IMMUNO-SUPPRESSIVE HYDROGELS FOR STEM CELL THERAPY AFTER TRAUMATIC BRAIN INJURY

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To my family for all their love, sacrifice, and unconditional support.

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"Caminante no hay camino, se hace camino al andar"

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LIST OF SYMBOLS AND ABBREVIATIONS

TBI	Traumatic Brain Injury
MSC	Mesenchymal Stem Cell
NSC	Neural Stem Cell
BBB	Blood-Brain Barrier
NGF	Neuronal Growth Factor
BDNF	Brain-derived Neuronal Factor
IGF	Insulin-like Growth Factor
FasL	Fas Ligand
CSF	Cerebrospinal Fluid
CNS	Central Nervous System
TNF-α	Tumor Necrosis Factor Alpha
IL-1β	Interleukin 1-Beta
IL-1β-RA	Interleukin 1-Beta Receptor Antagonist
IL-10	Interleukin-10
TGF-β	Transforming Growth Factor Beta

SUMMARY

During a traumatic brain injury (TBI) an external force disrupts the brain tissue and the proper functioning of neuronal pathways. This initial insult activates multiple cellular mechanisms that further propagate the tissue damage causing a secondary injury that exacerbates neurological deficits. This phase, known as the secondary injury, opens a therapeutic window in which neuroprotective treatments that successfully contain the propagation of the initial damage could significantly reduce neurological deficits associated with TBI. Mesenchymal stem cell transplantation (MSC) after TBI has been found to ameliorate neurological deficits due to the ability of the stem cells to modulate inflammation and immune cells and to increase the expression of neurotrophic factors that promote the survival of the neuronal tissue surrounding the injury site. However, the active rejection of the transplanted MSC by the host immune system could strongly diminish the stem cell's survival and therapeutic effect.

In this thesis, we used immunosuppressive hydrogels, specifically designed to induce the apoptosis of cytotoxic $CD8^+$ T cells, to enhance the survival of transplanted MSC in the injured brain. We demonstrated that creating localized immunosuppression near the MSC transplantation site resulted in a higher presence of MSC near the injury site. We also demonstrate that enhancing MSC survival by using immunosuppressive hydrogels increased the protein expression of the IL-1β receptor antagonist and the neurotrophic factors NGF and BDNF, which could lead to reduced neuronal damage. Therefore, the development of immune-suppressive hydrogels for stem cell transplantation could be a successful approach to enhance stem cell therapy after TBI.

INTRODUCTION

Traumatic brain injury (TBI) is defined as the disruption of the normal brain tissue and neuronal networks due to an external force [1]. In 2014, according to a CDC report, approximately 2.8 million visits to the emergency department, hospitalizations, and deaths in the United States were associated with TBI [1]. After the initial insult during a TBI, a complex biochemical and cellular cascade cause a secondary injury that further propagates the neuronal damage around the injury site. The mechanisms involved in the secondary injury include brain swelling, the disruption of the blood-brain barrier which can lead to a hypoxic environment and limited nutrients, the activation of an immune/inflammatory response, and the increase of oxidative stress, among others [2-4]. In addition to the complex brain pathology, the random nature of the events that cause TBI results in variable degrees of injury severity between TBI patients making it difficult to develop successful therapies that can ameliorate neurological deficits [2].

Mesenchymal stem cell (MSC) transplantation is a promising treatment for TBI due to the MSC's ability to coordinate the response of multiple cell types such as immune cells, endothelial cells, neuroglia, and neural progenitor cells to promote tissue repair [5]. Systemic delivery of MSCs after brain injury has been shown to alter the host peripheral immune profile and indirectly increase the regulatory T cell population near the injury site, which has been correlated with lower inflammation and decreased neuronal deficits [6-8]. Similarly, MSC has been shown to influence non-neuronal glial cells, such as astrocytes and microglia, which results in reduced inflammation and the expression of neurotrophic factors that promote the survival of the neuronal tissue surrounding the injury site [8-10].

Moreover, a mechanism commonly reported in the literature to mediate the MSC neuroprotective effect is the increased expression of neurotrophic and growth factors [11-14]. The MSC's ability to target multiple mechanisms involved in the secondary injury makes stem cell therapy an attractive candidate for the treatment of brain injury and other neurodegenerative diseases.

In order to maximize the MSC therapeutic effect, it is necessary to deliver a large number of stem cells within a short therapeutic window following injury and to ensure its viability for a reasonably long period of time so as to reduce the deleterious impact of the secondary injury [15]. Unfortunately, the active rejection of the transplanted MSC by the host immune response could diminish stem cell survival and in consequence, its therapeutic impact. It has been demonstrated that MSC transplantation in the adult intact brain resulted in early graft rejection and graft infiltration by microglia and astrocytes along with few $CD8^+$ cytotoxic T cells [16-18]. However, unlike the intact brain, the immune cell composition in the injured brain milieu includes a higher presence of peripheral leukocytes such as neutrophils, macrophages, and T cells due to the disruption of the blood-brain barrier [19]. The impact of the injured brain's immune composition on the survival and behavior of transplanted stem cells, including MSC, has not been completely elucidated. Therefore, systematic research is needed to understand how inflammatory signals and/or immune cells alter the MSC therapeutic efficacy in order to incorporate stem cell therapy as an alternative treatment during a brain injury acute phase.

In this study, we explored the hypothesis that designing immunosuppressive hydrogels as stem cell carriers can enhance allogeneic MSC survival and therapeutic effect after transplantation in the injured brain of immunocompetent rats. Specifically, we sought to

determine if the targeted reduction of the cytotoxic $CD8^+$ T cell population at the transplantation site could enhance the survival of the transplanted MSC near the injury site. In order to achieve this, we used an agarose based hydrogel to release FasL, a protein that plays an important role in T cell regulation by inducing the apoptosis of activated cytotoxic $CD8^+$ T cells. We hypothesized that co-delivering FasL with allogeneic MSC will result in a decrease of the cytotoxic $CD8^+$ T cell population near the transplantation site, which in consequence will lead to an increase in the MSC's survival. We also investigated if enhancing MSC survival after transplantation resulted in a decreased neuronal pathology supporting the hypothesis that early MSC transplantation could be hindered by the harsh brain milieu.

Our specific aims are as follows:

Specific Aim 1: Determine the effect of FasL-hydrogels on the survival of the transplanted MSC. Our working hypothesis was that the survival of transplanted allogeneic MSC can be enhanced by suppressing the host immune response, specifically the cytotoxic $CD8^+$ T cell response. Our objectives for this aim were to design a FasL releasing agarose based hydrogel that could induce the apoptosis of cytotoxic $CD8^+$ T cells and to investigate the hydrogel effect on the survival of allogeneic MSC after transplantation in the injured brain of immunocompetent rats. The outcomes of this aim are discussed in Chapter 3.

Specific Aim 2: Determine the effect of MSC transplantation using FasL-hydrogels on the injured brain environment. Our working hypothesis was that by using FasL hydrogels to enhance MSC survival after transplantation, we could enhance the MSC

neuroprotective effects in the injured brain. Our objective for this aim was to investigate if the use of FasL releasing hydrogels as carriers for MSC delivery could alter multiple regenerative signals such as the expression of neurotrophic factors, the volume size of the injured area, and the neuronal degeneration around the injury site. The outcomes of this aim are discussed in Chapter 4.

LITERATURE REVIEW

During a traumatic brain injury (TBI) an external force disrupts the neuronal tissue and the proper functioning of neuronal pathways [1, 4]. The initial insult triggers multiple cellular mechanisms that propagate the initial damage for a period of weeks or months exacerbating neurological deficits [2-4]. This phase, known as the secondary injury, creates a therapeutic window in which neuroprotective treatments that successfully contain the propagation of the secondary injury could significantly reduce the neurological deficits associated with TBI. Yet, the complex environment created by multiple cellular and biochemical pathways activated after a brain injury requires a multifunctional treatment approach that modulates multiple pathways simultaneously [20, 21]. Mesenchymal Stem cells (MSCs) have been shown to poses multiple neuroprotective capabilities making stem cell therapy an attractive candidate to treat diseases involving sustained neuronal degeneration such as TBI. Transplanted MSCs interact with the injured environment and release multiple cytokines and growth factors that enhance endogenous reparative mechanisms [11-14]. However, in order to develop a successful treatment, it is important to deliver a high number of stem cells within a short time frame after an injury and to ensure its viability after transplantation [15]. Early MSC transplantation after a brain injury could result in the limited survival of the transplanted stem cell due to the harsh environment near the site of injury which includes the presence of peripheral immune cells not usually abundant in the brain [19]. Therefore, the interaction between transplanted MSCs and the injured brain immune composition needs to be elucidated in order to promote the survival and healing phenotype of the transplanted stem cells.

In this chapter, we review the current scientific literature regarding the use of stem cell therapy as a treatment for TBI with an emphasis on the use of MSCs. In addition, we evaluate the literature available on the host immune response toward transplanted stem cells and its effect on stem cell survival. Finally, we explore the current use of biomaterials that modulate the immune response and/or create immunosuppressive environments, which could be potentially used as stem cell carriers for TBI.

1.1 Traumatic brain injury (TBI)

TBI is caused when an external force such as a blow or jolt to the head disrupt normal neuronal pathways and brain tissue; thus, creating a variety of neurological deficits depending on the severity and localization of the initial injury site [1, 4]. A TBI is usually divided into two phases known as primary injury and secondary injury [4]. During the primary injury or initial insult, a series of biochemical and cellular events are activated which leads to the propagation of neuronal damage for a period of weeks or months. These events include the disruption of the blood-brain barrier (BBB), the infiltration of peripheral leukocytes and increased inflammatory response, and neuronal overstimulation and apoptosis due to the spilled glutamate from the disrupted cells (Fig. 2.1) [2-4]. Therapies that modulate one or more mechanisms that mediate the secondary injury could potentially limit neuronal damage and the severity of neurological deficits.

