Infusion into MCA	<7 days	NCT00535197
Autologous Bone Marrow mononuclear stem cell	IV	24-72 hours	NCT00859014
CTX0E03 neural stem cells	local injection	6 months- 5 years	NCT01151124
SB623 modified stem cell	na	6-36 months	NCT01287936
Allogeneic Adult Mesenchymal bone marrow stem cells	IV	>6 months	NCT01297413
MultiStem	IV	1-2 days	NCT01436487
Allogenic CD34+ Umbilical cord blood Stem Cells	Brain Implant	6-60 months	NCT01438593
Autologous peripheral hematopoietic stem cell	Cerebral Artery Transplant	<1 year	NCT01518231
Autologous Human Umbilical Cord Blood	IV	6 weeks-6 years old, history of prenatal stroke	NCT01700166
Allogenic Mesenchymal Stem Cells from adipose tissue	IV	<12 hours	NCT01678534
Adipose-derived stem cells	IV and ICA	na	NCT01453829
Autologous Bone Marrow Stem Cells	IA/IV	>3 days and <90 days	NCT00473057
Autologous BMMCs and Marrow Stromal Cells	IV	<72 hours	NCT00908856
Ex-vivo cultures Adult allogenic MSCs	IV	<10 days	NCT01091701

CONCLUSIONS

There is a wealth of evidence supporting the use of stem cell therapies for ischemic stroke, but the mechanisms by which these cells exert their neuroprotective effects have yet to be fully elucidated. It must also be determined if different cell types have distinct mechanisms of action, as well as what risks are posed by each cell type and delivery method. With a more complete understanding of the mechanisms by which cell based therapies are able to ameliorate stroke injury, we will be able to successfully transition these therapies from the lab bench into the clinic.

Acknowledgments

We would like to thank Kyle Schaible for his administrative support.

Conflict of Interest

The authors declare no conflict of interest.

Chapter 2

Amelioration of Ischemic Brain Injury with Human Umbilical Cord Blood Stem Cells: Mechanisms of Action

ABSTRACT

Despite the high prevalence and devastating outcome of ischemic stroke, there remain few options for treatment following stroke onset. The treatments that are currently available remain limited both in the time window following stroke in which they are effective, as well as their success in ameliorating stroke injury in the brain and limiting functional impairment. Current therapies for stroke, such as tissue plasminogen activator (tPA) are only effective up to 4.5 hours following stroke onset. We have tested a human umbilical cord blood-derived stem cell line that has shown both a significant reduction in stroke infarct volume as well as improved functional recovery following stroke in the rat when administered 48 hours following stroke onset. In the present study we have compared high vs. low passage non-hematopoietic umbilical cord blood stem cells (nh-UCBSCs) to determine whether highly expanded nh-UCBSCs are as therapeutically effective as low passaged cells, and their mechanisms of action. Using the middle cerebral arterial occlusion (MCAO) model of stroke in Sprague-Dawley rats, we administered nh-UCBSC by intra-venous administration two days following stroke induction. These human cells were injected into rats without any immune suppression, and no adverse reactions were detected. Both behavioral and histological analyses have shown that the administration of these cells reduces the infarct volume by 50% as well as improve the functional

outcome of these rats following stroke for both high and low passaged nh-UCBSC. The brain transcriptome was compared between normal rats, and those with ischemic brain injury, and injury with nh-UCBSC treatment using next generation RNAseq analysis. Differences in the brain transcriptome revealed that ischemic brain injury was associated with significant increases in transcripts related to macrophage, T cell, and microglia function. Flow cytometry analysis of immune cells present in the brains of animals, in each of these three groups, confirmed infiltration of macrophages and T cells consequent to ischemia reduction to normal levels with nh-UCBSC treatment. Flow cytometry also revealed a restoration of normal levels of microglia in the brain following treatment. Overall, these data show a blunting of immune cell migration and activation that are typically activated in the brain following stroke, suggesting that nh-UCBSCs may act by inhibiting immune cell migration into the brain from the periphery, and possibly by inhibition of immune cell activation within the brain. Non-hematopoietic umbilical cord blood stem cells exhibit great potential to provide a novel therapy for stroke with no known ill-effects, and are effective at later time points following stroke than methods that are currently available in the clinic.

INTRODUCTION

Stroke is the fourth leading cause of death and the primary cause of long-term disability in the United States. Each year, more than 795,000 people have a

stroke, the majority of which are first time occurrences (Go, et al. 2014). Among all types of stroke 87% are ischemic strokes, which are caused by either a transient or permanent reduction of blood flow and oxygen supply to the brain, resulting in death of neural tissue. This reduction is most commonly due to arterial blockage due to clotting or narrowing of the blood vessels that supply the brain.

Despite the high prevalence of stroke there are very few options for treatment in existence today. One of the primary therapies available to stroke patients is tissue plasminogen activator (tPA), which is a thrombolytic agent, and is only effective within 4.5 hours following stroke onset (Lansberg, et al. 2009). Due to the narrow therapeutic time window, only 3-5% of stroke patients are able to receive this treatment (Go, et al. 2014). As yet, there are no therapies that are able to provide neuroprotection to the affected brain tissue following stroke.

Cell-based therapies are emerging as a promising therapy that can be administered within the first few days (compared to hours) following stroke onset. A variety of stem cell types have been tested as potential therapies for ischemic stroke, each with unique advantages and limitations. Embryonic stem cells (ESCs) are considered the gold standard for pluripotency as they are able to differentiate into any cell type. However, the administration of undifferentiated ESCs leads to teratoma formation, and it is extremely difficult to create a homogenous ESC culture that is completely free of undifferentiated cells (Seminatore, et al. 2010). ESCs that have been transplanted into the brain

following stroke have been shown to exhibit neuronal markers (Buhnemann, et al. 2006; Takagi, et al. 2005), though studies have not found these transplanted cells to exhibit functional connections (Ideguchi, 2010).

Induced pluripotent stem cells (iPSCs) are another potential cell-based therapy for ischemic stroke. These cells are adult fibroblasts that are genetically reprogrammed into an embryonic stem cell-like state (Takahashi, et al. 2007; Yu, et al, 2007). One of the primary benefits of these cells is the elimination of the need for immune suppression, as the fibroblasts can be taken from the patient needing the treatment. Unfortunately, studies have shown that despite this, epigenetic changes to these cells can still result in immune rejection (Pera, et al. 2011). Another drawback of using reprogrammed adult fibroblasts is the possibility of teratoma formation following administration (Kawai, et al. 2010).

Neural stem cells (NSCs) can be isolated from the subventricular zone and subgranular zone in both embryos and adults (Arvidsson, et al. 2002), and are not pluripotent, but are able to differentiate into many types of CNS cells. Administration of NSCs following stroke did show improvement in stroke outcome, though the survival rate of the administered cells was extremely low – at around 1-3% of transplanted cells (Wu, et al. 2010).

Several types of bone marrow derived stem cells are being studied as potential stroke therapies. Marrow stromal cells (MSCs) have been shown to migrate towards a site of injury *in vitro* (Menon, et al. 2007), though these cells show poor survival rates when administered *in vivo* (Coyne, et al, 2006).

Multipotent adult progenitor cells (MAPCs) are also bone marrow derived, and have been shown to be able to regenerate into neurons (Serafini, et al. 2007). Administration of these cells has been shown to improve recovery following stroke (Zhao, et al. 2002), though they also show low engraftment rates and have the potential for teratoma formation (Burns, et al. 2006).

Cells derived from the mononuclear fraction of human umbilical cord blood (UCB) are becoming prominently studied as a potential therapeutic agent for stroke because they overcome many of the limitations presented by the other cells types discussed above. Many studies have shown that administration of varying fractions of UCB provides an improvement in stroke outcome when administered within 72 hours following stroke (Rowe, et al. 2012; Cui, et al, 2012; Boltze, et al, 2012). A limitation of UCB is that the quantity available from each donor may require multiple donors for treating an individual patient. In a previous study we addressed this issue by identifying and characterizing CD34-negative stem cells within UCB that could be expanded *in vitro* (Xiao et al., 2005). This cell fraction also expresses the stem cell markers Sox-2, Rex-1, and Oct-4 (Xiao, et al., 2005). We refer to these cells as non-hematopoietic umbilical cord blood stem cells (nh-UCBSCs). We demonstrated that systemic administration of nh-UCBSCs 48 hours after ischemic brain injury could significantly reduce infarct volume and ameliorate associated neurological deficits. In the current report we describe mechanisms of action underlying the neuroprotective effects of nh-UCBSCs in treating ischemic brain injury.

MATERIALS AND METHODS

Animal Subjects

Female Sprague-Dawley rats, 275-300 grams, from Charles-River Laboratory. All animals were housed and maintained according to the guidelines of the University of Minnesota Animal Care and Use Committee (IACUC). Clean bedding and fresh food and water was regularly provided by Research Animal Resources (RAR) staff. All animals received regular veterinary care by RAR.

Surgical Induction of Stroke

Rats are anesthetized with Ketamine/Xylazine cocktail (0.85mL/kg; 75mg/ml Ketamine, 10mg/mL Xylazine) intramuscularly. The rat's head is stabilized in a Koph head-holder in a supine position after the fur of the neck area is shaved and sterilized. Midline skin in the neck is incised and retracted laterally to expose subdermal structures. The right common carotid artery is exposed after the digastric and sternomastoid muscles are retracted. After carefully separating the right vagus nerve, the right common carotid artery (rCCA) is traced distally to its bifurcation, the superficial branch of which is the external carotid artery (ECA). While tracing distally along it, the endpoint is reached which branches out into the superior thyroid artery, external maxillary artery and terminal lingual artery. The right ECA is freed by cauterization and cut at this point, so that ECA stem is long enough to be able to introduce the thread occluder for the temporary ligation. The right internal carotid artery (rICA) has an extracranial branch before it enters the cranium - the pterygopalatine artery - which is temporarily ligated for

a complete ischemic lesion. The rECA is cut at its distal stem so that a silicon-coated surgical thread can be inserted into its lumen. The thread is inserted into the bifurcation, and up into rICA. The occluder is kept in place for 1 hour before removal. The open arteries are then cauterized, the wound closed, and the animal monitored until fully sternal and recovered.

Laser Doppler

In order to ensure that the MCA occlusion is having the same effect on all animals, we used MoorLab laser doppler to monitor blood flow of the right hemisphere of the brain (Moor Instruments; Wilmington, DE). Once baseline blood flow is established, the MCA is occluded as described above. Blood flow must be maintained at 20-25% of baseline during the occlusion period in order for individual animals to be kept in the study.

Behavioral Analysis

The Neurological Severity Score (NSS) (modified from De Ryck) were used to evaluate neurological deficit following MCAO. The NSS test consists of eight subtests evaluating motor and sensorimotor function of the animals' limbs. Left and right side limbs are each scored as either 2 (normal function), 1 (impaired function), or 0 (unable to perform). The scores from these subtests were tallied for the right side and left side limbs. The animals' functional score is presented as a ratio of the total score of the left side limbs to the right side limbs.

Measurement of Infarct Size

PFA-perfused brains were parrafinized and cut into 5μm sections by microtome and mounted sequentially on gelatin-coated slides. A 5μm slice was mounted every 25μm throughout the length of the brain, slicing rostral to caudal. The slides were then stained with Hematoxylin and Eosin to determine infarct size. Briefly, slides were deparaffinized through 3 changes of xylene (3 minutes each), hydrated through graded alcohols for 30 seconds each, and then run through a gentle flow of tap water for 1 minute. Slides were stained with hematoxylin for 3 minutes, rinsed with water for one minute, acid water for 15 seconds, water for one minute, ammonium water for 15 seconds, tap water for one minute, and Eosin for 1 minute. Slides were then dehydrated through graded alcohols to 100% alcohol, and cleared in 3 changes of xylene (30 seconds each). Slides were coverslipped with Permount and allowed to air dry. Images of sequential sections from each brain were collected using a Nikon Eclipse E600 equipped with SPOT (Diagnostic Instruments Inc., Sterling Heights, MI). ImageJ (NIH) was used to obtain volume measurements. Infarct volume was measured by quantifying the infarct area of each mounted slice, and then multiplying the area measurement by the section thickness to create a measure of volume for that section. The volume of the infarct for all sequential sections was summed to obtain the total infarct volume.

Preparation and Administration of nh-UCBSC

Human Umbilical Cord Blood Stem Cells (UCBSC) were obtained and grown as described in our previous publications (Xiao, et al. 2005). The cell suspension

injectate was made immediately before use with a final concentration of $2x10^6$ cells/mL in sterile saline. Those animals chosen randomly to be in the treatment group received 1 million cells in 0.5mL sterile saline by way of the saphenous vein. Injected animals were briefly anesthetized with isofluorane gas for the duration of the injection (<1 minute), and monitored until fully awake and sternal.

Flow Cytometry

Brain mononuclear cells were isolated using a density gradient as described in Pino, et al. (2011). Briefly, whole brains were homogenized and suspended in a 30% Percoll solution with media. This mixture was gently pipetted over 1mL of 70% Percoll to create the density gradient. After centrifugation, the cell interface was removed. Once the mononuclear fraction was isolated and counted, a 96 well plate was seeded with $5X10^5$ cells per well. The seeded cells were then blocked using a mouse anti-rat Fc block for 5 minutes at 4º C. Cell surface antibodies were then added and incubated for 15 minutes at 4º C. Wells were then spun down and washed three times with PBS (w/2% FBS). Cells were fixed using the cytofix/cytoperm kit (BD Biosciences) according to manufacturer's directions. Cells were washed again three times. Intracellular antibodies were added and incubated for 20 minutes at 4ºC. Cells were washed three more times, and resuspended in FACS buffer with AccuCount beads (Spherotech; Lake Forest, IL) for analysis on the flow cytometer (BD FACSCanto). Cells were stained using CD45 and CD11b (BD Biosciences; San Jose, CA) to identify microglia and macrophages, CD3, CD4, and CD8a (eBiosciences; San Diego,

CA) to identify T cells and their subtypes. Tregs were identified by the expression of CD3 (+) CD4 (+) FoxP3 (+) on brain immunocytes. CD25 was used as an activation marker to identify activated CD3+ T lymphocytes. Mature B cells were identified using a triple color reagent to select CD3-CD161-CD45RA+ cells (ABD Serotec, Kidlington, UK). Isotype specific antibodies were used for all dyes and panel combinations to control for nonspecific antibody binding. Immunostained cell populations were analyzed using FlowJo software (TreeStar; Ashland, OR).

RNA isolation

RNA was isolated from the right cerebra of all animals using a modified method of the RNeasy Plus RNA Isolation Protocol (Qiagen; Germantown, MD). Tissue was disrupted using a mortar and pestle pre-cooled with liquid nitrogen. 600 µl RLT buffer + beta-mE were added to each sample and the sample was further homogenized using an electronic pestle. Each sample was run through a 28G needle to confirm homogenization. Once fully homogenized, the samples were run through gDNA Eliminator Mini Spin Columns (Qiagen; Germantown, MD) for two minutes at 10,000RPM or until the column was dry. 600 µl of 70% ethanol was added to each sample. Samples were then run through RNeasy Mini spin columns (Qiagen; Germantown, MD) two times for 15 seconds at 10,000RPM. Flow through was discarded. 700µl of RWT buffer was added to the RNeasy spin column, and the flow through was discarded. 500µl RPE buffer was added to the RNeasy spin column and the flow through was discarded. This step was repeated a second time. 50µl RNase-free water was added to the spin

columns and let sit for 10 minutes. Columns were then spun down and RNA was measured using a spectrophotometer.

RNAseq

RNA from the three experimental groups (control, stroke, stroke + UCBSC treatment) was submitted for sequencing at the University of Minnesota Genomics Center using the Illumina HiSEq 2000 sequencer. The gene expression profile for each group was analyzed using the following parameters: 50 cycle read length; paired-end read type; 20 million reads; 50-100 nucleotide fragment size. Genes with significant increases in expression for the MCAO model ($P<.01$) were identified using Galaxy. A subset of genes within this group was identified that revealed a significant decrease in expression for the therapy models as compared to the stroke models. These genes were categorized by cell type in which they are expressed.

Statistical Analysis

ANOVA was used to determine differences between groups. P values less than 0.05 were considered significant. Outliers in all data sets were determined using Grubb's test and excluded from analysis. The Bonferroni-Holm post-hoc test was used to determine significance between groups. All data is plotted as means with SEM. Statistics were calculated using GraphPad PRISM software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

High passage nh-UCBSC administration reduces infarct volume and improves functional recovery

In our previous study investigating the efficacy of nh-UCBSC in ischemic stroke (Xiao et al., 2005) we used cells that were passaged to generate approximately 8 population doublings. In the present study we also used nh-UCBSC at 25 population doublings to determine if the expanded cells maintained their therapeutic efficacy. As in the previous study human nh-UCBSCs were administered intravenously 48 hours following MCAO surgery. Animals were sacrificed 7 days following MCAO surgery for histological analysis of infarct size (Fig 1A, B). Quantification of infarct size showed a significant decrease in infarct volume in both nh-UCBSC groups as compared to the saline treated groups (Fig 1C). Behavioral function was assessed 24 hours and seven days following MCAO surgery with the Neurological Severity Score (NSS). At 24 hours following stroke, all treatment groups showed equivalent functional impairment. At seven days following MCAO surgery, the saline treated group showed no functional improvement. In contrast, the nh-UCBSC treated animals showed significant functional improvement in the NSS test (Fig 1D).

To determine whether the expansion of the cells diminished the therapeutic efficacy of nh-UCBSCs, cells at eight population doublings and 25 population doublings were compared. There was no significant difference

observed between the two groups in terms of reducing infarct size (Fig 1C) and improving neurological functional recovery (Fig 1D).

Changes in gene expression in the brain due to stroke are normalized with UCBSC treatment

Next generation sequencing RNAseq was used to determine transcripts which have significantly altered expression in the brain seven days following stroke onset. A total of 240 genes showed significantly altered expression levels following stroke that were then normalized with nh-UCBSC treatment (Fig 2). The genes that increased in expression level following stroke are predominantly related to inflammation and immune function. The genes that decreased in expression following stroke are predominantly related to neuronal function.

nh-UCBSC treatment reduces the number of macrophages in the brain following stroke

RNAseq analysis showed an increase in the expression of several macrophage activation markers in the ischemic brain, 7 days following stroke, and the expression levels were reduced to levels seen in non-ischemic brains with nh-UCBSC treatment. The genes that were significantly affected were FABP4 - a macrophage marker (Fig 3A), LPB – LPS binding protein, which binds to CD14 and TLR4 on macrophages, neutrophils and dendritic cells (Fig 3B), CCL6 - a marker of neutrophils and macrophages (Fig 3C), A2M – a protein

synthesized by macrophages (Fig 3D), MPEG1 – expressed by macrophages (Fig 3E), WAS – expressed by hematopoietic cells (Fig 3F), CD14 – a TLR4 co-receptor which is recognized by several pathogen associated molecular patterns (PAMPs) (Fig 3G), LGALS3BP – binds to macrophage associated lectin MAC-2 (Fig 3H), and MRC2 – mannose receptor found on macrophages and dendritic cells, involved in the recognition of pathogens (Fig 3I).

To determine alterations in the number of macrophages in the brain following ischemia and nh-UCBSC treatment, flow cytometry analysis were conducted. Flow cytometric analysis of brain tissue shows a significant increase in the number of CD11b high/CD45 high macrophages in stroke animals (Fig 4). This population is reduced in the nh-UCBSC treated animals, though the macrophage population is still significantly higher than that of the control group (Fig 4D).

nh-UCBSC treatment reduces the number of microglia in the brain following stroke

Flow cytometry was also conducted to determine the number of microglia in the brain after ischemia and treatment with nh-UCBSCs. Flow cytometry analysis of brain tissue revealed an increase in the absolute numbers of CD11b high/ CD45 intermediate microglia in the stroke brain (Fig 5). This population decreased in cell number in the nh-UCBSC treated group, but these changes were not statistically significant (Fig 5D).

nh-UCBSC treatment normalizes the number of T cells in the brain following stroke

RNAseq analysis a revealed a significant increase in the expression levels of several genes related to T cells and their function in the ischemic brain 7 days following stroke. These levels were reduced to numbers seen in non-ischemic brains in the nh-UCBSC treated animals. The genes that were significantly affected were: LCP2 – a T cell marker (Fig 6A), CD4 – a marker of Helper T cells (Fig 6B), GM2A – involved in T cell activation (Fig 6C), and SH2B3 – which is involved in T cell activation (Fig 6D).

Flow cytometric analysis was conducted to quantify T cell infiltration into the brain after ischemia and treatment with nh-UCBSCs (Fig 7). Analysis of CD3+ T cells showed a significant increase in the total numbers of CD3+ T cells in ischemic animals 7 days following MCAO surgery, and a reduction to levels seen in non-ischemic brains of the CD3+ T cell population in the nh-UCBSC treated animals at 7 days (Fig 7D).

Flow cytometry showed a significant increase in CD4+ T cells in the stroke brain 7 days following MCAO (Fig 8A-C). The number of CD4+ T cells was reduced to non-ischemic levels with nhUCBSC treatment (Fig 9D). In contrast, no changes in the absolute number of CD8a+ cytotoxic T cells were observed among the treatment groups (Fig 8E).

CD4+ helper T cells were co-stained with the activation marker CD25 to determine their activation profile during ischemic stroke and with nh-UCBSC

treatment (Fig 9). Flow cytometry analysis showed the absolute number of CD4+/CD25+ activated helper T cells significantly increased in the stroke brain at 7 days, and the number of these cells was reduced to non-ischemic levels in the nh-UCBSC treated group (Fig 9D).

CD8a+ cytotoxic T cells were co-stained with the activation marker CD25 (Fig 10). Flow cytometry analysis showed that there was no change in the number of activated cytotoxic T cells in the brain across treatment groups (Fig 10D).

Flow cytometry was used to evaluate the presence of regulatory T cell population by gating for CD3+, CD4+, FoxP3+ cells (Fig 11). Quantification of this population shows an increase in the absolute number of regulatory T cells in the stroke brain 7 days following MCAO, and a reduction in the cell number of this population in the UCBSC treated group. These changes in the Treg population were not statistically significant (Fig 11D).

UCBSC treatment normalized the number of B cells in the brain following stroke

Flow cytometry analysis of mature B cells shows a decrease in the absolute number of B cells in the ischemic brain 7 days following MCAO, and B cells numbers increased towards the control levels of B cell numbers in the UCBSC treated group (Fig 12A-C). The changes in B cells numbers were not statistically significant (Fig 13D).

DISCUSSION

Our results confirm that administration of nh-UCBSCs 48 hours after stroke onset significantly reduces infarct size and improves functional recovery in a rat model of transient focal ischemia. In order to determine the practical potential of transitioning nh-UCBSC therapy into the clinic, we determined that nh-UCBSCs do not lose therapeutic efficacy when expanded in culture for an extended period of time. This would allow clinicians to expand cells from one donor to be able to treat multiple patients – giving nh-UCBSC therapy a distinct advantage over other proposed cord blood cell-based therapies which require a new donor for each patient, and tissue matching.

The therapeutic benefit of nh-UCBSCs when delivered at 48 hours after stroke is consistent with our previous study (Xiao et al., 2005) using low passage nh-UCBSCs. Several groups have studied different time periods of administration of the mononuclear fraction of umbilical cord blood stem cells following stroke in order to determine the optimal therapeutic time window. Newcomb, et al (2006) found functional improvement and reduced infarct size was optimized when cells were administered 48 hours following ischemic stroke. They also found that the time of administration was more important to an improved stroke outcome than cell dose, as the optimal cell dose at any other time point tested did not show significant improvement. Boltze, et al. (2012) confirmed these results, and found that improvements in functional recovery and

significant decreases in infarct size were observed if the mononuclear fraction of umbilical cord blood stem cells were administered within 72 hours following stroke onset. Together these results demonstrate the ability for cell therapy to greatly expand on the current time window of therapy with tPA at 4.5 hours following stroke (Lansberg, et al. 2009).

Our results also demonstrated that the neuroprotective effects of nh-UCBSC administration were achieved without the utilization of any immune suppressive agent. This observation is consistent with the work by Pan and colleagues (Pan, et al.; 2005) showing that immunosuppressive agents are not required for achieving therapeutic efficacy with human cord blood in cases of a rat model of ischemic stroke. They compared MHC levels in animals that received injections of UCBSCs with and without Cyclosporin A. They found that there was no significant difference in the expression of MHC I between groups, and there was no difference in cell survival between groups. Vendrame et al (2005 and 2006) also assessed the efficacy of the mononuclear fraction of cord blood in ischemic brain injury and found neuroprotective effects without the use of immunosuppressive compounds. Thus, immune suppression is not required to prevent acute rejection of nh-UCBSCs in a rat model of ischemic stroke. nh-UCBSCs were also tested as a therapy for myocardial infarction by Chen, et al. (2013). This group administered the cell therapy at 48 hours following ischemia, and did not use any immune-suppressive agents. They found that the administration of nh-UCBSCs showed an increase in overall heart function and

preserved fiber orientation in the injured tissue area, similar to that of the sham operated animals.

As cell-based therapies are gaining traction in the field of novel stroke treatments, multiple cell types have been tested. One of the most commonly investigated cell types is marrow stromal cells. These cells have been shown to migrate to the site of injury post-injection, though they often have very poor rates of cell survival. Despite this, multiple studies have observed improved stroke outcome following marrow stromal cell administration (Coyne, et al. 2006; Willing, et al. 2003). Multipotent Adult Progenitor Cells (MAPC) have also demonstrated an ability to improve stroke outcome following administration, though no evidence of engraftment into the injured tissue has been found (Zhao, et al. 2002; Burns, et al. 2006). Several groups have studied umbilical cord blood stem cells, and all have found these cells to improve stroke outcome, but as with previously discussed cell types, little to no evidence of engraftment of these cells in the injured brain tissue has been found (Cui, et al. 2012; Rowe, et al. 2012; Xiao, et al. 2005).