1.1.1 Immune and inflammatory response after TBI

The brain is considered an immune-privileged site due to its low abundance of peripheral immune cells and limited access due to the blood-brain barrier (BBB), a layer of tightly

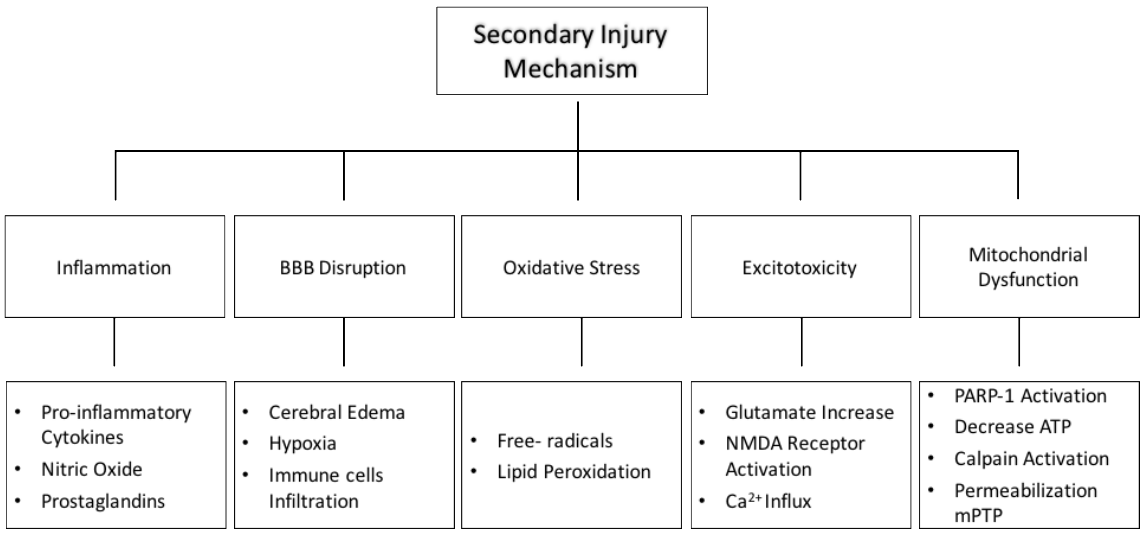


Figure 2.1: Summary of the mechanisms involved in the propagation of the secondary injury following TBI. Multiple mechanisms are involved in the propagation of the secondary injury which significantly contribute to the development of neurological deficits. The release of cytoplasmic content into the extracellular matrix initiate multiple processes including the uncontrolled release of neurotransmitters (excitotoxicity), lipid degradation, mitochondrial dysfunction, and oxidative stress. Also, the disruption of the blood-brain barrier (BBB) result in the formation of an edema (increased water content) which can increase the intracranial pressure and reduce brain oxygen levels due to limited blood flow. BBB disruption also lead to the infiltration of peripheral immune cells which can increase the expression of inflammatory proteins. Successful treatment for TBI needs to target multiple mechanism of the secondary injury in order to contain the initial damage.

connected endothelial cells that control the entry of circulating immune cells, proteins, pathogens and other molecules from the blood into the CNS [22]. The main immune population in the healthy brain is the microglia, a specialized tissue macrophage usually confined in an immunosuppressive environment in the normal brain but highly activated and involved in the removal of dead tissue after a brain injury[22, 23]. In addition to microglia, peripheral macrophages have been found in the choroid plexus, meninges,

perivascular space, and ventricles while the cerebrospinal fluid (CSF) is highly rich in T cells [22]. It is believed that despite the limited immune presence, the brain is constantly monitored by macrophage and T cell sampling of the CSF [22].

After TBI, the BBB is temporary disrupted, which leads to the formation of edema that can increase the intracranial pressure and create a hypoxic environment [3]. BBB disruption also results in the infiltration of peripheral immune cells that can potentially increase the expression of inflammatory cytokines in the brain causing further damage [2, 24, 25]. The infiltration of peripheral immune cells into the brain is also elicited by the release of cytoplasmic content from the disrupted neuronal and glial cells into the ECM [26]. These molecules, such as S100B proteins and high mobility group box 1 (HMGB1), are known as damage-associated molecular pattern molecules (DAMPs) and alert innate immune cells to initiate a process to clear damaged cellular debris and to sequester tissue-damaging irritants [24, 26, 27]. The initial response by resident glial cells and peripheral immune cells plays a significant role in the initiation of repair mechanisms and the containment of damage [24, 27]. However, sustained immune activation could lead to a prolonged inflammatory state and exacerbate the secondary injury.

The infiltration of peripheral leukocytes such as neutrophils, T cells, and macrophages have been found to peak around 1-3 days post-injury in several brain injury rodent models [16, 19, 28-30]. Neutrophils are recruited early to the injury site after TBI and have been shown to increase plasma leakage and induce edema formation during acute inflammation in peripheral tissue [30-32]. Kenne et al showed that neutrophil depletion decreases cell apoptosis in the brain cortex and attenuates microglia/macrophage activation after TBI [30]. On the other hand, the T cell effect on the development of a secondary injury depends

on the role of the different $CD3^+$ T cell subtypes. T cells are classified as $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells, and $CD4^+CD25^+FoxP3^+$ regulatory T cells according to their function. Total depletion of peripheral T cells, including both $CD8^+$ and $CD4^+$ T cells, has been shown to increase the infiltration of innate immune cells, such as neutrophils and inflammatory macrophages, in a hypoxia-ischemia brain injury model [33]. Moreover, it has been found that Regulatory T cells play an essential role in the downregulation of the inflammatory/immune response after brain injury [7, 8]. Specific depletion of regulatory T cells in a stroke model resulted in increased brain damage, deteriorated functional outcome, and increased activation of resident and invading inflammatory cells [8]. The neuroprotective role of $CD4^+$ regulatory T cells was also shown in a focal cerebral ischemia model wherein systemic administration of these cells decreased cerebral inflammation and infiltration of peripheral inflammatory cells thus resulting in smaller infarct size [7]. On the other hand, the depletion of $CD8^+$ cytotoxic T cells improved neurobehavioral performance and increased cortical neuronal density in a model of inflammation-induced perinatal brain injury [34]. Other findings suggesting the implication of the immune system in the pathophysiology of TBI were observed in rats with ischemic brain injury that also underwent splenectomy or splenic irradiation. These rats showed a decrease in the size of the injury infarct cavity, which could be an indication of reduced neurological deficit [35, 36] . Thus, the contribution of the multiple components of the peripheral immune response can be beneficial or detrimental to the brain after an injury, which offer multiple targets that could be exploited for the development of treatments.

The increased infiltration of immune cells into the injured brain alters the brain milieu by increasing the expression of multiple inflammatory/anti-inflammatory cytokines. Studies

have shown that the mRNA expression of inflammatory/anti-inflammatory cytokines such as IFN-γy IL-1β, TNF-α, IL-6, IL-13, IL-4, and IL-10 is increased in the brain and cerebrospinal fluid (CSF) during the first 12-24 hours after TBI [37, 38]. Most of these cytokines reached near basal levels around 3 days post-injury [37, 38]. A common approach to ameliorate the secondary injury is the use of bioactive molecules that modulate the expression of cytokines, specifically to inhibit inflammatory proteins or to increase anti-inflammatory proteins. Extensive research has demonstrated that IL-1β contributes to neuronal damage after TBI [38-42]. Multiple studies have found that delivering the IL-1 receptor antagonist (IL-1βRA) or antibodies against IL-β reduced neuronal damage and improved behavioral outcomes after brain injury [38-42]. On the other hand, IL-10 is an anti-inflammatory protein involved in the resolution of the inflammatory response. Delivering IL-10 after a brain injury has been found to improve neurological outcomes [43-45]. Therefore, efforts focused on the development of therapies that can shift the cytokine expression to create an anti-inflammatory milieu could be beneficial in ameliorating the secondary injury.

1.2 Controlled cortical impact as an animal model of TBI

One of the limitations during the development of treatments for TBI is the heterogeneous pathophysiology observed in patients, which can be the result of multiple factors such as the location, nature, and severity of the injury [46]. Various animal models have been developed in order to understand specific aspects of the secondary injury and to test potential treatments (Figure 2.2) [46-63]. In order to obtain a replicable secondary injury in an animal model, it is necessary to strictly control multiple parameters such as age, gender, genetic background, and injury parameters (strength, duration, localization) [46].

Therefore, the ability of each TBI model to mimic most of the aspects of a human brain injury is limited and might restraint the translation of pre-clinical treatments to successful clinical trials.

In this thesis, we used a controlled cortical impact (CCI) model to study allogeneic stem cell survival after TBI. In the CCI model, an impact tip controlled by a pneumatic or electromagnetic piston hits the exposed intact dura on the brain cortex of the animal [47, 48]. The severity of the injury caused by the CCI model depends on various parameters including speed, impact tip diameter, depth of injury, duration of injury, and localization [53, 54]. An advantage of this injury model over other TBI models is the ease at which mechanical parameters (speed, depth of injury, duration, etc) can be controlled, which allows the adjustment of the injury severity to obtain the pathophysiological damage required for the experimental design. Some of the pathophysiological features that have been reported using the CCI model include cortical tissue loss, cavity formation, acute subdural hematoma, axonal injury, BBB dysfunction, neuroinflammation, and oxidative damage [47, 48, 58, 64-68]. The motor, cognitive and emotional deficits caused by the CCI model have been extensively characterized. Using the Morris water maze test, CCI has been reported to cause spatial memory deficits in mice and rats, which has been correlated to both the depth of deformation and the velocity of the impact [63, 69-71]. Also, emotional deficits after CCI have been found using the forced swim test, and elevated-plus maze [69]. Similarly, motor deficits have been identified in the CCI model as quantified by the rotarod and beam walk test [70]. The model replicability, easy adjustment of parameters, and extensively characterized pathophysiology and functional deficits make the CCI model a good system for the initial testing of therapeutics for the injured brain.

TBI Model	Methodology	Pathophysiology
Controlled Cortical Impact	After a craniotomy, a pneumatic or electromagnetic impact device is used to control an impact tip that hit the exposed intact dura.	Tissue deformation, hemorrhage, brain swelling, continuous gray matter degeneration, neuroinflammation, motor & cognitive impairment.
Fluid percussion injury	After a craniotomy, a pendulum is used to strike the piston of a fluid reservoir and generate a fluid pressure pulse to the intact dura.	Hemorrhage, brain swelling, continuous gray matter degeneration, motor & cognitive impairment.
Weight drop model	Can be performed with or without a craniotomy. The skull or brain tissue is exposed and impacted with a free falling, guided weight.	Hemorrhage, continuous gray matter degeneration, diffuse axonal injury, BBB disruption, neuroinflammation, motor & cognitive deficits.
Maryland's model	Modification of the weight drop model to mimic TBI damage associated with motor vehicle and sports accidents. The impact force is applied to the anterior part of the cranium in order to cause anterior-posterior plus sagittal rotational acceleration of the brain inside the intact cranium.	Skull fractures, prolonged apnea, petechial hemorrhages, diffuse axonal injury, absence of cortical contusions and absence of mortality.
Blast model	A compression-driven shock tube in which compressed air create a blast wave is used to simulate blast effects.	Diffuse cerebral brain oedema, extreme hyperemia, delayed vasospasm, diffuse axonal injury, and absence of macroscopic tissue damage or hemorrhage.
Penetrating ballistic-like brain injury	Original model: An inflatable penetrating probe is used to create a permanent injury track and to mimic the temporary cavity induced by a penetrating bullet. Recent version: A modified air-rifle accelerates a pellet, which hits a small probe that penetrates the animal's brain.	Cavity formation, white matter degeneration, hemorrhage, brain swelling, gliosis, BBB disruption, neuroinflammation, motor and cognitive deficits.