Despite the repeated success of cell based therapies in pre-clinical trials the mechanism of action of these cells is widely debated. Initially, it was thought that by introducing stem cells to the post-ischemic brain, these new cells would be able to engraft into the damaged area and replace lost tissue, thus recovering function in the damaged areas of the brain. While some studies have shown evidence of these exogenous cells in the post-ischemic brain after treatment, the

evidence that these cells engraft to become functional cells is limited. What is more commonly found is that systemically administered cells show a benefit to stroke outcome, but are generally not found - or only found in small numbers - in the brain after administration (Rosado, et al. 2013). This continued finding has suggested that the mechanism of action is not likely due to engraftment and cell replacement, but due to some other mechanism. More recently, evidence has shown that a possible explanation for the mechanism of action of cell-based therapies is due to these cells exhibiting an influence on the post ischemic environment via the immune response and the inflammatory cascade (Walker, et al. 2011).

Our analysis of mRNA in animals with ischemic stroke demonstrated a robust immune response in the ischemic brain associated with the infiltration of macrophages and T cells, as well as the proliferation of microglia. This observation is consistent with our current understanding of the role the immune system plays in the post-stroke brain. Ischemic stroke causes a disruption of the blood brain barrier, thus allowing peripheral immune cells to infiltrate into the brain and influence the local immune response. These results are congruent with studies in both animals and humans that have found similar increases in these cells in the circulating blood as well as the brain (Garcia, et al.; 1994; Urra, et al, 2009).

T cell trafficking into the brain has been shown at 24-48 hours after ischemia (Kleinschnitz, et al; 2010). This involvement of the perivascular space

activates resident macrophages and microglia which begin to clear dead cells, thus increasing inflammation and stimulating the influx of more leukocytes. The ischemic cell death that occurs in the brain following stroke is another source of increased inflammatory signaling. These observations combined with studies in which T cell deficient mice are protected from stroke damage suggest that infiltration of peripheral T cells play a crucial role in the development of stroke injury (Kamel, et al.; 2012). T cells, but not B cells have been shown to be involved in the evolution of the brain infarction following stroke (Kleinschnitz, et al., 2010; Liesz, et al, 2011). These infiltrating T cells are a major source of IFNγ, which causes neurotoxicity (Yilmaz, et al; 2006; Shichita et al; 2009). The results of our study show the aforementioned influx of T cells in the ischemic brain. This influx has been reported to occur at approximately the same time period at which we administer nh-UCBSC treatment – following which we do not see the characteristic increase of T cells present in the ischemic brain. This suggests that the mechanism by which the nh-UCBSC treatment works involves the inhibition of the influx of T cells - at 48 hours following stroke – though whether this effect is direct or indirect has yet to be determined. If these cells are achieving the amelioration of stroke deficit by blunting the influx of immune cells to the brain following stroke, it would also prevent the propagation of an inflammatory microenvironment surrounding the ischemic area.

Monocytes from bone marrow which can differentiate into either macrophages or dendritic cells have been shown to migrate into to infarct areas

in the brain following ischemic stroke (Amantea, et al. 2009). These cells are found in the border of the infarct area within 24 hours following stroke, and mature phagocytes are found directly within the infarct core (Gliem, et al. 2012). Studies have shown that the phagocytes found within the infarct core are resident microglia that have transformed into phagocytes (Tanaka, et al. 2003; Schilling, et al. 2003). Infiltrating peripheral macrophages are most abundant in the brain at 3-7 days following focal ischemia (Schilling, et al. 2003; Breckwoldt, et al. 2008). These results suggest that resident microglial activation precedes peripheral monocyte and macrophage infiltration, and that the peripheral macrophages are likely to contribute to the delayed phase of inflammation and brain injury that occurs at 3-7 days following focal ischemia. The results of our studies corroborate these findings, showing an influx of macrophages in the brain as shown by flow cytometry, as well as an increase in the expression of macrophage-related mRNA transcripts in our stroke animals at 7 days following stroke. The timing of our nh-UCBSC treatment at 48 hours following stroke coincides with the time period during which peripheral monocytes are infiltrating into the ischemic brain. As our results show a significant decrease in the number of macrophages in the brain at 7 days following stroke in our nh-UCBSC treated animals, this suggests that the treatment has some effect on the infiltration of these peripheral cells, thus blunting the added inflammation and injury that typically results from the infiltration of these cells.

One of the earliest cellular responses to ischemic stroke is the activation of resident microglia, which occurs within the first hour following stroke onset. There are two subtypes of activated microglia: the classically activated M1 and the alternatively activated M2. Ischemic neurons prime the polarization of microglia towards the M1 subtype, which acts as a pro-inflammatory mediator, and enhances neural injury. In contrast, the M2 phenotype has been shown to provide neural protection via the promotion of an anti-inflammatory microenvironment (Hu, et al. 2012). Our results show the characteristic increase in activated microglia in the ischemic brain 7 days following stroke. The population of microglial cells decreases in the brains treated with nh-UCBSCs, although this decrease is not significant. This decrease suggests that the nh-UCBSC treatment either directly or indirectly ameliorated the activation of microglia in the post-ischemic brain.

Our body of data suggests that the changes we observe in immune cell populations and their related mRNA transcripts following nh-UCBSC treatment following stroke show that one of the major potential mechanisms of action is the inhibition of the recruitment of peripheral immune cells into the ischemic brain. This could occur via the inhibition of MCP-1 and CCR2, both of which are involved in the migration of peripheral inflammatory cells following stroke. Studies of mice that are deficient in both MCP-1 and CCR2 have shown a significant reduction in infiltrating macrophages following stroke (Schuette-Nuetgen, et al. 2012). Another possibility is that nh-UCBSCs are able to blunt the polarization of

microglia and macrophages to their pro-inflammatory M1 phenotype. Studies of adult marrow derived mesenchymal stromal cells have been shown to secrete low levels of immunomodulatory molecules such as CCL2 and IL-6 – both of which promote M2 macrophages (Dao, et al. 2013). Our mRNA results show an increase in expression of LBP (LPS Binding Protein), CD14, and TLR4, all of which are involved in the LPS-based activation of M1 activation. The expression of these genes is completely normalized following treatment with nh-UCBSCs, suggesting an influence on the molecular mechanism by which the M1 phenotype is achieved.

Our results also showed a decrease in the number of mature B cells in the brain following stroke, and that the number of mature B cells seemed to be restored to control levels following nh-UCBSC treatment. While the role of B cells in the development of stroke is still widely debated, regulatory B cells have been recently reported to have benefits in the ischemic brain 24-48 hours following stroke (Ren, et al; 2011). In addition, it has been shown that the absence of B cells leads to an increased number of activated T cells, microglia, and monocytes in the brain. The IV administration of B cells prior to stroke resulted in a decrease in infarct size as well as a decrease in the number of activated immune cells in the brain (Bodhankar, et al. 2013). A possible mechanism for the protective effect of B cells is the production of the anti-inflammatory cytokine IL-10 in the stroke brain (Li, et al. 2014) These results, combined with our findings suggest that at least some fraction of B cells play a role in protecting the ischemic brain –

possibly by combatting the inflammatory cascade with the release of anti-inflammatory signals.

The body of our work suggests that the administration of nh-UCBSC following stroke has a profound effect on the immune response to ischemia, reflected largely in the alteration of immune cell phenotypes present in the brain following stroke. One of the primary mediators of the release and activation of immune cells following brain injury is the spleen. Seifert and colleagues (2012) have shown that NK cells and monocytes from the spleen traffic to the brain 48 hours following injury, and that T cells migrate to the brain by 96 hours following injury. Offner, et al. (2006) reported similar findings, noting an increase in macrophages and CD4+FoxP3+ T cells in the blood stream at 96 hours following stroke, as well as a decrease in B cells, suggesting the migration of immune cells from the spleen to the site of injury following stroke. In addition, multiple studies have reported a reduction in spleen size in the acute phase of ischemic stroke, corresponding to the migration of immune cells to the brain, as shown by a decrease in CD8+ T cells in the spleen (Vendrame, et al. 2006). When these animals were treated with HUCBC, the spleen showed a decrease in expression of the pro-inflammatory cytokine TNFα, and an increase in the expression of the anti-inflammatory cytokine IL-10, suggesting a switch from a Th1 to Th2 response in the spleen. A human study also showed a decrease in spleen size in the acute phase following stroke, followed in some cases by spleen expansion.

Patients that showed splenic expansion following stroke correlated to good clinical recovery (Sahota, et al. 2013).

To further support the importance of the role of the spleen in the immune response to stroke, several studies have shown that animals that received a splenectomy prior to stroke showed a decreased immune response, and improved stroke outcome (Ostrowski, et al. 2012; Zhang, et al. 2013; Rasouli, et al. 2011; Fathali, et al. 2013). In addition, studies have shown that treatment with the mononuclear fraction of HUCB following stroke reverses the stroke related changes in the spleen, such as a decrease in T cell number in the spleen, decrease in monocytes in the blood stream, and a decrease in the macrophages and microglia in the brain (Golden, et al. 2012). These findings suggest that the inhibition of the immune response propagated by the spleen following ischemic injury could be crucial to ameliorating stroke related brain injury. It is likely that the administration of nh-UCBSCs at 48 hours following stroke plays a role in the inhibition of the splenic response, resulting in improved stroke outcome.

In conclusion, our results have further demonstrated the potential for nh-UCBSCs to provide effective therapy for stroke when administered 48 hours following the onset of ischemia. Our analyses of mRNA transcripts and immune cell phenotyping in the post-stroke brain have shown that nh-UCBSC treatment ameliorates the upregulation of immune cell presence in the brain. These results suggest that the influx of immune cells at 48 hours following stroke play a major

role in the development of stroke infarction, and the blunting of these cell populations in the brain following stroke effectively ameliorates stroke deficit.

ACKNOWLEDGEMENTS

These studies were funded in part by NIH Grants T32 DA007097, T32 AG029796, and R41 NS056626.

Chapter 3

Summary, and Future Directions

Summary of Results

Our body of work has shown the ability for systemic administration of nh-UCBSCs 48 hours following MCAO in a rat to improve functional recovery and reduce the volume of the infarct in the rat brain. These results were achieved without the use of an immunosuppressive agent. Evidence from previous studies has shown that the mechanism by which nh-UCBSCs exert their effects is not due to engraftment into existing tissue, and that the survival of injected cells is very low as soon as 48 hours following administration. Given this information, we deemed the use of an immunosuppressive agent to be unnecessary.

In addition, we tested nh-UCBSCs that had been expanded in culture for extended periods of time to determine if they are able to maintain their therapeutic efficacy. Our results show that extended time in culture does not affect the therapeutic efficacy of nh-UCBSCs to treat stroke, and that there are no major genetic transformations occurring following this extended time in culture. These results suggest that nh-UCBSCs obtained from a single donor can have the ability to be expanded extensively to provide treatment for multiple patients. The lack of need for immunosuppression in addition to the ability for nh-UCBSCs to maintain their therapeutic efficacy following extended culture time gives this cell line a distinct advantage over other cell-based therapies in terms of clinical applications.

Our study has also shown evidence that the administration of nh-UCBSCs acts by blunting the immune response following ischemia that typically results in the influx of peripheral immune cells such as macrophages and T cells. These infiltrating cells secrete pro-inflammatory and cytotoxic factors that contribute to the non-specific cell killing that contributes to the growing stroke injury in the brain. Our results also suggest nh-UCBSCs play a role in decreasing microglial activation within the brain, which is another source of the propagation of the pro-inflammatory environment and cell killing following stroke.

Limitations of Study

While our study utilized many of the gold standards for stroke research in rodents (i.e. MCAO model of stroke, NSS score for behavior, healthy rat), there are several limitations to the parameters of our study.

Firstly, our animal model utilized young, healthy female rats. While the use of young, healthy animals is an extremely common model for stroke research for several pragmatic reasons, it also presents several problems when attempting to translate any results gained via this model to humans. We chose to use female rats because despite strokes occurring in both males and females, the vast majority of pre-clinical stroke research is conducted in male research animals. There is a possibility that the estrous cycle of our female test subjects could have a confounding effect on our results, and it was not accounted for in this study.

Most strokes occur in elderly humans, but we use young animals due to

their good survival rates following MCAO surgery. In addition, most stroke patients are not healthy adults whose only health concern is the stroke itself – nearly all present with one, if not more co-morbidities which can contribute to the patients' immune response, inflammatory state, and ability to recover functionally.

We also only used a single model of temporary focal ischemia that resulted in a reproducible injury in all of our animal subjects and had a high rate of survival. This is a practical approach for experimental studies, but does not reflect the highly variable nature of strokes occurring in humans. Strokes vary in size and duration, and some are chronic and do not result in reperfusion, as was the case in our current animal model. The severity of stroke could change the extent of the immune response, BBB permeability, and inflammatory microenvironment following stroke. While current research shows that nh-UCBSC therapy to be effective at 48 hours following stroke, this time point might be able to be extended when being applied to instances of chronic stroke. In these cases, there is a prolonged state of inflammation in the brain, which could be benefitted by nh-UCBSC treatment. It is also possible that testing multiple administrations of nh-UCBSC could provide improved outcome in instances of chronic stroke.

Another limitation of our study is that we do not know if nh-UCBSCs indiscriminately blunt the immune response, or if it is somehow selective in only blunting the aspects of the immune response in stroke that are detrimental. More

work would have to be completed that more thoroughly characterized how each aspect of the immune response following stroke is being affected by the administration of nh-UCBSCs.