Figure 2.2: Summary of the multiple models developed to study TBI. Each model aims to mimic specific biomechanical aspects of a TBI. The strict control of the biomechanical parameters difficult the models ability to reproduce all aspect of the human TBI pathophysiology.

1.3 Pre-clinical treatments for TBI

Currently, emergency medical treatments for TBI focus on interventions to monitor and reduce intracranial cerebral pressure in order to stabilize cerebral perfusion [72]. If the intracranial pressure is not normalized and stabilized within a short span following injury,

it can reduce cerebral blood perfusion and oxygen supply to the brain which has been associated with poor outcomes for the patients. Efforts to develop neuroprotective therapies for brain injury focus on the delivery of steroids to reduce the neuronal death caused by the secondary injury. Progesterone, a sex-related hormone, has been reported to attenuate neurological deficits and/or behavioral anxiety in rat models of TBI [73-77]. The neuroprotective effect of progesterone has been attributed to its anti-inflammatory properties as it has been shown that progesterone delivery after a brain injury reduces the expression of inflammatory cytokines such as IL-1β and TNF-α and the presence of cells related to neuroinflammation such as astrocytes and macrophages [73, 78]. Another hormone of interest for the treatment of brain injury is erythropoietin (EPO). Although EPO is commonly known as a key regulator of erythropoiesis, it also has been shown to have anti-apoptotic and immune-modulatory properties [79]. Studies have shown that early local or systemic EPO delivery has neuroprotective effects after brain injury reducing the volume of the injured area and increase neuronal density [46, 51, 80-82]. However, the beneficial effect found using EPO in pre-clinical models required an early delivery up to 6 hours after an injury, which is a short window that difficult the translation of EPO delivery as a treatment for TBI [80, 82].

Despite the extended scientific knowledge obtained using animal models, most drugs/treatments tested in clinical trials have failed to clearly show efficacy [83]. The diversity in brain injuries and the complexity of the biochemical and cellular processes that follow an injury are factors that challenge the translation of animal studies into successful treatments [20]. Hence, the use of combinatory treatments and/or stem cell therapy that can

target multiple aspects of the secondary injury are of interest in order to ameliorate neurological deficits associated with TBI.

1.4 Stem Cell Therapy for TBI

Stem cell (SC) transplantation has shown successful therapeutic consequences in TBI and stroke [21, 84]. SC are multipotent cells able to self-renew and to differentiate into multiple cell types. Immortalized progenitor cells, embryonic rodent and human neural stem cells, and bone marrow stem cells have been successfully transplanted in experimental models of TBI and have been shown to ameliorate the neurological status of the injury site [84]. In recent years there has been an increased interest in the use of MSC for transplantation after TBI due to the MSC's ability to ameliorate neurological deficits [21, 25, 84, 85]. Although MSC transplantation has been shown to partially improve functional outcome following TBI in rodents, this may not be due to cell replacement since only a small population of transplanted MSC get engrafted in the brain tissue and among them, only a few differentiate into neurons [13, 86]. Thus, the MSC therapeutic effect is greatly mediated by a paracrine effect that alters the environment to protect the tissue surrounding the injury site. An important mechanism that mediates the MSC therapeutic effect is the increased protein expression of growth factors that promote neuronal survival around the injury site. MSCs have been shown to increase the expression of neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and GDNF [11, 14]. The neuroprotective effect of NGF and BDNF in multiple models of brain injury has been extensively studied. Exogenous NGF administration has been correlated with reduced neurologic deficits and reduced neuronal cell damage/death [87-91]. Similarly, BDNF

delivery has also shown therapeutic effect after brain injury by promoting neuronal survival and neurogenesis [92-94].

MSCs have also been found to modulate the cellular behavior of multiple cell types. Systemic MSC transplantation by intravenous injections alters the host peripheral immune profile increasing the number of regulatory T cells and indirectly increases the infiltration of regulatory T cells within the injury, which has been shown to be beneficial after brain injury [7, 8]. In addition to the immune response, non-neuronal brain resident cells such as microglia and astrocytes play important roles in tissue protection and repair. Microglia, similar to macrophages, is considered to have two opposite polarization, part of a spectrum, known as M1 (classically activated, inflammatory) and M2 (alternatively activated, anti-inflammatory) [95]. Zanier et al found that MSC transplantation into the injured brain modulates microglia response towards an M2 phenotype, which is considered beneficial due to its pro-healing anti-inflammatory properties [10]. Also, *in vitro* studies confirm the MSC's ability to reduce inflammatory cytokines on LPS activated microglia [9]. On the other hand, most studies on MSC-astrocytes interactions have been done *in vitro*. MSCs have been shown to reduce astrocyte apoptosis and to upregulate the astrocyte's gene expression of trophic factors after an *in vitro* anaerobic insult [96]. *In vivo*, the transplantation of MSC after stroke has been correlated to an altered glial scar composition and the increased expression of glial-derived neurotrophic factor (GDNF) [12, 97]. The MSC's ability to modulate multiple cell types makes MSC therapy a promising multifunctional approach for TBI.

1.4.1 Host Response after MSC Transplantation

Although the use of autologous MSC for brain injury would be an ideal scenario, harvesting and expanding patient-specific MSC has logistic, timing and economic constraints, and can introduce differences in cell therapeutic potency related to the patient's age [98, 99]. Therefore, allogeneic MSC transplantation would be beneficial to ensure that stem cells possess an optimal therapeutic potential and are delivered into the brain in a timely manner after an injury. However, allogeneic MSCs could be susceptible to rejection by the host immune response thereby diminishing the beneficial effect of this treatment. Eliopoulos et al showed that allogeneic MSCs have a limited survival after transplantation within the skin since they are eliminated by CD8+ T cells, Natural killer T (NKT) and NK cells that infiltrate the graft [100]. Other studies using MSC as a treatment for Graft-versus-Host-Disease (GVHD) showed that MSC transplantation failed to ameliorate GVHD and instead elicited a T cell response [101, 102]. Within the brain, various studies have shown that MSC transplantation in the non-injured adult brain results in graft rejection approximately 14 days post-transplantation [16-18]. The MSC grafts were infiltrated mostly by microglia and astrocytes along with few $CD8^+$ cytotoxic T cells [16-18]. Although T cell infiltration has been reported to be low in the intact brain, its infiltration into the injury site increases early after an injury thus making these cells a potential contributor to stem cell rejection after transplantation.

1.5 Immuno-modulatory hydrogels for stem cell transplantation

1.5.1 General Characteristics of Hydrogels

Hydrogels are cross-linked polymers capable of absorbing high amounts of water thus serving as an appropriate bioengineering material that can support stem cell viability after

transplantation [103]. The high-water content promotes the exchange of ions, nutrients, and metabolites with the surrounding tissue, thus helping to maintain cell viability [104]. In addition, the facilitated diffusion allows the communication between the host tissue and the embedded cells by the release of cytokines and bioactive molecules. Hydrogels can also serve as delivery vehicles for bioactive molecules or chemical compounds, via passive diffusion or chemical conjugation [105]. Interestingly, hydrogels provide the ability to control the presentation of ligands or bioactive molecules which can direct stem cell behavior [106-108]. For example, it has been reported that RGD functionalized alginate hydrogels can alter MSC spheroids migration and osteogenic differentiation *in vitro* depending on the RGD density [109]. Another characteristic of hydrogels that can be used to alter stem cell fate is the matrix stiffness which can be tuned by either changing the concentrations of precursor and/or cross-linkers. Hydrogels with brain-like elasticity have been able to influence neural stem cell differentiation into neurons [110]. The ability to support cell viability and tunable characteristics make hydrogels a great platform to desing carriers for stem cell trasnplantation.

1.5.3 Hydrogels for protein delivery

In addition to stem cell delivery, hydrogels have been used to deliver proteins that ameliorate neuronal damage after TBI. An important concern during protein delivery to treat brain injury or to enhance the survival of transplanted stem cells is to ensure the protein bioactivity and prolonged release to maximize its therapeutic effect. Hydrogels provide an excellent platform to design protein delivery systems due to the gentle preparation procedures usually used (aqueous environment, room temperature) that help to conserve the protein bioactivity [111]. Proteins can be physically incorporated in the

hydrogel matrix and their release is mediated by multiple mechanisms such as diffusion, swelling, degradation or the use of reversible protein−polymer interaction [111]. Hydrogels used for the controlled delivery of protein could be classified into two main categories: affinity-based delivery systems and reservoir-based delivery systems. In an affinity-based delivery system, the protein release is controlled by pre-existing or introduced functional binding sites in the hydrogel that interact with the protein using non-covalent bonding, hydrogen bonding, ionic bonding, and van der Waals forces [112]. On the other hand, reservoir-based delivery systems use physical barriers such as a hydrogel pore size to slow the diffusion rate of the proteins thereby slowing its release [112].

Affinity-based delivery systems control protein release by using the binding affinity between the protein and hydrogel matrix [112]. This advantage allows tuning a protein release profile by testing binding sites with various affinities while minimally compromising other hydrogel characteristics such as stiffness or porosity. A common approach to create affinity-based delivery systems is to incorporate heparin, a sulfated glycosaminoglycan that is able to bind to many growth factors, into a hydrogel matrix [113, 114]. *In vitro*, the incorporation of heparin to fibrin-based hydrogels matrixes has successfully slowed the release of NGF despite the lower affinity of NGF (in comparison to other growth factors) requiring an excess of heparin [115, 116]. Using a similar approach, Li et al developed an heparin-poloxamer hydrogel to sustain the release of bFGF or NGF after a peripheral nerve injury, which resulted in an enhanced axonal regeneration and recovery of motor function [113]. Recently, the design of hydrogels using chondroitin sulfate (CS) as a matrix has gained interest. The CS negative charge can electrostatically sequester positively charged growth factors [117, 118]. CS particles have been successfully

synthesized to control the release of TGF-β1 *in vitro* [118]. In addition, CS-based hydrogels have been shown to control the release of FGF-β *in vitro* and to enhance the survival of neural stem cells after transplantation in the injured brain [119, 120]. However, the use of CS-based hydrogels to simultaneously deliver growth factors and stem cells in the injured brain has not been reported.

In reservoir-based delivery systems, the physical characteristics of a hydrogel are controlled to create barriers that slow a protein diffusion rate [112]. Wang et al created a multicomponent (nanoparticles/microparticles/hydrogel) system to induce the sequential delivery of EGF and EPO in order to enhance endogenous neural stem cell repair mechanisms after a stroke brain injury [121]. They used a hyaluronan methylcellulose (HAMC) hydrogel to simultaneously deliver two sets of particles into the injured brain cortex: poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with pegylated endothelial growth factor (EGF-PEG), and biphasic microparticles comprised of a PLGA core loaded with EPO and a poly(sebacic acid) coating. This delivery system induced an initial release of EGF followed by the release of the EPO protein, which enhanced the proliferation of endogenous NSC and reduced neuronal damage [121]. Another approach using a multicomponent hydrogel system was developed by Meilander et al, in which lipid-based microtubes were used to sustain the release of multiple proteins including NGF *in vitro* [122]. Lyophilized lipid microtubes can be rehydrated with a solution containing a high concentration of the desired protein in order to trap some of the protein inside the microtubes during the water absorption process [122]. Jain et al used this lipid microtubes/agarose hydrogel system to sustain the delivery of BDNF into the injured spinal cord [123]. An advantage of using lipid microtubes to sustain protein release is the system

flexibility which allows the easy loading of different proteins in a gentle procedure that preserves the protein's bioactivity.

1.5.4 In situ gelling *hydrogels for stem cell delivery in the injured brain*

In several injury situations, the injury site receiving stem cell transplantation has an irregular shape due to the initial insult and subsequent degenerative process. Therefore, the use of premade hydrogels is limited due to the hydrogel's inability to completely fill the space and minimize gaps between the hydrogels and the host tissue. Different variables such a temperature or pH can be used to control the hydrogel's gelling process allowing the formation of the hydrogel *in situ* [124-126]. The *in-situ* gelling process results in the conformational filling of the transplantation site, which increases the hydrogel-tissue interface thereby improving the hydrogel integration with the host tissue. [124-126]. In addition, the design of hydrogels that gel *in situ* could allow the delivery of stem cells into the brain with minimal invasion minimizing the exacerbation of the secondary injury [127, 128]. Multiple *in situ* gelling hydrogels have been developed for stem cell delivery in various diseases (Figure 2.3) [127, 129-136]. In the context of brain injury, Tate et al developed thermosensitive collagen-based hydrogels functionalized with fibronectin or laminin proteins that enhanced the survival of neural stem cells delivered in the injured brain [127]. Further optimization of *in situ* gelling hydrogels to sustain or control protein delivery could lead to the development of minimally invasive multifunctional hydrogel carriers for stem cell delivery in the central nervous system.

1.5.5 Hydrogels for stem cell delivery after brain injury

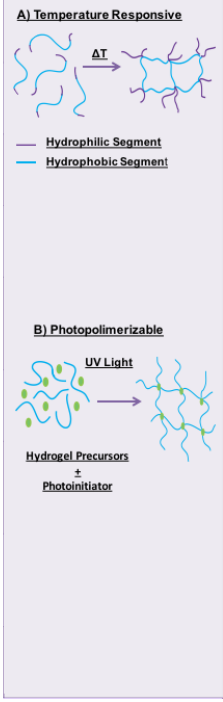
In situ Hydrogels	Stem Cell Transplantation	Material	Comments
	Primary Fetal – derived Neural Stem cells	Collagen I/laminin or Collagen I/fibronectin	Enhanced cell survival and migration, and improved behavioral recovery after traumatic brain injury mice.
	Embryonic Stem Cells	Chitosan–GP	Improved heart function, wall thickness, and microvessel densities in a rat model of myocardial infarction.
	Adipose- derived Mesenchymal Stem Cells	Chitosan–GP	Improvement of renal function, microvessel density, and tubular cell proliferation in a rat model of acute kidney injury.
	Muscle-Derived Stem cells	Triblock co-polymer PEG–PLGA–PEG	Increased engraftment of muscle-derived stem cells, and enhanced wound in a diabetic mouse model.
	Mesenchymal Stem Cells	p(NiPAAm-co-Aac) and TGF β-3 or bFGF	Hydrogel scaffolds with TGF β-3 favored cartilage tissue formation by increasing the cells proliferation rate and cartilage-specific ECM production.
	Retinal Stem-Progenitor Cells	HAMC (blend of hyaluronan and methylcellulose)	Enhanced survival and even distribution of cells in the sub-retinal space. Increased cell integration into retinal pigment epithelium.
	Mesenchymal Stem Cells	poly(ethylene oxide) diacrylate /TGF β-3	Enhanced cartilage production by increasing the production of proteoglycan and collagen II.
	Satellite Cells	Hyaluronan	Improved muscle structure and number of new my fibers, which promoted functional recovery after a partial ablation of the tibialis anterior muscle in mice.
	Mesoangioblasts Stem Cells	Polyethylene glycol-fibrinogen	Increased cell survival and differentiation of the transplanted cells in the tibialis anterior muscle of dystrophic mice.

Figure 2.3: *In situ* gelling hydrogels for stem cell delivery in vivo. Thermoresponsive and photopolimerizable hydrogels have been widely used as injectable scaffolds for stem cell delivery. The *in-situ* gelling process allows the conformational filling of the transplantation site increasing the hydrogel integration with the host tissue. A great variety of hydrogel materials and stem cells have been successfully tested in different *in vivo* scenarios.

The survival of transplanted stem cells into the injured brain could be limited by multiple biochemical processes of the secondary injury such as the development of glutamate excitotoxicity, oxidative stress and the inflammatory response [137]. The use of hydrogels as stem cell carriers for transplantation provides the opportunity to tune or control the local stem cell microenvironment to enhance the cell's survival and/or therapeutic effect [138]. Efforts using hydrogels to enhance stem cell delivery have focused on the development of hydrogel matrixes that incorporate cues from the extracellular matrix (ECM), which can provide signals to enhance the survival, proliferation, and migration of the transplanted

stem cells or provide resistance to detrimental signals present in the injured milieu [138, 139].

Several strategies to develop biomimetic hydrogels have used hyaluronan (HA) and chondroitin sulfate (CS), two glycosaminoglycans that are major components of the brain ECM, as a matrix [140]. HA is a long, negatively charged, and heavily hydrated glycosaminoglycan that has been known to have a beneficial role in wound healing and that also plays an important role in the formation of CNS [141, 142]. The development of hyaluronan based hydrogels for neural tissue repair has been of interest due to the hydrogel's mechanical properties similar to the brain tissue, and ability to promote angiogenesis and to reduce scar formation due to astrocyte activation [143]. Also, the binding of HA to the cells surface receptors, such as the cluster determinant 44 (CD44) and the receptor for hyaluronan-mediated motility (RHAMM), can modulate stem cell proliferation and survival [139, 144]. In the intact brain, injectable HA-Gelatin hydrogels improved the survival of xenogeneic human neural stem cells after transplantation in immunodeficient mice [145]. Zhong et al tested the use of HA-Gelatin-Heparin hydrogels as neural stem cell (NSC) carriers in a stroke brain injury model [146]. He found that HA-Gelatin-Heparin hydrogels can promote the survival of nutrient and growth factor deprived NSCs *in vitro* and also enhance the survival of NSCs *in vivo* after transplantation within the infarct cavity of a stroke [146]. In addition, it was found that the HA-Gelatin-Heparin hydrogels were able to reduce the infiltration of microglia within the NSC graft [146]. Therefore, the ability of HA-based hydrogels to enhance stem cell survival could be mediated by a modulation of the brain neuroglia immune-like response in addition to the HA binding to CD44 and RHAMM receptors on the transplanted stem cells.

Another component of the brain ECM recently explored to design stem cell carriers is the glycosaminoglycan chondroitin sulfate (CS). CS has been found to regulate NSC self-renewal and proliferation by facilitating the endogenous enrichment and presentation of growth factors to the stem cells [120, 147]. Betancur et al studied the effect of CS-based hydrogels on the survival of allogeneic NSCs after transplantation into the injured brain [119]. The study found that using CS-based hydrogels increased the presence of NSC near the transplantation site and also increased the percentage of transplanted cells showing the proliferation marker ki67 and stem cell markers (nestin and Sox1) [119]. The effect of CS-based hydrogels on the NSC survival and proliferation was correlated with a higher binding of the endogenous growth factor FGF-b to the matrix, which promoted the survival and undifferentiation of the transplanted stem cells [119]. As the stem cell's therapeutic effect is considered to be mediated by the release of cytokines and growth factors from the undifferentiated stem cells, developing hydrogel matrixes that can maintain the cell's "stemness" *in vivo* could be beneficial to enhance stem cell therapy after brain injury [148].

1.5.6 Immuno-modulatory hydrogels for cell transplantation

During the stem cell transplantation process, the host immune response could be induced by various factors such as the delivery process, and the biomaterial and/or transplanted stem cells immunogenicity. Therefore, the development of biomaterials that can modulate the immune response in a localized manner is of interest in order to enhance the survival and efficacy of transplanted cells. Our body provides us various examples of localized immunosuppression such as the eye and testis, which induce localized immunosuppression by the release of soluble cytokines like TGF-β2 and IL-10 among others, and the expression of FasL [35-37]. In addition, various types of stem cells such as mesenchymal stem cells

(MSC) and retinal progenitor cells (RPC) exhibit immune-modulatory properties [149-151]. Although the mechanisms for MSC-mediated immune-modulation are not fully understood, it is known that MSCs express low levels of human MHC class I and lack human MHC class II, two important molecules in the antigen presentation pathways [150, 151]. RPCs transplanted into a kidney pouch model using poly (lactic-glycolic acid) (PLGA) polymers showed an enhanced survival even in the presence of the pro-inflammatory cytokine interferon γ (IFNγ) [149]. The survival of RPCs has been shown to be due to the production of immune-suppressive factors such as TGF-β2, and Fas ligand [149]. All these examples found in nature serve as inspiration for the development of immune-modulatory biomaterials.