Future Research Directions

Currently, the work presented here is providing the groundwork for several follow-up studies. We are aiming to reproduce the results from this study in a canine model of stroke. The rat and canine data will then be used to submit to the FDA for the approval of a human clinical trial using nh-UCBSCs as a treatment for ischemic stroke. We are also beginning a clinical study that looks at the alteration of immune cell subsets in peripheral blood following ischemic stroke. Once these immune cell subsets have been characterized, data will be collected to determine if the characteristics of these immune cell populations correlate to stroke severity, and could potentially be used as biomarkers for stroke severity and recovery. Based on our existing data, we would expect to see higher numbers of T cells and macrophages in the blood of patients with severe stroke, and smaller populations of these cells in circulation in patients with less severe cases. In addition, monitoring these cell populations could provide a valuable way to monitor a patients' improvement following treatment.

Conclusion

Our study has provided valuable data supporting the use of nh-UCBSC as a cell-based therapy for ischemic stroke. In addition to strengthening the body of data supporting the therapeutic efficacy of these cells as a stroke treatment, we have also provided novel data suggesting the mechanism of action by which these cells provide the improved stroke outcome that has been repeatedly observed in pre-clinical research. This study has laid the groundwork for additional animal studies as well as several clinical studies, and has improved our understanding of how alterations in the immune response following stroke can have a beneficial effect on stroke outcome.

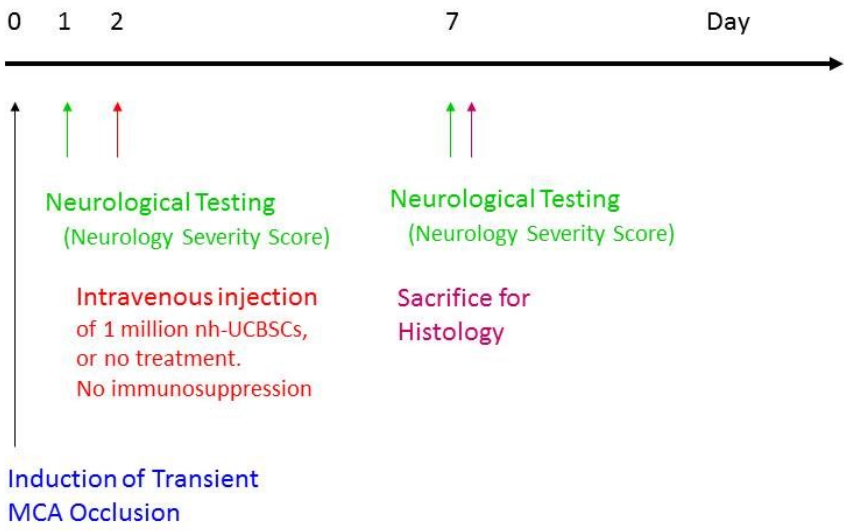

Figure 1. Experimental Timeline. Temporary focal ischemia was induced by MCAO surgery on day 0. Behavioral testing using the Neurological Severity Score (NSS) was conducted on day 1. On day 2, animals were given either an IV injection of 1 million nh-UCBSCs or no treatment without immunosuppression. On day 7 following MCAO surgery, behavioral testing was conducted again, and animal subjects were sacrificed for histological assessment.

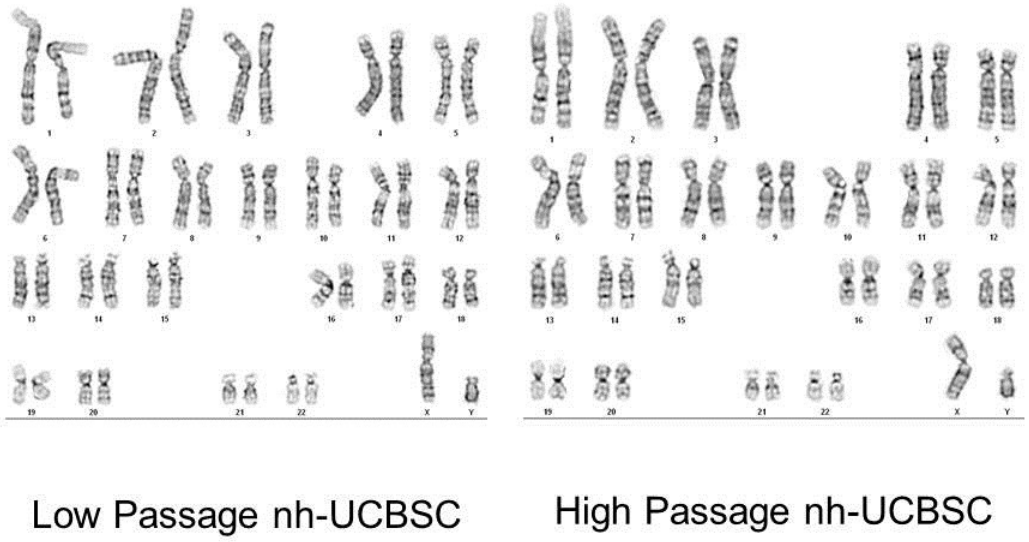

Figure 2. Karyotyping reveals no genetic transformation of nh-UCBSCs following extended time in culture.

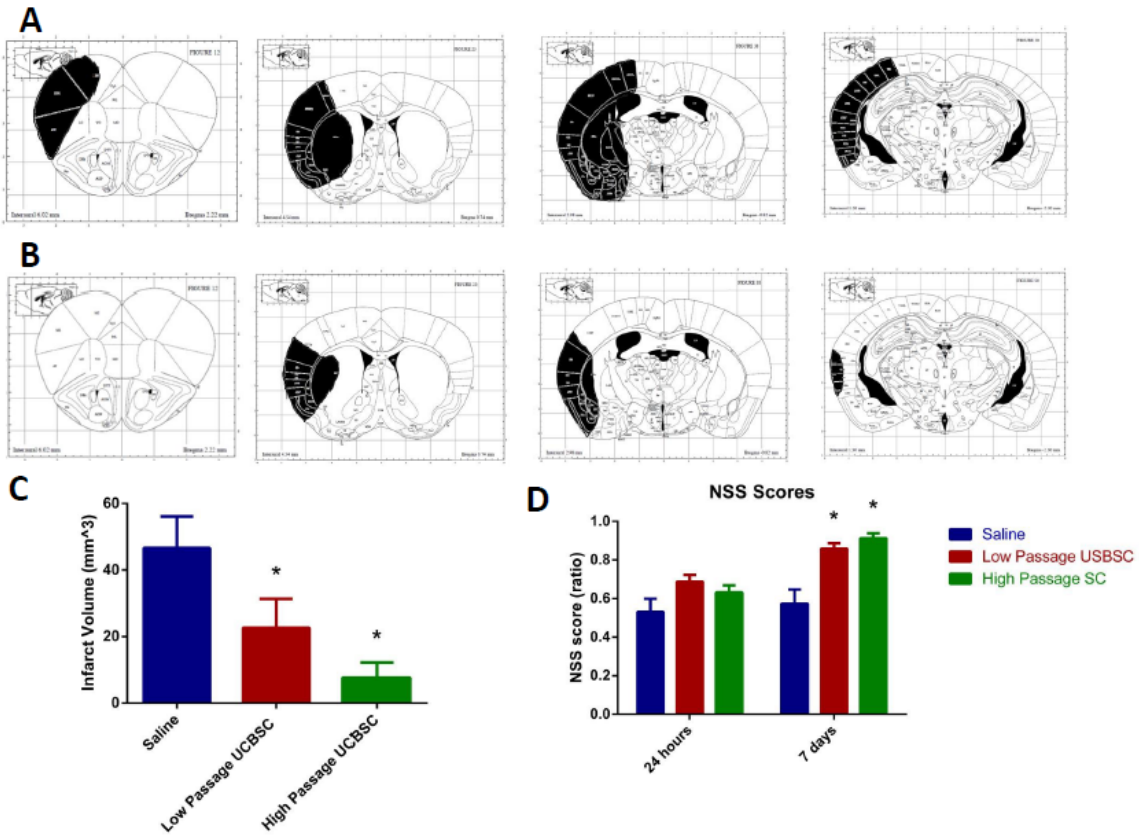

Figure 3. Stroke infarct volume measured by H&E staining. Staining was done 7 days following MCAO. Representative coronal sections of A) saline treated and B) nh-UCBSC treated MCAO animals. C) Quantification of infarct volume in saline treated, low passage UCBSC treated, and high-passage UCBSC treated animals (n=6). Low passage and high passage UCBSC groups have significantly smaller infarct volumes than saline treated animals ($p<0.05$). Average values (±SEM) from pooled data are presented. D) Treatment with high and low passage UCBSC improves functional recovery 7 days following MCAO. Behavior assessed by the Neurological Severity Score (NSS) is represented as a ratio of the scores of the left and right limbs. Both low and high passage UCBSC treated animals showed significant improvement in motor function compared to the saline group which showed no improvement at 7 days ($p<0.05$). Average values (±SEM) from pooled data are presented.

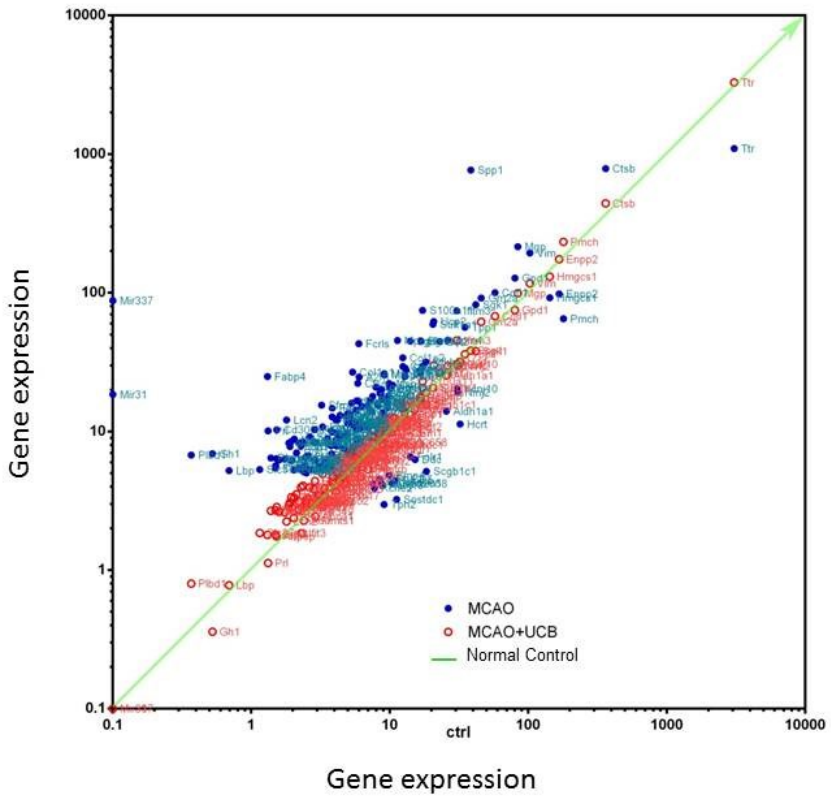

Figure 4. Scatterplot of gene expression levels across treatment groups obtained by RNASeq. Solid line represents gene expression levels (FPKM) in healthy control animals. Blue dots represent gene expression levels of stroke animals 7 days following MCAO surgery, and red dots represent gene expression levels of nh-UCBSC treated stroke animals 7 days following MCAO surgery. Genes shown in this scatterplot have significantly different expression levels ($p<0.05$) in the stroke group as compared to the healthy control group, and nh-UCBSC treatment normalized the expression levels of these genes.

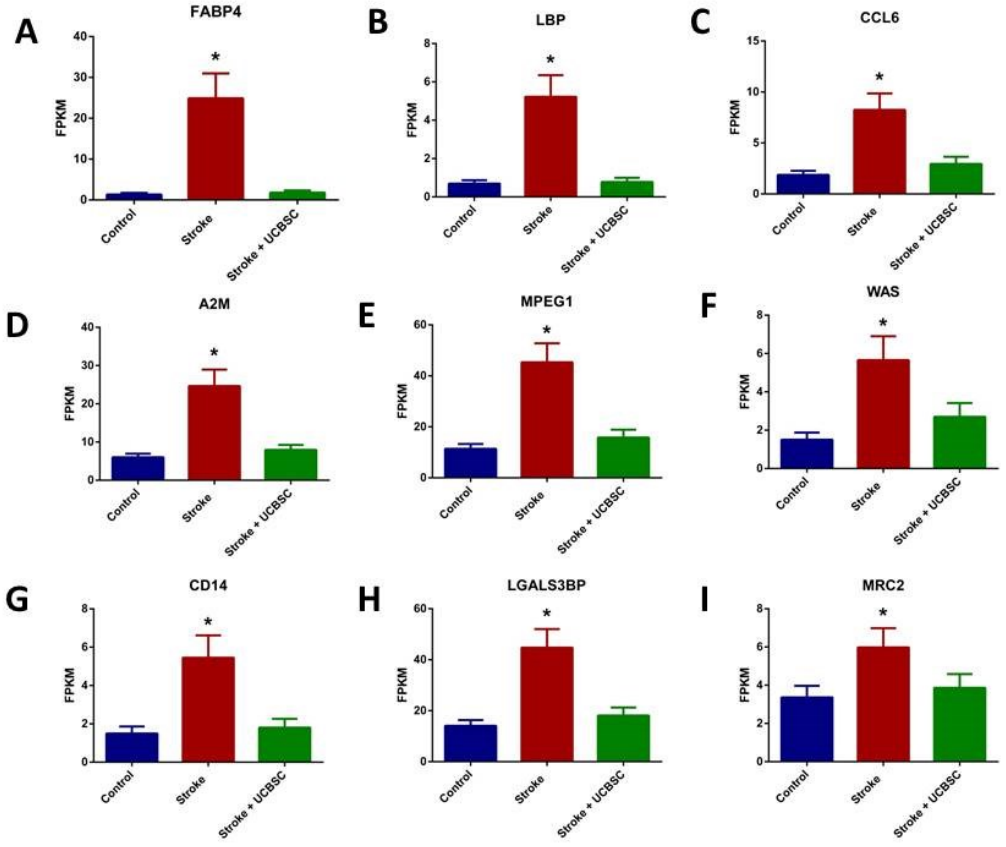

Figure 5. RNA sequencing shows an increase in the expression of a number of macrophage markers in the ischemic brain 7 days following stroke, and a normalization of expression in the nh-UCBSC treated brains. This pattern of expression if shown in A) FABP4, a macrophage marker, B) LBP, LPS binding protein that binds to CD14 and TLR4, C) CCL6, a marker of macrophages and neutrophils, D) A2M, a protein synthesized by macrophages, E) MPEG1, which is expressed by macrophages, F) WAS, which is expressed by hematopoietic cells, G) CD14, a TLR4 co-receptor, H) LGALS3BP, which binds to macrophage associated lectin, and I) MRC2, which is a mannose receptor found on macrophages and dendritic cells.