During the development of an immune response, various checkpoints could be targeted to direct the immune response towards the desired phenotype (e.g. inflammatory versus anti-inflammatory milieu) (Figure 2.4). For instance, an important step during an immune response is the presentation of antigens to the T-cells by antigen-presenting cells (APCs) such as macrophages and dendritic cells. The generation of the T-cells to maintain a tolerogenic state towards a specific antigen can be achieved by the presentation of the antigen in the presence of stimulatory molecules such as TGF-β1 and interleukin-10 (IL-10) [152]. On the other hand, the antigen presentation in the presence of cytokines like interleukin-6 (IL-6) and interleukin-23 (IL-23) leads to an immunogenic T-cell response [152]. Hume et al demonstrated that functionalized poly (ethylene glycol) hydrogels with immobilized TGF-β1 and IL-10 decreased activation markers on dendritic cells and reduced their ability to activate T cells *in vitro* [153]. Thus, functionalizing hydrogels with cytokines that promote tolerogenic responses could be a tool to create localized immune-

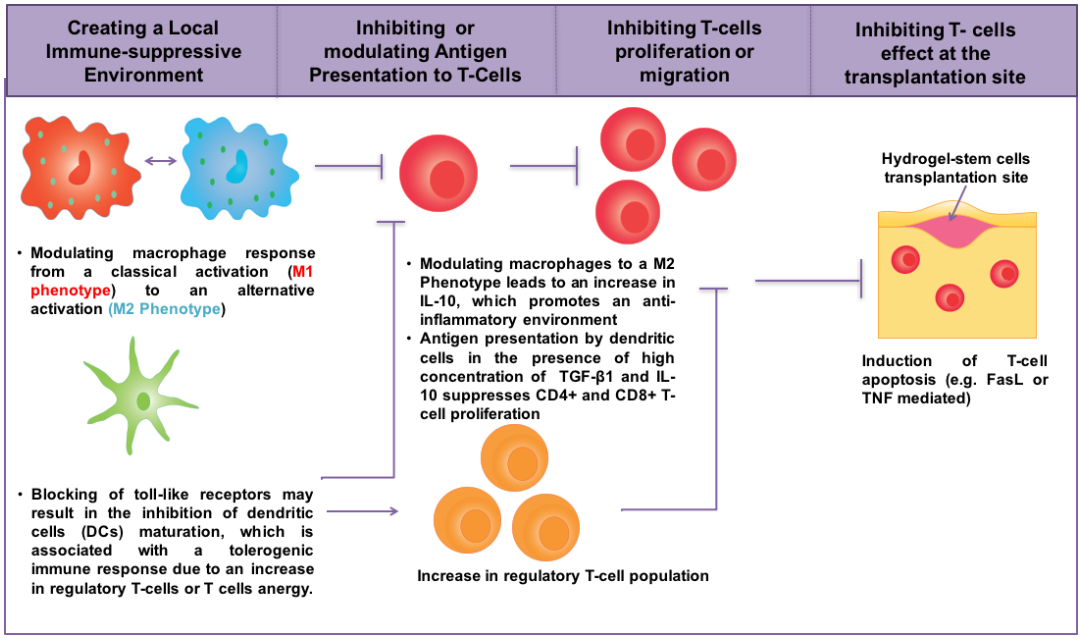


Figure 2.4: Checkpoints during the immune response that can be used to suppress the immune response and to enhance stem cell survival. An important step during the immune response is the activation of antigen presenting cells, such as macrophages and dendritic cells, and the subsequent antigen presentation to T-cells. Different cytokines can be used to shift the macrophages phenotypes from a pro-inflammatory (classically activated, M1) to an anti-inflammatory (alternative activated, M2) phenotype. In addition, blocking to cells receptor can decrease the dendritic cells maturation, which promotes the formation of regulatory T-cells. These T-cells play an important role suppressing effector T-cells (cytotoxic) in order to avoid an exacerbated immune reaction that may damage healthy body tissue. Another approach to obtain a localized immune response is the synthesis of immune-barrier around the transplanted cells. For example, hydrogels coated with FasL can induce the apoptosis of effector T-cells at the transplantation site.

privileged zones at the stem cell implantation site in order to enhance the survival of the transplanted stem cells.

The design of hydrogels to create immune-barriers has been explored in the transplantation of pancreatic islets to treat Diabetes Mellitus Type I. Initial efforts to immune-isolate pancreatic islets were focused on the use of hydrogels as physical barriers that constraint the infiltration of immune cells and/or inflammatory cytokines [154, 155]. The physical isolation of pancreatic islets is mainly achieved by reducing the hydrogel pore size which limits the infiltration of cells by size exclusion. However, reducing the hydrogels pore sizes not only can result in the poor transfer of nutrients but also limits the interaction of the transplanted cells with the host. Within the context of stem cell therapy, the therapeutic effect of transplanted stem cells could be hindered if the needed molecules, cytokines or growth factors, cannot reach the target tissue. In order to overcome this, scientists have focused on the development of bioactive hydrogels that can suppress immune cells with special attention to the suppression of cytotoxic $CD8^+$ T cells. Hume et al designed immune-active polymer coatings on poly (ethylene glycol) (PEG) hydrogels to create immune-protective carriers for cell encapsulation and delivery [156]. In this study, a bifunctional coating was synthesized functionalizing PEG hydrogels with an anti-Fas antibody and the cell adhesion molecule ICAM-1, which resulted in the apoptosis of Jurkat T-cells [156]. Similarly, Shendi et al designed anti-Fas functionalized hyaluronic acid-based hydrogels for the encapsulation of neural stem cells [157]. These hydrogels were able to maintain NSC viability while inducing the apoptosis of Jurkat T cell *in vitro* [157]. Despite the successful development of immuno-suppressive hydrogels for cell encapsulation, there are few reports of their use *in vivo*. Headen et al pioneered the use of

immune-suppressive hydrogel to enhance the survival of pancreatic islets *in vivo* [158]. The study showed that using FasL functionalized hydrogels for pancreatic islets encapsulation prolonged the survival and function of the pancreatic islets in a diabetic mice model [158]. The development of hydrogels to created localized immune-suppression at a transplantation site may potentially reduce or eliminate the need for systemic immune suppression, which has multiple side effects including the susceptibility to pathogens and the development of cardiovascular diseases [153, 159, 160]. However, it is critical to elucidate the short and long-term interactions between the transplanted cells and the host tissue and to design immunomodulatory hydrogels that could target multiple aspects of the complex *in vivo* immune response that include a variety of immune and non-immune cells (e.g. neuroglia). In this thesis, we explored the use of FasL releasing hydrogels to enhance the survival of transplanted allogeneic MSC in the injured brain of immunocompetent rats. Our goal is to examine if targeting the $CD8^+$ cytotoxic T cell population near the transplantation site could enhance the viability and therapeutic effect of the transplanted MSCs.

FASL-RELEASING HYDROGELS PROMOTE THE SURVIVAL OF TRANSPLANTED MSC AND MODULATE HOST T-CELL AND INFLAMMATORY RESPONSE IN TBI

1.6 Introduction

The development of multifunctional therapies that can target multiple pathways involved in the propagation of a TBI secondary injury could potentially ameliorate neurological deficits after brain injury [21]. The development of stem cell therapies as a treatment for TBI has gained the interest of the scientific community due to the functional plasticity of multiple stem cell types [21, 148]. Mesenchymal stem cell (MSC) transplantation is a promising treatment for TBI due to the cell's ability to modulate multiple pathways that can potentially minimize the propagation of a secondary injury after TBI. MSC have been shown to increase the expression of neurotrophic factors that promote neuronal survival around the injury site [11, 13, 161]. In addition, MSC has been shown to modulate the activation of the brain neuroglia (astrocytes and microglia) and peripheral immune cells which result in a reduced inflammatory response after TBI [6, 9, 10, 162]. In order to maximize the neuroprotective effect of transplanted MSC, it is important to deliver a high number of stem cells within a short time frame after injury and to ensure its viability after transplantation [15]. However, multiple factors such as the stem cell delivery process, the harsh environment of the injured brain (hypoxic conditions, limited nutrients) and the active rejection by the host immune system could potentially limit the survival of transplanted MSC in the injured brain [137].

Although the use of autologous MSC for brain injury would be an ideal scenario, harvesting and expanding patient-specific MSC has logistic, timing and economic constraints, and can introduce differences in cell therapeutic potency related to the patient's age [98, 99]. Therefore, allogeneic MSC transplantation provides an opportunity to ensure that stem cells of interest have an optimal therapeutic potential and are delivered in a timely manner into the brain after an injury. However, allogeneic MSC could be susceptible to rejection by the host immune response thereby diminishing the beneficial effect of this treatment [17, 163-165]. Within the brain, various studies have shown that MSC transplantation in the non-injured adult brain results in graft rejection approximately 14 days post-transplantation [16-18]. MSC grafts were infiltrated mostly by microglia and astrocytes along with few $CD8^+$ cytotoxic T cells [16-18]. Similarly, allogeneic MSC transplantation within the spinal cord also induced an immune rejection by the host that could be ameliorated by using the immunosuppressant cyclosporin A [164]. Outside the central nervous system, Eliopoulos et al showed a limited survival of allogeneic MSCs after transplantation within the skin, which was correlated with high infiltration of CD8+ T cells, Natural killer T (NKT), and NK within the graft [100]. Another study using MSC as a treatment for Graft-versus-Host-Disease (GVHD) showed that MSC transplantation failed to ameliorate GVHD and instead elicited a T cell response [100-102]. Therefore, the role of the host T cell response after MSC transplantation in the injured brain still needs to be elucidated in order to understand its contribution to the MSC survival after transplantation in the injured brain.

The development of stem cell carriers that can improve the survival of MSC during and after transplantation in the injured brain could potentially enhance the beneficial effect of

stem cell therapy after TBI. Hydrogels are cross-linked polymers with multiple characteristics that make them suitable to bioengineer stem cell carriers. The hydrogel's high-water content allows the exchange of, oxygen, nutrients, and biomolecules between the host tissue and transplanted stem cells, which facilitate an active interaction that influences the phenotypic cellular behavior of both the host and the transplanted cells [103-105]. An important characteristic of hydrogels is their ability to be optimized to deliver bioactive molecules either by passive diffusion, non-covalent binding or chemical conjugation [103-105]. Therefore, hydrogels offer a great platform to design immuno-modulatory stem cell carriers that modulate or suppress the local immune response at an injury and/or transplantation site.

The immune system has multiple checkpoints that researchers have exploited to modulate the host immune response in order to favor endogenous repair processes or to enhance cell transplantation [156, 158, 166, 167]. In order to enhance peripheral nerve regeneration, Mokarram et al used agarose hydrogels to deliver IL4 or fractalkine which enriched the anti-inflammatory (M2) macrophage population near the site of injury and created a permissive environment for tissue repair [166, 167]. The use of hydrogels to create immunosuppressive environments has also been tested. For example, Hume et al decreased activation markers on dendritic cells in order to reduce the ability of these cells to activate T cells by functionalizing poly (ethylene glycol) hydrogels with TGF-β1 and IL-10 [41].