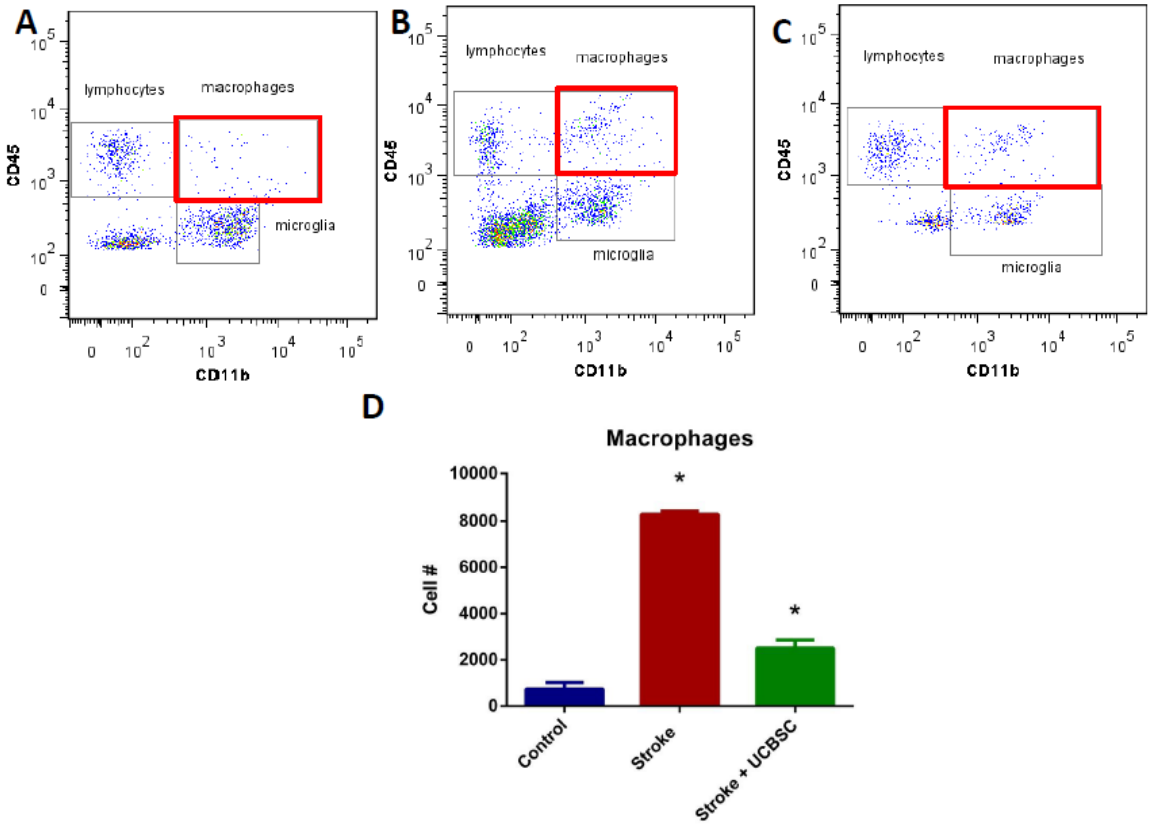

Figure 6. Number of macrophages in the stroke brain are significantly increased at 7 days following stroke, and UCBSC treatment reduces the total number of macrophages in the brain at 7 days following stroke. Flow cytometry showing macrophage populations in A) healthy control brain, B) saline treated stroke brain, and C) nh-UCBSC treated brain 7 days following stroke. Gates were established based on isotype stained brain samples. D) Quantification of macrophage cell numbers in each treatment group showed a significant increase in macrophages in the stroke brain 7 days following stroke, and a decrease in the UCBSC treated stroke group, though there is no significant difference between the stroke and UCBSC treated groups ($p<0.05$). Average values (±SEM) from pooled data (n=5) are presented.

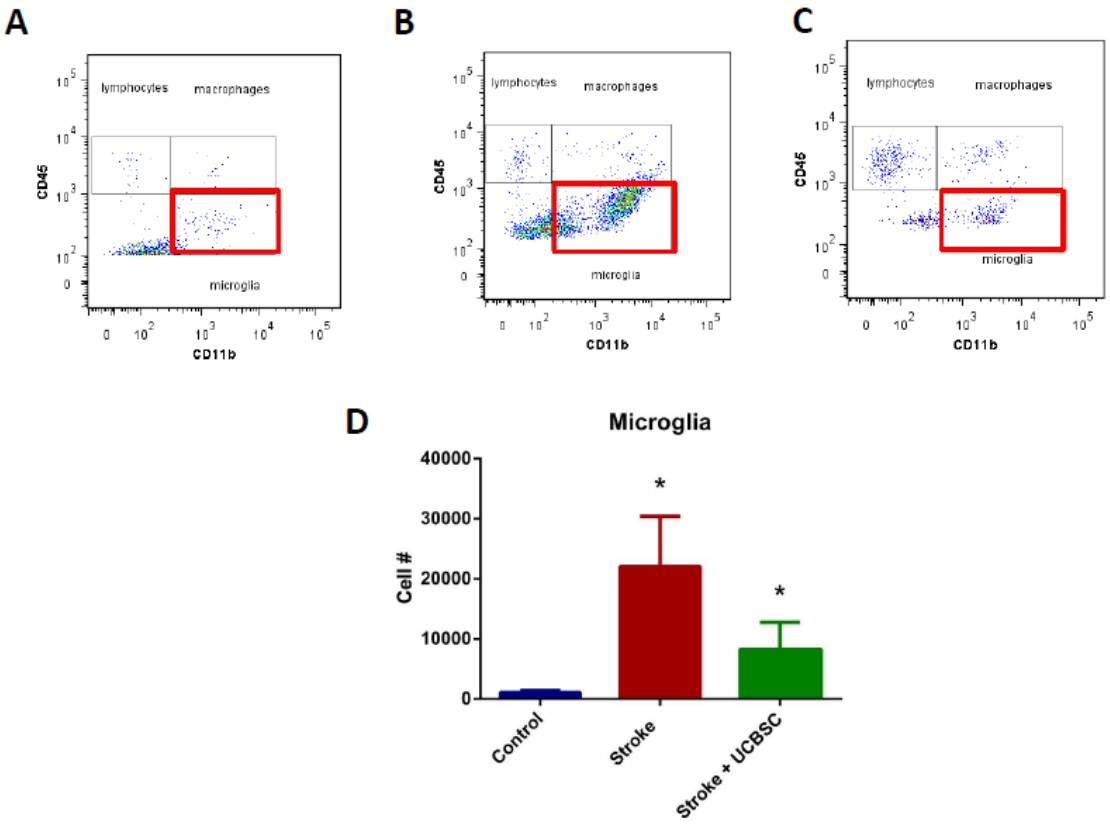

Figure 7. Population of Microglia increases in the ischemic brain 7 days following stroke, and is reduced in UCBSC treated stroke brains. Flow cytometry showing microglia populations in A) healthy control brain, B) stroke brain, and C) nh-UCBSC treated brain 7 days following stroke (n=5). Quantification of the absolute numbers of microglia in each treatment group shows an increase in microglia in stroke brains 7 days following stroke, and a decrease in the nh-UCBSC treated group, but these changes were not statistically significant ($p<0.05$). Average values (±SEM) from pooled data are presented.

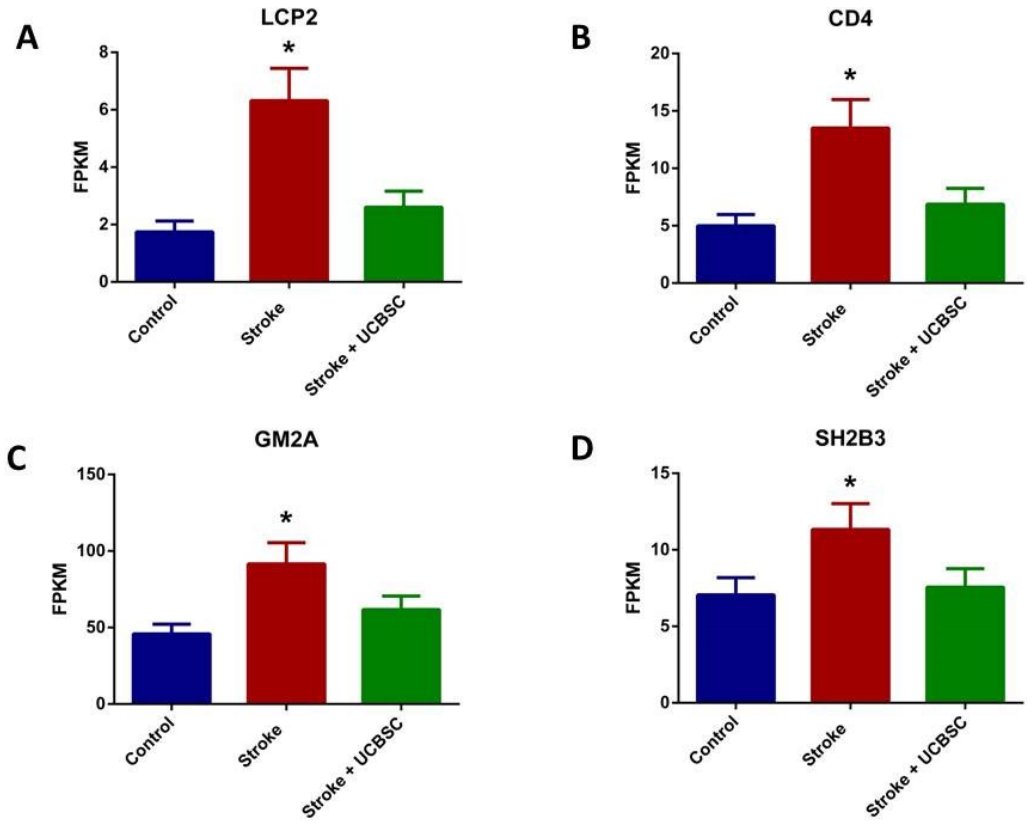

Figure 8. UCBSC treatment normalizes the expression of T cell related RNA transcripts at 7 days following stroke. A) RNA expression of LCP2 is significantly increased 7 days following stroke and normalized in the animals treated with UCBSC. B) RNA expression of CD4 is significantly increased 7 days following stroke and is normalized in the animals treated with UCBSC. C) RNA expression of GM2A is significantly increased 7 days following stroke and is normalized in the animals treated with UCBSC. D) RNA expression of SH2B3 is significantly increased 7 days following stroke and is normalized in the animals treated with UCBSC.

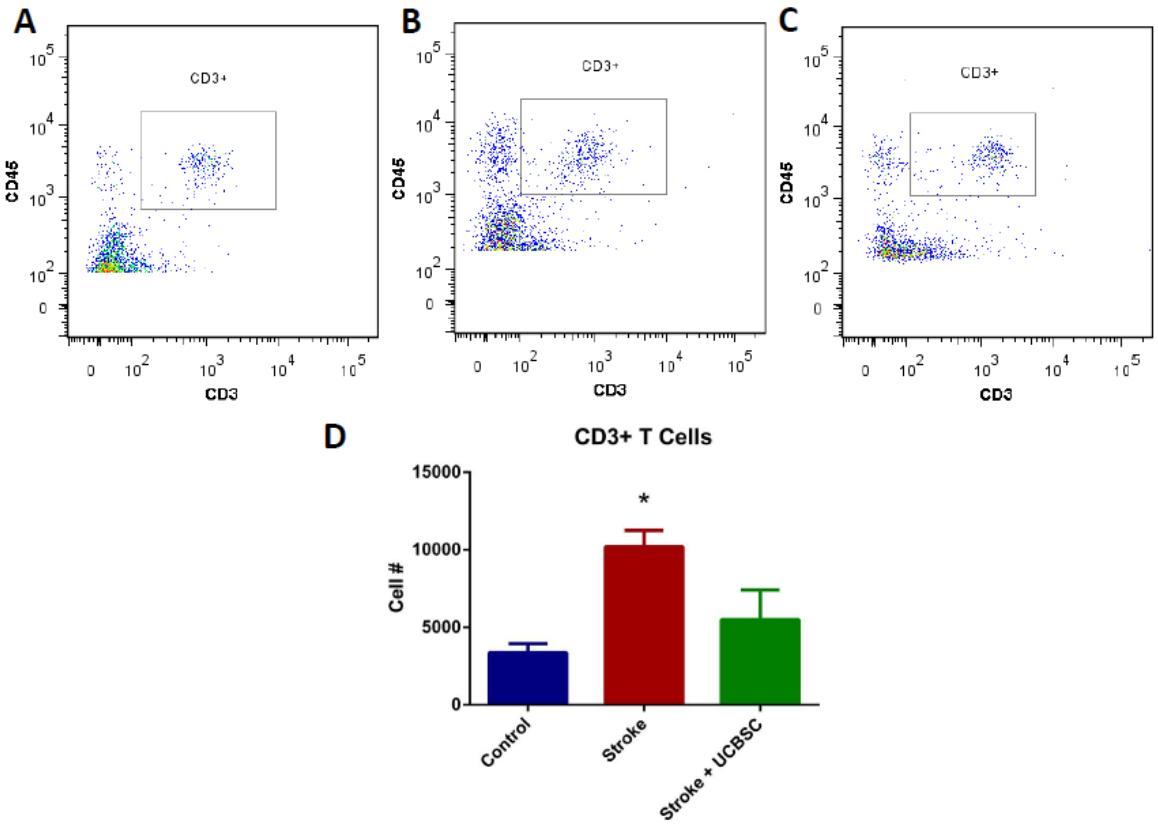

Figure 9. Administration of nh-UCBSC normalizes the number of CD3+ T cells present in the brain 7 days following MCAO. Flow cytometry shows the CD3+ T cell population in the brain of A) healthy control rats, B) stroke animals, and C) nh-UCBSC treated stroke animals. (n=5) Gating was established based on isotype stained brain samples. D) Quantification of the absolute number of CD3+ T cells in control, stroke, and UCBSC treated stroke brains shows a significant increase in the total number of CD3+ T cells in the brain 7 days following MCAO ($p<0.05$), and a normalization of the T cell population in the UCBSC treated animals. Average values (±SEM) from pooled data are presented.

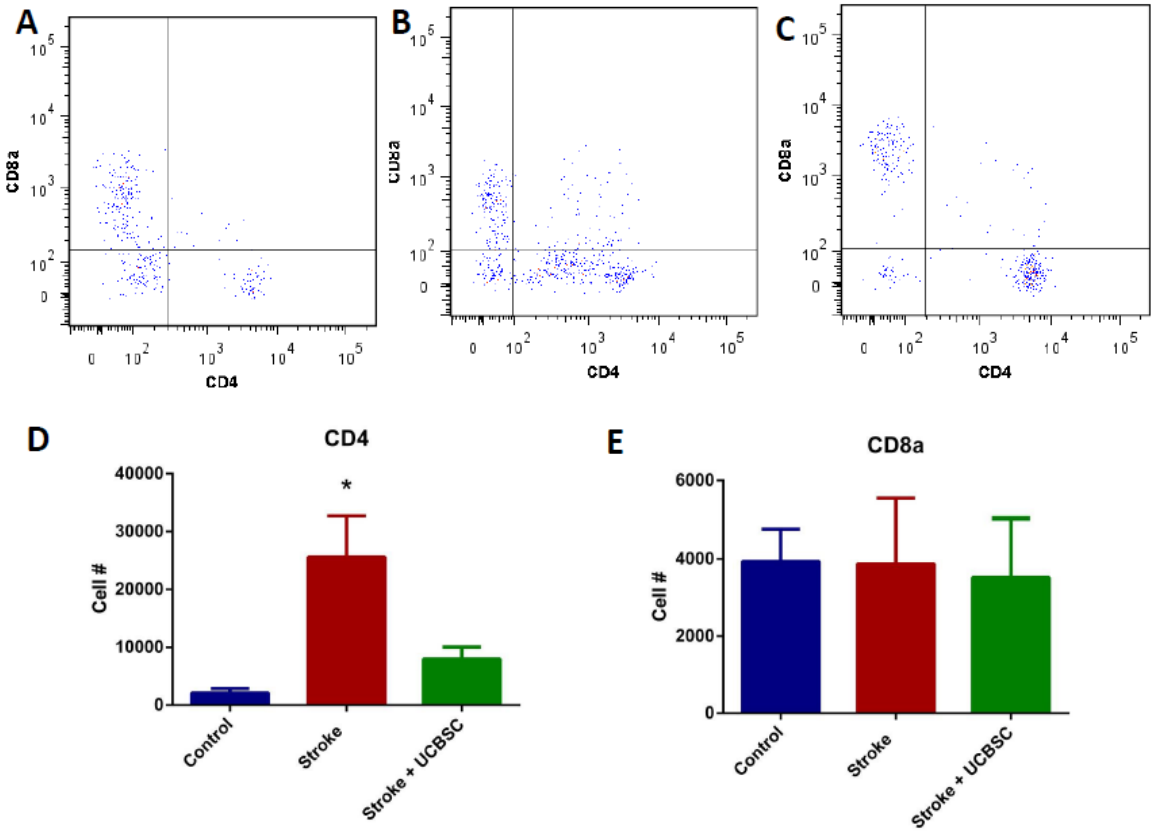

Figure 10. nh-UCBSC treatment normalizes the number of CD4+ Helper T cells 7 days following stroke. Population of CD4 (defined as CD3+CD8a-CD4+) and CD8a (defined as CD3+CD4-CD8a+) T cells in A) healthy control brain B) MCAO brain and C) nh-UCBSC treated MCAO brain 7 days following stroke. (n=5) Quad gates were established based on isotype stained brain samples. D) Quantification of CD4+ T cell populations show a significant increase in cell number in the MCAO group ($p<0.05$), and a normalization of the population with nh-UCBSC treatment. E) Quantification of CD8a+ cytotoxic T cells showed no significant difference in cell number between treatment groups. All gates were determined using isotype stained control tissue. Average values (±SEM) from pooled data are presented.

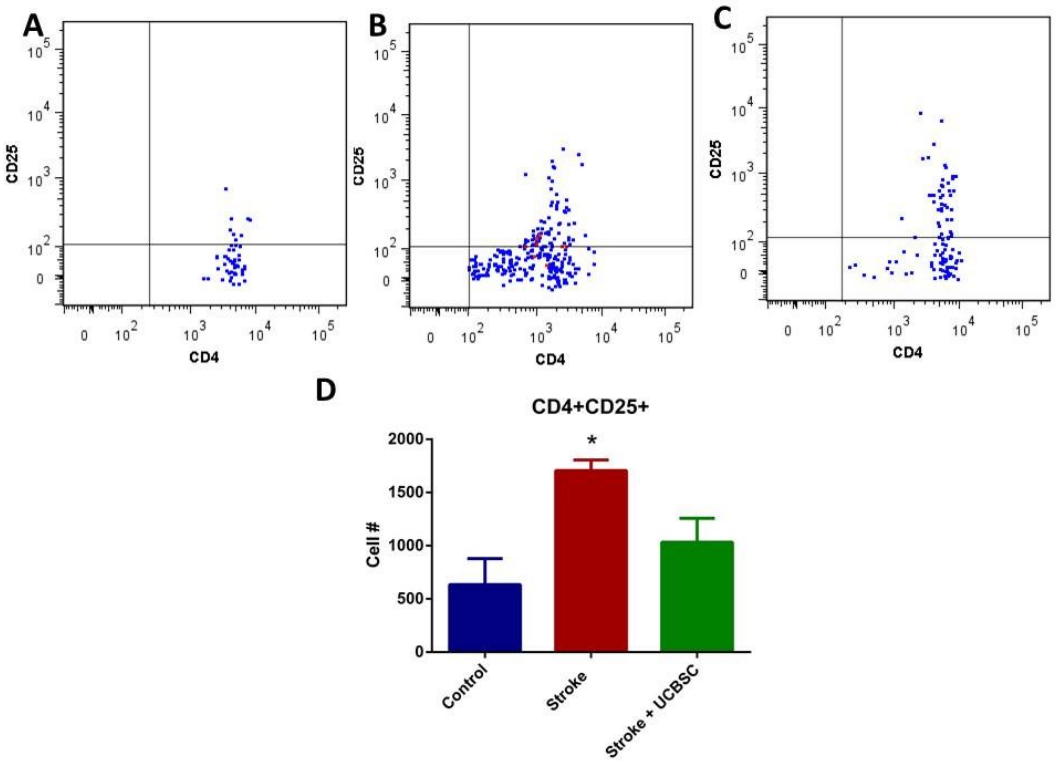

Figure 11. Population of activated CD3+CD8a-CD4+ CD25+ Helper T cells are significantly increased in the brain 7 days following stroke, and normalized in nh-UCBSC treated stroke brains. Flow cytometry of CD4+CD25+ cell populations in A) Healthy control brain B) Ischemic brain, and D) nh-UCBSC treated stroke brains 7 days following MCAO. (n=5) Quad gates were established based on isotype stained brain samples. C) Quantification of CD4+CD25+ cell populations. Cell number is significantly increased in stroke brains 7 days following stroke ($p<0.05$) and is normalized with nh-UCBSC treatment. All gates were defined using isotype stained control tissue. Average values (±SEM) from pooled data are presented.

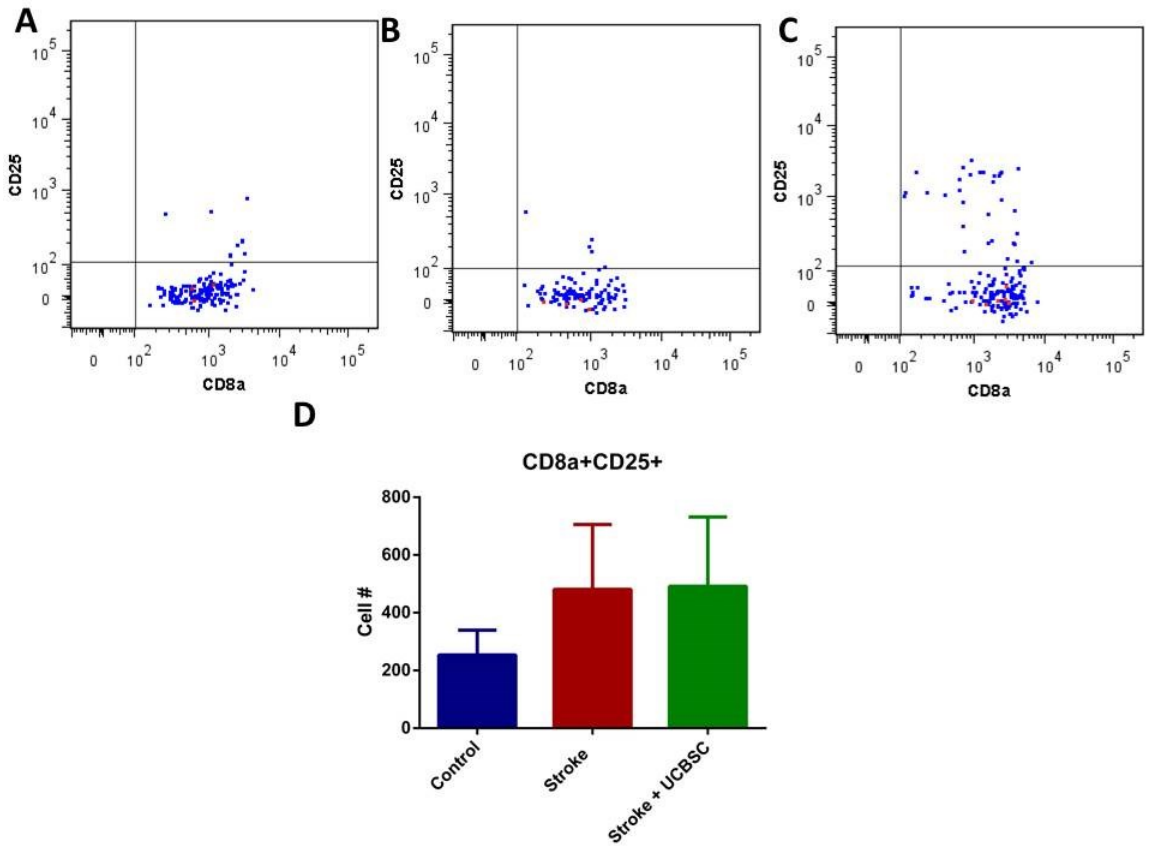

Figure 13. Populations of CD3+CD4-CD8a+CD25+ activated Cytotoxic T cells do not change significantly 7 days following ischemia. Flow cytometry of CD8a+CD25+ cell populations in A) Healthy control brains, B) stroke brains, and D) nh-UCBSC treated brains 7 days following stroke. (n=5) Quad gates were established based on identically stained brain samples with isotype specific control antibodies. C) Quantification of CD8a+CD25+ cell population in the brain 7 days following stroke. Populations showed no significant difference between treatment groups ($p<0.05$). All gates were determined using isotype stained control tissue. Average values (±SEM) from pooled data are presented.