Another approach that has been tested is the development of bioactive polymer/hydrogels that exploit the Fas/FasL apoptotic pathway to induce T cell apoptosis [156-158]. The Fas receptor (also known as CD95) is a Type I integral membrane protein expressed in the cell surface of multiple immune cells including T cells, NK cells, neutrophils, and

macrophages, and is capable of inducing apoptosis once it binds to its cognate ligand FasL [166-172]. The Fas/FasL pathway eliminates excess effector T cells during an immune response as part of a negative feedback mechanism called propriocidal regulation [172-174]. Thus, this mechanism plays an essential role in balancing the proliferation of activated effector immune cells that could be damaging to the host during the development of an immune response against a pathogen [172]. Various approaches have been used to use hydrogels to engage the Fas receptor pathway in order to suppress an immune response against transplanted allogeneic cells. Hume et al designed immune-active polymer coatings by attaching an anti-Fas monoclonal IgG antibody into poly (ethylene glycol) hydrogels, which successfully induced the apoptosis of Jurkat T cells *in vitro*. *In vivo*, Headen et al used FasL presenting hydrogels to enhance the survival of allogeneic pancreatic islets in a diabetic mice model [158]. The use of hydrogels that deliver FasL for stem cell transplantation in the injured brain has not been tested. In this project, we aim to determine if FasL delivering hydrogels could create an immunosuppressive environment near the stem cell transplantation site and enhance the survival of transplanted MSCs in the injured brain (Fig 3.1)

In order to achieve our goal, we used agarose hydrogels embedded with FasL loaded lipid microtubes as MSC carriers for transplantation in the injured brain of immunocompetent rats (Figure 3.2). In order to induce apoptosis, a FasL trimer triggers the formation of Fas signaling-competent trimers and the assembly of the death-inducing signaling complex (DISC) [172]. *In vivo*, FasL protein exists in two versions: a membrane-bound FasL version and a soluble FasL version. Soluble FasL is not efficient inducing cell apoptosis but has been associated with other functions such as the chemotactic recruitment of

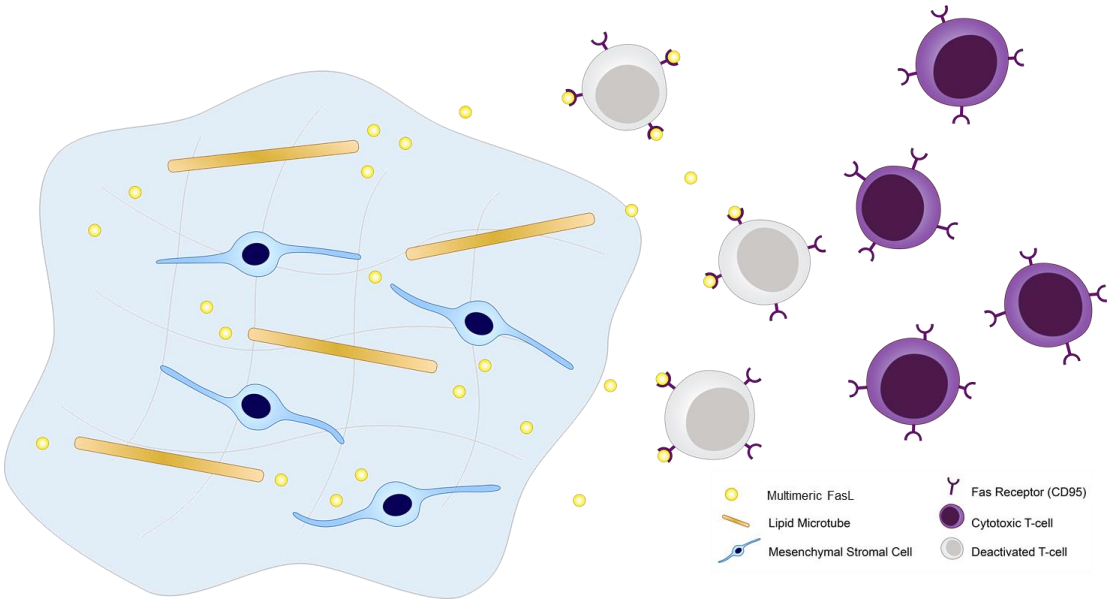


Figure 3.1: Immune-suppressive hydrogels for MSC delivery into the injured brain. Cytotoxic T cells near the injury and/or transplantation site could reduce the survival of transplanted MSC. Using hydrogels stem cells carriers that release FasL, a protein that induce the apoptosis of activated cytotoxic T cells, near the transplantation site could could reduce the cytotoxic T cell population near the transplantation site, and in consequence, potentially increase the survival of the transplanted MSC.

neutrophils *in vivo* [172, 175]. In this project, we used an engineered FasL protein in which two FasL trimers are fused using the collagen domain of ACRP30 [176]. This soluble FasL protein, commercially known as multimeric FasL (Adipogen Company), is highly efficient inducing the apoptosis of Jurkat T cells (Appendix A.1). In order to slow the diffusion of multimeric FasL and maximize its immunosuppressive effect with used an agarose/lipid microtubes system previously developed in our lab [122]. Lipid microtubes are self-assembled structures in which water-soluble compounds can be loaded in the hollow inner part of the tubes by capillarity; a process without heat that keeps the protein bioactivity

[122, 177]. Our lab has previously used lipid microtubes to sustain the release of multiple proteins such as chondroitinase ABC, Rho GTPases, and BDNF within the spinal cord after an injury [123, 178]. Rat MSCs and FasL loaded microtubes were embedded within injectable agarose hydrogels and delivered into the injured brain of immunocompetent rats. We explored the effect of these FasL releasing agarose hydrogels on the survival of the transplanted MSC and the host T cell response. Also, we determined if altering the host T cell response and/or MSC survival could indirectly alter the expression of pro-inflammatory and anti-inflammatory cytokines near the site of injury, which could

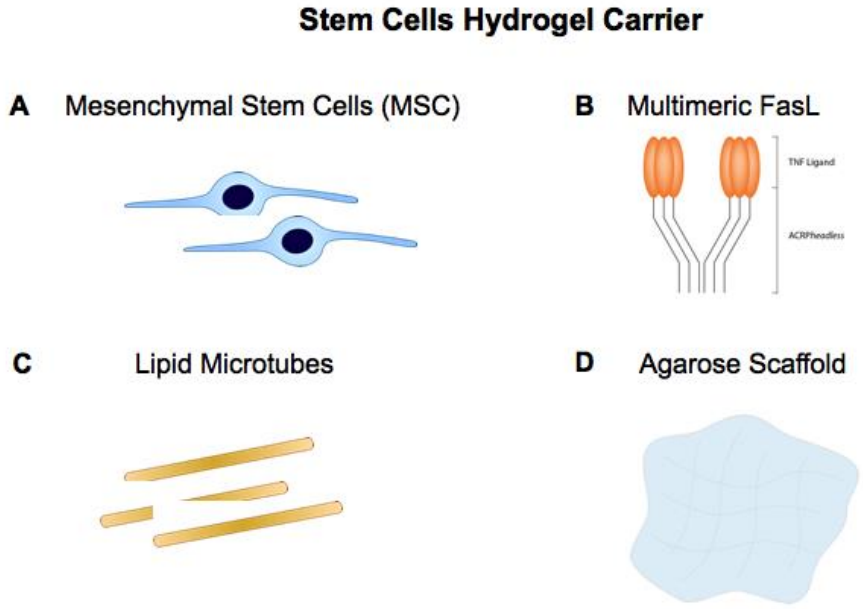


Figure 3.2: Immune-suppressive hydrogels for MSC delivery into the injured brain. Cytotoxic T cells near the injury and/or transplantation site could reduce the survival of transplanted MSC. Using hydrogels stem cells carriers that release FasL, a protein that induce the apoptosis of activated cytotoxic T cells, near the transplantation site could could reduce the cytotoxic T cell population near the transplantation site, and in consequence, potentially increase the survival of the transplanted MSC.

influence the development of a secondary injury after TBI.

1.7 Methods

1.7.1 FasL release from Lipid Microtubes embedded in Agarose hydrogel

Lipid Microtubes Synthesis

Lipid microtubes were fabricated as previously described [122]. Briefly, 1,2-bis-(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine (DC8,9PC, Avanti Polar Lipids, Alabaster, AL) was dissolved in 70% ethanol at a concentration of 1 mg/mL. The lipid was placed in a water bath with the temperature programmed to decrease from 50°C to 20°C over 48 hours and then stored at room temperature to facilitate self-assembly of lipid microtubes. Trehalose (18.9 mg/mL) was added to the lipid microtubes solution. Then, the lipid microtubes were lyophilized and stored at room temperature until further use.

FasL release form lipid microtubes

In order to study the release of FasL in our Lipid microtubes/Agarose hydrogel system four experimental groups were used. The first group, known as "*In vivo* parameters," shows FasL release as we used it in our *in vivo* studies, in which we did not remove the FasL not absorbed by the lipid microtubes during the rehydration process. In the second group, "Lipid microtubes", we removed most of the FasL not absorbed by the lipid microtubes during the rehydration process in order to determine the contribution of the lipid microtubes to FasL release from the Lipid microtubes/Agarose hydrogel system. Because the protein uptake by the lipid microtubes is around 60-70% of the initial protein, we used two agarose controls in which 60% or 80% of the initial total protein (4ug) mixed with plain agarose (without lipid microtubes).

"In vivo parameters" experimental group preparation:

In this group, .375mg of lipid microtubes were reconstituted using 15uL of an 800ug/mL FasL solution and left in ice for one hour. Then, 15uL of plain DMEM media was added to the lipid microtubes in order to obtain a total volume of 30uL. This 30uL solution was mixed with 30uL of 3% agarose in order to obtain 1.5% lipid microtubes/agarose hydrogels. The 60uL of the 1.5% lipid microtubes/agarose hydrogel solution was aliquoted in three separate wells of a 96 well plate (20uL per well). The plate was placed at 4C degrees for 3 minutes to promote the gelling of the agarose mix. Finally, 100uL of a 3% BSA solution was used to wash the hydrogels overnight. The washing solution was changed every day for 13 days.

"Lipid microtubes" experimental group preparation:

Similarly, .375mg of lipid microtubes were reconstituted using 15uL of an 800ug/mL FasL solution and left in ice for one hour. Then, the solution was centrifuged at 3,000g for 5 minutes and the supernatant was removed as much as possible. The pellet was reconstituted in 30uL of plain DMEM media and mixed with 30uL of a 3% agarose solution in order to obtain 1.5% lipid microtubes/agarose hydrogels. The 60uL of the 1.5% lipid microtubes/agarose hydrogel solution was aliquoted in three separate wells of a 96 well plate (20uL per well). The plate was placed at 4C degrees for 3 minutes to promote the gelling of the agarose mix. The washing step was done exactly as explained in the "*In vivo* parameters" experimental group.