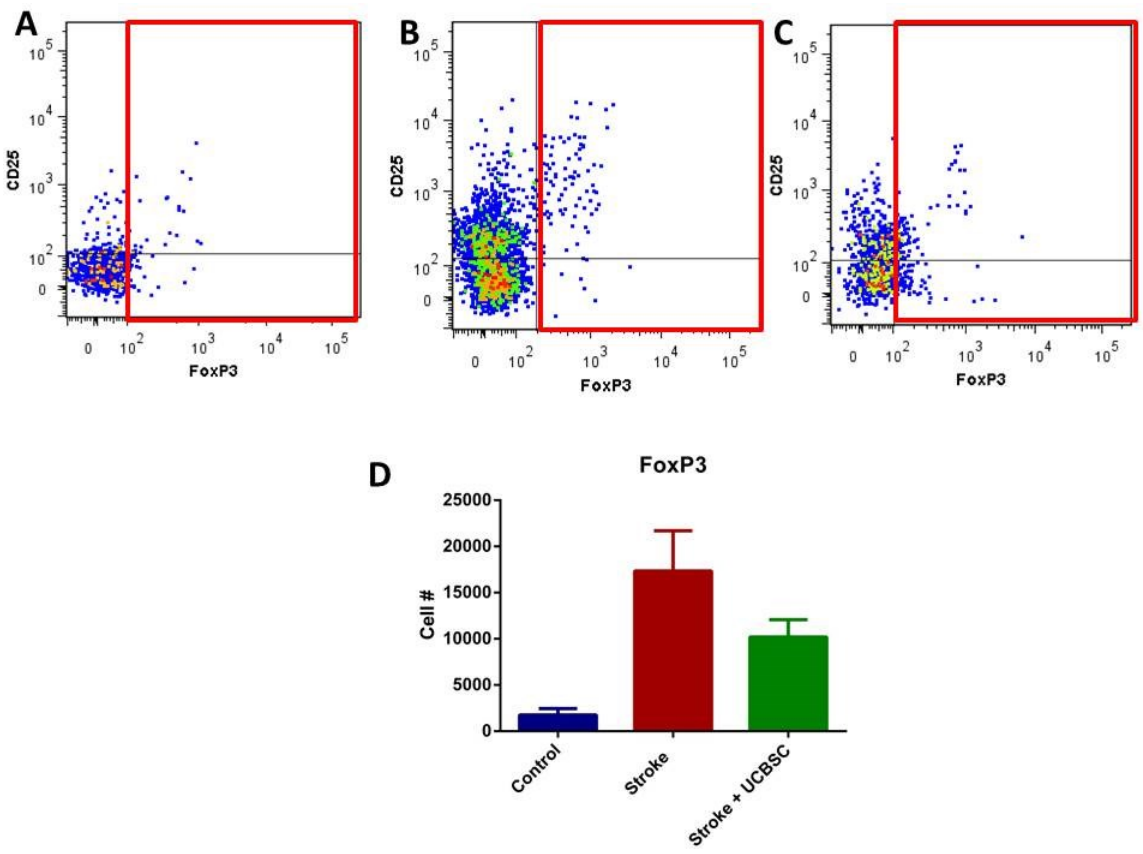

Figure 14. nh-UCBSC treatment normalizes CD3+CD4+FoxP3+ expression 7 days following stroke. Flow cytometry of A) healthy control brain, B) stroke brain, and C) nh-UCBSC treated brain 7 days following MCAO surgery. (n=5) Quad gates were established based on isotype stained brain samples. D) Quantification of FoxP3+ expression in all treatment groups shows an increase in expression in the ischemic brain, though that change is not statistically significant ($p<0.05$). Average values (±SEM) from pooled data are presented.

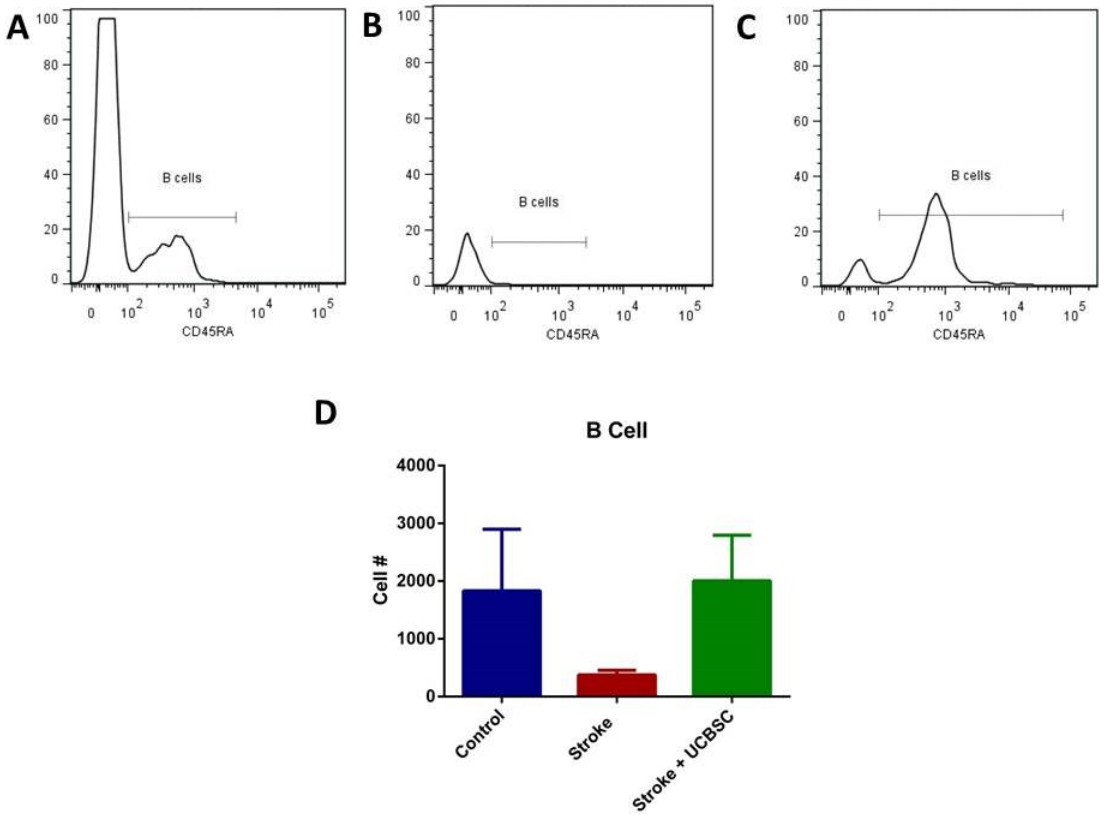

Figure 15. Total number of mature B cells (defined as CD3-CD161-CD45RA+) present in the brain decreases 7 days following stroke and normalizes with nh-UCBSC treatment. Flow cytometry of mature B cells in A) healthy control brain, B) stroke brain, and C) nh-UCBSC treated stroke brain 7 days following MCAO surgery. (n=5) Gating was established based on isotype stained brain samples. D) Quantification of total cell number of mature B cells in the brain in all treatment groups shows a decrease in cell number in the ischemic brain and a normalization with nh-UCBSC treatment, though these changes are not statistically significant ($p<0.05$). Average values of pooled data (±SEM) are presented.

Bibliography

Altman J, Das G (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. The Journal of Comparative Neurology 124:319-335.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8:963-970.

Bang OY, Lee JS, Lee PH, Lee G (2005) Autologous mesenchymal stem cell transplantation in stroke patients. Ann Neurol 57:874-882.

Bodhankar S, Chen Y, Vandenbark AA, Murphy SJ, Offner H (2013) IL-10-producing B-cells limit CNS inflammation and infarct volume in experimental stroke. Metabolic Brain Disease 28:375-386.

Boltze J, Schmidt UR, Reich DM, Kranz A, Reymann KG, Strassburger M, Lobsien D, Wagner DC, Forschler A, Schabitz WR (2012) Determination of the therapeutic time window for human umbilical cord blood mononuclear cell transplantation following experimental stroke in rats. Cell Transplantation 21:1199-1211.

Borlongan CV, Hess DC (2006) New hope for stroke patients: mobilization of endogenous stem cells. CMAJ 174:954-955.

Bose A, Henkes H, Alfke K, Reith W, Mayer TE, Berlis A, Branca V, Po Sit S (2008) The Penumbra System: A Mechanical Device for the Treatment of Acute Stroke due to Thromboembolism. American journal of Neuroradiology 29:1409-1413.

Buhnemann C, Scholz A, Bernreuther C, Malik C, Braun H, Schachner M, Reymann K, Dihne M (2006) Neuronal differentiation of transplanted embryonic stem cell-derived precursors in stroke lesions of adult rats. Brain 129:3238-3248.

Burns T, Ortiz-Gonzalez X, Gutierrez-Perez M, Keene C, Sharda R, Demorest Z, Jiang Y, Nelson-Holte M, Soriano M, Nakagawa Y, Luquin M, Garcia-Verdugo J, Prosper F, Low W, Verfaillie C (2006) Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: a word of caution. Stem Cells 24:1121-1127.

Chen Y, Ye L, Zhong J, Li X, Yan C, Chandler MP, Calvin S, Xiao F, Negia M, Low WC, Zhang J, Yu X (2013) The structural basis of functional

improvement in response to human umbilical cord blood stem cell transplantation. Cell Transplantation.

Colucci-D'Amato L, Bonavita V, di Porzio U (2006) The end of the central dogma of neurobiology: stem cells and neurogenesis in adult CNS. Neurological Sciences 27:266-270.

Coyne T, Marcus A, Woodbury D, Black I (2006) Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 24:2483-2492.

Cui X, Chopp M, Shehadah A, Zacharek A, Kuzmin-Nichols N, Sanberg D, Dai J, Zhang C, Ueno Y, Roberts C, Chen J (2012) Therapeutic benefit of treatment of stroke with Simvastin and human umbilical cord blood cells: neurogenesis, synaptic plasticity, and axon growth. Cell Transplantation 21:845-856.

Damjanov I (1990) Teratocarcinoma stem cells. Cancer surveys 9:303-319.

Dao MA, Nolta JA, Case CC (2013) Immunosuppressive activity of adult marrow mesenchymal stromal cells on innate immune cells in the central nervous system. Advances in Neuroimmune Biology 4:177-185.

De Ryck M (1990) Animal models of cerebral stroke: pharmacological protection of function. Eur Neurol 30:21-27.

Doetsch F (2003) A niche for adult neural stem cells. Current Opinion in Genetics and Development 13:543-550.

Donnan GA, Howells DW, Markus R, Toni D, Davis SM (2003) Can the time window for the administration of thrombolytics in stroke be increased? CNS Drugs 17:995-1011.

Fathali N, Ostrowski RP, Hasegawa Y, Lekic T, Tang J, Zhang JH (2013) Splenic immune cells in experimental neonatal hypoxia-ischemia. Translational Stroke Research 4:208-219.

Fischer U, Harting M, Jimenez F, Monzon-Posadas W, Xue H, Savitz S, Laine G, Cox C (2009) Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells and Development 18:683-691.

Garcia JH, Liu KF, Yoshida Y, Lian J, Chen S, Del Zoppo GJ (1994) Influx of leukocytes and platelets in an evolving brain infarct (Wistar rat). The American Journal of Pathology 144:188.

Go AS et al. (2014) Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 129:399-410.

Golden JE, Shahaduzzaman M, Wabritz A, Green S, Womble TA, Sanberg PR, Pennypacker KR, Willing AE (2012) Human umbilical cord blood cells alter blood and spleen cell populations after stroke. Translational Stroke Research 3:491-499.

Harting MT, Jimenez F, Adams SD, Mercer DW, Cox CS (2008) Acute, regional inflammatory response after traumatic brain injury: Implications for cellular therapy. Surgery 144:803-813.

Hayase M, Kitada M, Wakao S, Itozaku Y, Nozaki K, Hashimoto N, Takagi Y, Dezawa M (2009) Committed neural progenitor cells derived from genetically modified bone marrow stromal cells ameliorate deficits in a rat model of stroke. Journal of Cerebral Blood Flow & Metabolism 29:1409-1420.

Ideguchi M, Palmer T, Recht L, Weimann J (2010) Murine Embryonic Stem Cell-Derived Pyramidal Neurons Integrate into the Cerebral Cortex and Appropriately Project Axons to Subcortical Targets. The Journal of Neuroscience 30:894-904.

Jahan R (2010) Solitaire Flow-Restoration Device for Treatment of Acute Ischemic Stroke: Safety and Recanalization Efficacy Study in a Swine Vessel Occlusion Model. American Journal of Neuroradiology 31:1938-1943.

Kamel H, Iadecola C (2012) Brain-immune interactions and ischemic stroke: clinical implications. Archives of Neurology 69:576-581.

Kawai H, Yamashita T, Ohta Y, Deguchi K, Nagotani S, Zhang X, Ikeda Y, Matsuura T, Abe K (2010) Tridermal tumorigenesis of induced pluripotent stem cells transplanted in ischemic brain. Journal of Cerebral Blood Flow & Metabolism 30:1487-1493.