Agarose control experimental groups preparation:

In order to prepare the 60% and 80% agarose control groups, 7.2ug or 9.6ug of FasL were diluted to a total volume of 30uL using plain DMEM media. Each solution was mixed with 30uL of 3% agarose in order to obtain 1.5% lipid microtubes/agarose hydrogels. The 60uL

of the 1.5% lipid microtubes/agarose hydrogel solution was aliquoted in three separate wells of a 96 well plate (20uL per well). The plate was placed at 4C degrees for 3 minutes to promote the gelling of the agarose mix. The washing step was done exactly as explained in the "*In vivo* parameters" experimental group.

The wash supernatants from each experimental group were stored at -80C and a rat FasL ELISA kit (R&D Systems) was used to quantify the FasL concentration.

1.7.2 Lentiviral transduction of MSC to express GFP and transplantation of these cells after CCI

Rat MSC derived from the bone marrow of Fischer 344 rats were obtained from Cyagen and, cultured used the manufacturer recommended media kit (OriCell^{TM} Mesenchymal Stem Cell Growth Medium) at 37 °C and 5%CO_2. GFP expressing lentiviral preps were made at Duke Viral Vector Core Lab by transducing 293T cells with a lentiviral plasmid (pCCLc-MNDU3-Luciferase-PGK-EGFP-WPRE) that was purchased from Addgene (# 89608). The concentrated viral supernatant was used to transduce rat MSCs grown in a 24-well plate. Cells that were GFP positive were sorted using FACS and expanded. Aliquots of GFP^{+}MSC cells were frozen and kept in liquid nitrogen until further use.

Rat GFP^{+}MSCs were validated using flow cytometry to determine the expression of the surface markers CD90 and CD45. Also, a MSC functional differentiation kit (R&D Systems) was used to test the MSC multipotency *in vitro* by examining the expression of osteocalcin and FABP4, markers of osteogenic and adipogenic differentiation respectively. Briefly, MSCs were cultivated for 14 days using an osteogenic or adipogenic media according to the manufacturer's instructions. Then, the MSC were fixed with 4%

paraformaldehyde for 10 minutes and permeabilized with 4% goat serum blocking solution. The cells were stained with a mouse Anti-Human Osteocalcin or Goat Anti-Mouse FABP4 overnight at 4°C degrees followed by a 594 Alexa Fluor anti-mouse IGg or anti-goat IGg secondary antibody.

1.7.3 Controlled Cortical Impact (CCI) of adult Sprague Dawley rats

All procedures involving animals were performed according to the guidelines set forth in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, Pub no. 85-23, 1985) and was approved by the Georgia Institute of Technology's and Duke University's Institutional Animal Care and Use Committees. Male Sprague-Dawley rats (8 weeks; Charles River) were housed in plastic cages and kept on a 12-h light-dark cycle. Food and water were available ad libitum. Rats were induced into anesthesia using 5% isoflurane for 3-5 minutes and kept under 2-3% isoflurane for the duration of the surgical procedure. Rats were mounted in a stereotaxic device after shaving the head area. The incision area was cleaned using chlorhexidine and 70% ethanol. A sagittal incision was made in the scalp and the fascia retracted to expose the cranium. A 5-mm craniotomy was made over the left frontoparietal cortex using a 5mm diameter dental drill (center: -3.0 mm AP, +2.0 mm ML from bregma). After removal of the bone, unilateral contusions of the lateral frontoparietal cortex were created using a controlled cortical impact (CCI) device. Briefly. the injury was produced by activating a pneumatic piston (3mm diameter tip) positioned 10 grades from vertical in the coronal plane to a depth of 2 mm (4m/s velocity, 100ms duration). Following the injury, the wound cavity was thoroughly cleaned, and all bleeding stopped before suturing the incision.

1.7.4 GFP-MSC transplantation 2 days post-injury

Just before transplantation, GFP-MSC (passage 6) were harvested and counted and hemocytometer and Trypan Blue. 5uL aliquots containing approximately 500,000 cells were prepared and kept on ice until transplantation. The rats were randomly separated in the following experimental groups and 5uL of the MSC aliquot was mixed according to the described formulation: 1) **Agarose group**- 10uL of 2% agarose hydrogel + 5uL plain DMEM media, 2) **Agarose-FasL group**- 10uL of 2% agarose hydrogel + 5uL of FasL/Lipid microtubes (4ug FasL), 3) **Agarose-MSC group-** 10uL of 2% agarose hydrogel + 5uL of plain media, and 4) **Agarose-MSC-FasL group-**10uL of Agarose hydrogel + 5uL FasL/Lipid microtubes (4ug FasL). All the injections were done using a 50uL Hamilton syringe with a 26-gauge needle at a rate of 2 uL per minute and a 3mm depth in the middle of the injury site. The syringe was cooled using ice for 3 minutes before the injection in order to initiate the agarose gelling process. Following the injection, the wound cavity was thoroughly cleaned, and all bleeding (if any) stopped before suturing the incision.

1.7.5 Flow cytometry analysis

At 6 days post-transplantation, the brain tissue around the injury site was harvested after a PBS cardiac perfusion following appropriate protocols laid out by IACUC. The tissue was then processed to obtain a single-cell suspension. Briefly, the tissue was crushed into a 50mL tube using a 100um nylon cell strainer. The solution was centrifuged at 300g for 5 minutes, resuspended in 1mL of a Liberase low TM/PBS solution and incubated for 30 minutes at 37C. The samples were diluted using a DNAse/FBS in PBS solution (600U

DNAse, 10% FBS), passed through a 70um nylon cell strainer into a 50mL tube and thoroughly washed with a 10% FBS/PBS solution. The solution was centrifuged at 300g for 5 minutes, resuspended in a 25% isotonic Percoll solution, and centrifuged at 521g for 20 minutes. After centrifugation, the cell pellet was washed twice with 10mL of 10% FBS made in PBS. In order to remove red blood cells, the cell pellet was resuspended in 1mL of red blood cells lysis buffer for 1 minute, washed with a 10% FBS/PBS solution, and centrifuged for at 300g for 5 minutes. The resulting pellet was resuspended in 200uL of flow cytometry buffer and stained with the following antibodies CD3-APC, CD8-PE, CD4-APC-Cy7, and CD95-PE-Cy7 using 1ug of antibody per 100uL of flow buffer for 30 minutes in dark. After two washes with flow cytometry buffer, the cells were analyzed using a Novus Flow Cytometer.

1.7.6 RT-PCR Analysis

Brain tissue for RT-PCR analysis was obtained at two days post-transplantation (4 days after TBI) after cardiac perfusion using PBS and following appropriate protocols as laid out by IACUC. The harvested tissue from the ipsilateral side of the injury was rapidly frozen in liquid nitrogen. RNA was extracted using RNeasy Maxi Kit (Qiagen) according to the manufacturer's instruction. Reverse transcriptase PCR was performed to synthesize cDNA using the RT2 First Strand Kit (Qiagen). qRT-PCR was performed using SYBR green assay for genes encoding the following pro-inflammatory and anti-inflammatory cytokines: IFNγ, IL12β, TNFα, IL-1β, IL-4, IL-10, TGFβ and IL-1βRA. Primers against GADPH and HRPT were used as housekeeping genes. The ΔΔCt analysis method to obtain the relative mRNA expression with respect to a Naïve group.

1.7.7 Protein Analysis

Brain tissue for protein extraction was obtained at two days post-transplantation after cardiac perfusion using PBS and following the appropriate protocol laid out by IACUC. The harvested tissue was rapidly frozen in liquid nitrogen. For total protein extraction, we used a modified protocol adapted from methods previously described for the analysis of cytokine/chemokine in the brain [179]. The extracted brain tissue was weighed and homogenized with the Tissue Ruptor II (Qiagen) using 10mL/g of tissue using an extraction solution consisting of N-PER reagent with a protease inhibitor. The homogenized solution was centrifuged three times at 3000g for 15 minutes to remove broken cell debris. The final supernatant or homogenous tissue lysate was aliquoted in fresh tubes and stored at -80 until further use. For protein quantification, Bicinchoninic Acid (BCA) assay was used to calculate the total protein concentration. ELISA was performed after normalizing the amount of protein used for each sample. The following cytokines were estimated: IL-10, TNFα, IL-12β, TGF-β, IL-1β and IL-1βRA following the manufacturer's protocol.

1.7.8 Statistics

The statistical analysis used consisted of an initial Brown-Forsythe analysis to test the assumption of equal variances between the experimental groups. If the assumption of equal variances between the experimental groups was confirmed an ANOVA test was performed to identify any potential significant difference in an experimental data set. A Tukey's multiple comparison test was used to identify specific significant differences between experimental groups if the ANOVA result suggested a significant difference in the experimental data set. However, if the assumption of equal variances between the

experimental groups was rejected two data analyses options were used. In the first option, a Log10 transformation of the data set was used to eliminate the significantly different variances between the groups and it was confirmed by repeating a Brown-Forsythe test. If the variances between the groups were no longer significant, the data set was analyzed using an ANOVA and Tukey's multiple comparison test as explained above. In the second option, the original data set was analyzed using a non-parametric Kruskal-Wallis test to identify any potential significant difference in the data set. Then, a Dunn's multiple comparisons test was used to identify specific significant differences between experimental groups if the Kruskal-Wallis test result suggested a significant difference in the experimental data set.