Kim D, Jahan R, Starkman S, Abolian A, Kidwell CS, Vinuela F, Duckwiler GR, Ovbiagele B, Vespa PM, Selco S, Rajajee V, Saver JL (2006)

Endovascular Mechanical Clot Retrieval in a Broad Ischemic Stroke Cohort. American Journal of Neuroradiology 27:2048-2052.

Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H, Stoll G (2010) Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. Blood 115:3835-3842.

Kondziolka D, Steinberg G, Wechsler L, al e (2005) Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. J Neurosurg 103:38-45.

Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn K, Gebel J, Jannetta P, DeCesare S, Elder E, McGrogan M, Reitman M, Bynum L (2000) Transplantation of cultured human neuronal cells for patients with stroke. Neurology 55:565-569.

Kuh SU, Cho YE, Yoon DH, Kim KN, Ha Y (2005) Functional recovery after human umbilical cord blood cells transplantation with brain-derived neurotrophic factor into the spinal cord injured rat. Acta Neurochir 147:985-992.

Lansberg MG, Bluhmki E, Thijs VN (2009) Efficacy and safety of tissue plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a metaanalysis. Stroke 40:2438-2441.

Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY (2010) A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. Stem Cells 28:1099-1106.

Lee S, Chu K, Jung K, Kim S, Kim D, Kang K, Hong N, Kim J, Ban J, Park H, Kim S, Park C, Lee S, Kim M, Roh J (2008) Anti-inflammatory mechanism of intravascular neural stem cell transplantation in haemorrhagic stroke. Brain 131:616-629.

Li B, Piao C-S, Liu X-Y, Guo W-P, Xue Y-Q, Duan W-M, Gonzalez-Toledo M, Zhao L-R (2010a) Brain self-protection: The role of endogenous neural progenitor cells in adult brain after cerebral cortical ischemia. Brain Research 1327:91-102.

Li L, Jiang Q, Ding G, Zhang L, Zhang Z, Li Q, Panda S, Lu M, Ewing J, Chopp M (2010b) Effects of administration route on migration and distribution of

neural progenitor cells transplanted into rats with focal cerebral ischemia, an MRI study. J Cereb Blood Flow Metab 30:653-662.

Li P, Gan Y, Mao L, Leak R, Chen J, Hu X (2014) The critical roles of immune cells in acute brain injuries. Immunological Mechanisms and Therapies in Brain Injuries and Stroke: 9-25.

Liu H, Honmou O, Harada K, Nakamura K, Houkin K, Hamada H, Kocsis J (2006) Neuroprotection by PIGF gene-modified human mesenchymal stem cells after cerebral ischemia. Brain 129:2734-2745.

Liu J, Solway K, Messing RO, Sharp FR (1998) Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. Journal of Neuroscience 18:7768-7778.

Liu N, Chen R, Du H, Wang J, Zhang Y, Wen J (2009) Expression of IL-10 and TNF-a in rats with cerebral infarction after transplantation with mesenchymal stem cells. Cellular & Molecular Immunology 6:207-213.

Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nature Biotechnology 25:1177-1181.

Menon L, Picinich S, Koneru R, Gao H, Lin S, Koneru M, Mayer-Kuckuk P, Glod J, Banerjee D (2007) Differentiation gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells. Stem Cells 25:520-528.

Moniche F, Gonzalez A, Gonzalez-Marcos JR, Carmona M, Pinero P, Espigado I, Garcia-Solis D, Cayuela A, Montaner J, Boada C, Rosell A, Jimenez MD, Mayol A, Gil-Peralta A (2012) Intra-Arterial bone marrow mononuclear cells in ischemic stroke: A pilot clinical trial. Stroke 43:2242-2244.

Muller F, Snyder E, Loring J (2006) Gene therapy: can neural stem cells deliver? Nat Rev Neurosci 7:75-84.

Nemeth K, Leelahavinichkul A, Yuen P, Mayer B, Parmelee A, Doi K, Robey P, Leelahavinchkul K, Koller B, Brown J, Hu X, Jelinek I, Star R, Mezey E (2009) Bone marrow stromal cells attenuate sepsis via prostoglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 15:42-49.

Neuhoff S, Moers J, Rieks M, Grunwald T, Jensen A, Dermietzel R, Meier C (2007) Proliferation, differentiation, and cytokine secretion of human umbilical cord blood-derived mononuclear cells in vitro. Experimental Hematology 35:1119-1131.

Newcomb JD, Aimo Jr. CT, Sanberg CD, Sanberg PR, Pennypacker KR, Willing AE (2006) Timing of cord blood treatment after experimental stroke determines therapeutic efficacy. Cell Transplantation 15:213-223.

Nottebohm F (1989) From bird song to neurogenesis. Scientific American 260:74-79.

Offner H, Subramanian S, Parker SM, Wang C, Afentoulis ME, Lewis A, Vandenbark AA, Hurn PD (2006) Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. The Journal of Immunology 176:6523-6531.

Onda T, Honmou O, Harada K, Houkin K, Hamada H, Kocsis J (2008) Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. Journal of Cerebral Blood Flow and Metabolism 28:329-340.

Ostrowski RP, Schulte RW, Nie Y, Ling T, Lee T, Manaenko A, Gridley DS, Zhang JH (2012) Acute splenic irradiation reduces brain injury in the rat focal ischemic stroke model. Translational Stroke Research 3:473-481.

Pan Y, Nastay JB, Zhang H, Bretton RH, Panneton WM, Bicknese AR (2005) Engraftment of freshly isolated or cultured human umbilical cord blood cells and the effect of cyclosporin A on the outcome. Experimental Neurology 192:365-372.

Parent JM (2003) Injury-induced neurogenesis in the adult mammalian brain. Neuroscientist 4:261-272.

Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM (2002) Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann Neurol 52:802-813.

Pera M (2011) Stem cells: The dark side of induced pluripotency. Nature 471:46-47.

Pino PA, Cardona AE (2011) Isolation of brain and spinal cord mononuclear cells using percoll gradients. J Vis Exp 48.

Rasouli J, Lekhraj R, Ozbalik M, Lalezari P, Casper D (2011) Brain-spleen inflammatory coupling: a literature review. The Einstein Journal of Biology and Medicine 27:74.

Ren X, Akiyoshi K, Dziennis S, Vandenbark AA, Herson PS, Hum PD, Offner H (2011) Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke. The Journal of Neuroscience 31:8556-8563.

Roger V et al. (2012) Heart Disease and Stroke Statistics - 2012 Update: A Report From the American Heart Association. Circulation 125:e2-e220.

Rosado-de-Castro P, Schmidt FR, Battistella V, Lopes de Souza SA, Gutfilen B, Goldenberg RC, Kasai-Brunswick TH, Vairo L, Silva RM, Wajnberg E, Emmanuel do Brasil P, Gasparetto EL, Maiolino A, Alves-Leon SV, Andre C, Mendez-Otero R, Rodriguez de Freitas G, Barbosa da Fonesca LM (2013) Biodistribution of bone marrow mononuclear cells after intra-arterial or intravenous transplantation in subacute stroke patients. Regenerative Medicine 8:145-155.

Rowe D, Leonardo C, Recio J, Collier L, Willing A, Pennypacker K (2012) Human Umbilical Cord Blood Cells Protect Oligodendrocytes from Brain Ischemia through Akt Signal Transduction. The Journal of Biological Chemistry 287:4177-4187.

Roybon L, Zhi M, Asztely F, Fosum A, Jacobsen S, Brundin P, Li J (2006) Failure of transdifferentiation of adult hematopoietic stem cells into neurons. Stem Cells 24:1594-1604.

Sahota P, Vahidy F, Nguyen C, Bui TT, Yang B, Parsha K, Garrett J, Bambhroliya A, Barreto A, Grotta JC, Aronowski J, Rahbar MH, Savitz S (2013) Changes in spleen size in patients with acute ischemic stroke: a pilot observational study. International Journal of Stroke 8:60-67.

Savitz S, Dinsmore J, Wu J, Henderson G, Stieg P, Caplan L (2005) Neurotransplantation of fetal porcine cells in patients with basal ganglia infarcts: a preliminary safety and feasability study. Cerebrovasc Dis 20:101-107.

Seifert HA, Hall AA, Chapman CB, Collier LA, Willing AE, Pennypacker KR (2012) A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. Journal of Neuroimmune Pharmacology 7:1017-1024.

Seminatore C, Polentes J, Ellman D, Kozubenko N, Itier V, Tine S, Tritschler L, Brenot M, Guidou E, Blondeau J, Lhuillier M, Bugi A, Aubry L, Jendelova P, Sykova E, Perrier A, Finsen B, Onteniente B (2010a) The Postischemic Environment Differentially Impacts Teratoma or Tumor Formation After Transplantation of Human Embryonic Stem Cell-Derived Neural Progenitors. Stroke 41:153-159.

Seminatore C, Polentes J, Ellman D, Kozubenko N, Itier V, Tine S, Tritshler L, Brenot M, Guidou E, Blondeau J, Lhullier M, Bugi A, Aubry L, Jendelova P, Sykova E, Perrier A, Finsen B, Onteniente B (2010b) The postischemic environment differentially impacts teratoma or tumor formation after transplantation of human embryonic stem cell-derived neural progenitors. Stroke 41:153-159.

Serafini M, Dylla S, Oki M, Heremans Y, Tolar J, Jiang Y, Buckley S, Pelacho B, Burns T, Frommer S, Rossi D, Bryder D, Panoskaltsis-Mortari A, O'Shaughnessy M, Nelson-Holte M, Fine G, Weissman I, Blazar B, Verfaillie C (2007) Hematopoietic reconstitution by multipotent adult progenitor cells: precursors to long-term hematopoietic stem cells. J Exp Med 204:129-139.

Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A (2009) Pivotal role of cerebral interleukin-17-producing ydT cells in the delayed phase of ischemic brain injury. Nature Medicine 15:946-950.

Takagi Y, Takahashi J, Saiki H, Morizane A, Hayashi T, Kishi Y, Fukada H, Okamoto Y, Koyanagi M, Ideguchi M, Hayashi H, Imazoto T, Kawasaki H, Suemori H, Omachi S, Iida H, Itoh N, Nakatsuji N, Sasai Y, Hashimoto N (2005) Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. The Journal of Clinical Investigation 115:102-109.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861-872.

Toda H, Takahashi J, Iwakami N, Kimura T, Hoki S, Mozumi-Kitamura K, Ono S (2001) Grafting neural stem cells improved the impaired spatial recognition in ischemic rats. Neurosci Lett 316:9-12.

Urra X, Cervera A, Obach V, Climent N, Planas AM, Chamorro A (2009) Monocytes are major players in the prognosis and risk of infection after acute stroke. Stroke 40:1262-1268.

Vendrame M, Gemma C, Pennypacker KR, Bickford PC, Davis Sanberg C, Sanberg PR, Willing AE (2006) Cord blood rescues stroke-induced changes in splenocyte phenotype and function. Experimental Neurology 199:191-200.

Walczak P, Zhang J, Gilad A, Kedzioreck D, Ruiz-Cabello J, Young R, Pittenger M, van Zijl P, Huang J, Bulte J (2008) Dual modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. Stroke 39:1569-1574.

Walker P, Harting M, Jimenez F, Shah S, Pati S, Dash P, Cox C (2010) Direct intrathecal implantation of mesenchymal stromal cells leads to enhanced neuroprotection via NFkB-mediated increase in interleukin-6 production. Stem Cells and Development 19:867-876.

Walker PA, Letourneau PA, Bedi S, Shah SK, Jimenez F, Cox Jr. CS (2011) Progenitor cells as remote "bioreactors": Neuroprotection via modulation of the systemic inflammatory response. World Journal of Stem Cells 3:9.

Weiss S, Reynolds B, Vescovi A, Morshead C, Craig C, Van der Kooy D (1996) Is there a neural stem cell in the mammalian forebrain? Trends Neurosci 19:387-393.

Willing A, Vendrame M, Mallery J, Cassady C, Davis C, Sanchez-Ramos J, Sanberg P (2003) Mobilized peripheral blood cells administered intravenously produce functional recovery in stroke. Cell Transplant 12:449-454.

Wu W, Chen X, Hu C, Li J, Yu Z, Cai W (2010) Transplantation of neural stem cells expressing hypoxia-inducible factor-1alpha (HIF-1a) improves behavioral recovery in a rat stroke model. Journal of Clinical Neuroscience 17:92-95.

Xiao J, Nan Z, Motooka Y, Low WC (2005) Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury. Stem Cells and Development 14:722-733.

Yilmaz G, Arumugam TV, Stokes KY, Granger DN (2006) Role of T lymphocytes and interferon-y in ischemic stroke. Circulation 113:2105-2112.

Yu J, Vodyanik M, Smuga-Otto K, Antosiewicz-Bourget J, Frane J, Tian S, Nie J, Jonsdottir G, Ruotti V, Stewart R, Slukvin I, Thomson J (2007) Induced

pluripotent stem cell lines derived from human somatic cells. Science 318:1917-1920.

Zhang BJ, Men XJ, Lu ZQ, Li HY, Qiu W, Hu XQ (2013) Splenectomy protects experimental rats from cerebral damage after stroke due to anti-inflammatory effects. Chinese Medical Journal 126:2354-2360.

Zhao L, Duan W, Reyes M, Keene C, Verfaille C, Low W (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174:11-20.