1.8 Results

1.8.1 FasL release from lipid microtubes/agarose hydrogels

In order to maximize the effect of FasL after transplantation, we used lipid microtubes to slow the release of FasL from the agarose hydrogels (Figure 3.3.). Lyophilized lipid microtubes were reconstituted using 20uL of an 800ug/mL FasL solution and centrifuge after an hour to remove the supernatant with the FasL not absorbed by the microtubes. Then, the lipid microtubes were embedded in agarose hydrogels and washed every 24 hours using a BSA/PBS solution. We also used agarose hydrogels without lipid microtubes that were mixed with 60%, and 80% of the original total protein in order to determine the contribution of the agarose hydrogel to the FasL release. We found that the concentration

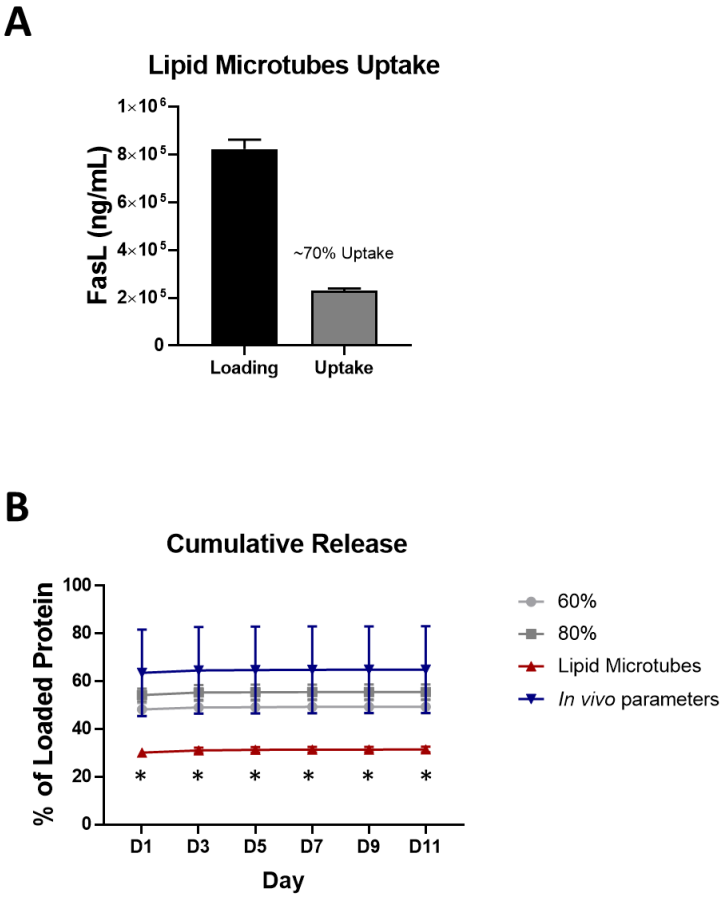


Figure 3.3: FasL release from the lipi/microtubes/agarose system used to as a stem cell carrier. A: According to the decreased protein concentration in the supernatant of the reconstituted lipid microtubes, around 70% of the total protein was trapped inside the lipid microtubes. B: The cumulative release from the lipid microtubes/agarose hydrogels was significantly lower than the 80% and in vivo parameters groups at all time points according to a Repeated measures two-way ANOVA ($p = .01$) and a Tukey's multiple comparison test (all p values were equal or less than .01).

of FasL from the original loading solution (800ug/mL) decreased to approximately 230ug/mL after the reconstitution of the lyophilized lipid microtubes. Thus, the lipid microtubes trapped around 71% of the total initial protein (8.55ug out of 12ug). In addition, we calculated the cumulative percentage of released protein with respect to the total protein initially loaded (Figure 3.3, Graph B). The lipid microtubes/agarose hydrogels released around 31% of the loaded protein, while the 60%, 80% agarose and *in vivo* parameter groups released 48%, 55%, and 64% respectively. The cumulative release from the lipid microtubes/agarose hydrogels was significantly lower than the 80% and *in vivo* parameters groups at all time points according to a two-way repeated-measures ANOVA ($p = .01$) and a Tukey's multiple comparison test (all p values were equal or less than .01).

1.8.2 FasL agarose hydrogels reduce the cytotoxic T-cells in vivo two days post-transplantation.

The effect of FasL hydrogels on the host T-cell response after a brain injury was tested using flow cytometry. Agarose-FasL or Agarose hydrogels (n=4 per experimental group) were transplanted two days post-injury and flow cytometry was used two days post-transplantation to profile the T cell population around the site of injury (Figure 3.5). The gating strategy used aimed to analyze the general T cell population using the pan marker CD3 and then, to determine the percentage of $CD8^+$ cytotoxic T cells and $CD4^+$ Helper T cells/Regulatory T cells within the total T cell population (Figure 3.4). Agarose-FasL hydrogels did not significantly change the percentage of the general $CD3^+$ T-cell population around the site of injury in comparison to Agarose hydrogels according to an unpaired t-test ($p=.66$). However, the percentage of $CD8^+$ cytotoxic T cells within the total

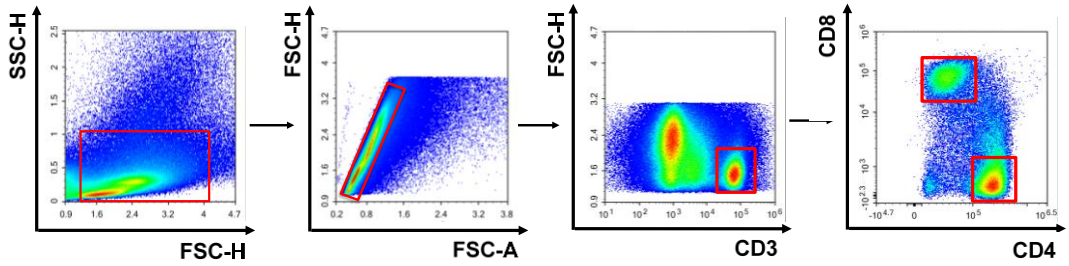


Figure 3.4: Flow cytometry gating strategy to determine host T-response near the transplantation site. An initial gating using a SSC-H versus FSC-H graph was used to removed cell debris followed by a second gating using a FSC-H versus FSC-A graph to remove cell duplets. The cells were gated using the $CD3^+$ biomarker to determine the general T cell population and within the $CD3^+$ T cell population the $CD8^+$ and $CD4^+$ markers were used to identify T cell subtypes.

T-cell population was significantly reduced according to an unpaired t-test (p= .02). Agarose FasL hydrogels did not significantly change the percentage of the $CD4^+$ t-cell population in comparison to Agarose hydrogels (p =.08).

1.8.3 FasL agarose hydrogels increase MSC presence near the injury site at 6 days post-transplantation

The effect of the immunosuppressive FasL-agarose hydrogels on the survival of transplanted MSC was studied in an allogeneic model in which rat GFP^+MSC were transplanted into immunocompetent Sprague-Dawley rats two days after TBI (Figure 3.7). The GFP^+MSCs used in these experiments were validated using flow cytometry and an *in vitro* functional differentiation test (Figure 3.6). As expected, the GFP^+MSCs showed negative staining of the pan leukocyte marker CD45 and were positive for the surface marker CD90. In addition, the GFP^+MSCs showed the expression of the differentiation

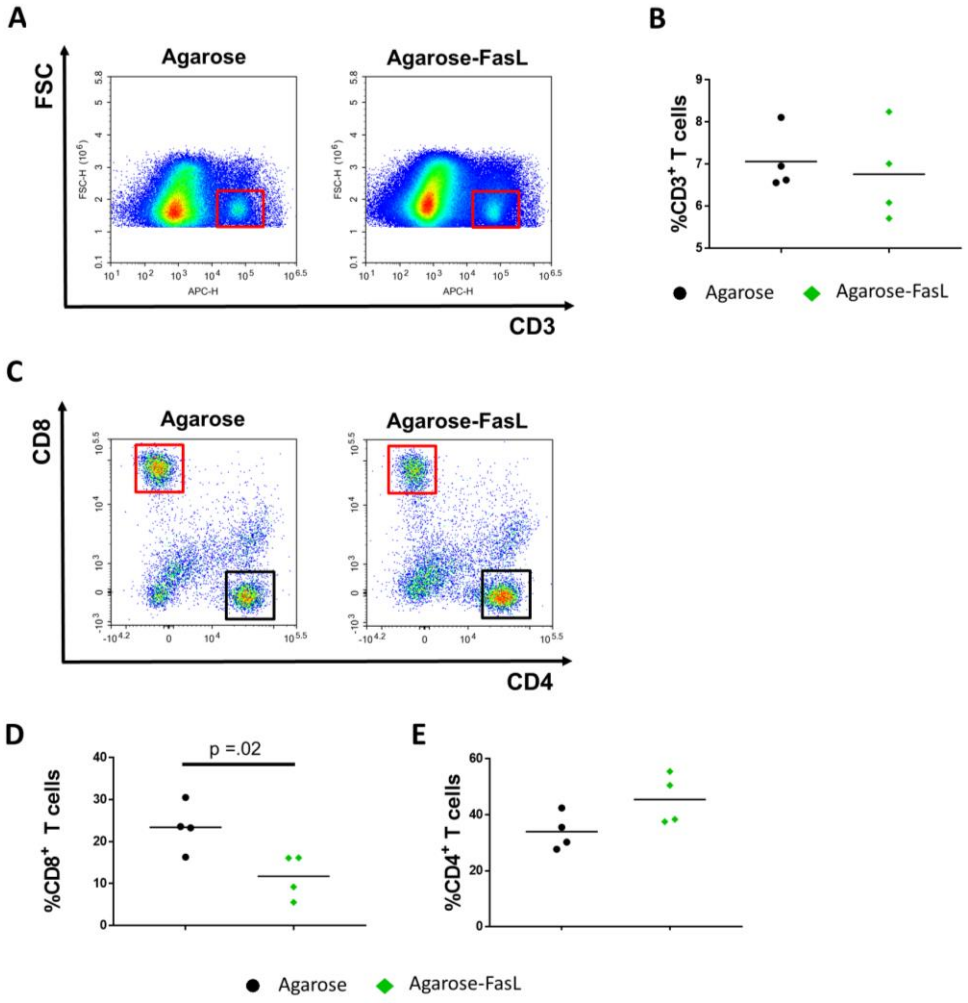


Figure 3.5: FasL hydrogels reduce the CD8+ cytotoxic T-cell population in vivo 2 days post-transplantation (4 days post-injury). A: Representative flow cytometry data showing the effect of FasL-hydrogels on the host general $CD3^+$ T cell population (red square) B: Quantification of the % of CD^+ T cells near the injury site. An unpaired t-test did not show a significant difference (p = .67) between the groups. C: Representative flow cytometry data showing the effect of FasL-hydrogels on the host $CD3^+CD8^+$ cytotoxic T-cell population (red square) and $CD3^+CD4^+$ T cell population (back square). D: Quantification of the % of CD8+ cytotoxic T cells within the general T cell population. An unpaired t-test showed a significant difference between the groups (p = .02). E: Quantification of the % of CD4 T cells within the general T cell population. An unpaired t-test did not show a significant difference (p = .08) between the groups.

markers osteocalcin or FABP4 (adipocyte) after exposure to an osteogenic or adipogenic differentiation media, respectively. The survival of transplanted GFP^+MSCs within the injury site was analyzed at 6 days post-transplantation using flow cytometry (n=8 per experimental group). The pan leukocyte marker CD45 was used to avoid the quantification of GFP positive signals coming from phagocytic cells, such as macrophages, that could potentially engulf dying MSC (Figure 3.7). The Agarose-MSC-FasL group showed an increased presence of $CD45^-GFP^+$ MSC cells (3.61 ± 0.51) compared to the Agarose-MSC (2.183 ± 0.59) experimental group according to an ANOVA (p=.0002) and Tukey's multiple comparison test (p=.01)(Figure 3.8). Due to the significantly different in the variance of the experimental groups, the data in Figure 3.8 was analyzed using a Log_{10} transformation of the original data. The original data and analysis are available in appendix A.3.

1.8.4 GFP^+MSC transplantation in the injured brain increases the general T cell population 6 days post-transplantation.

In order to determine if FasL hydrogels could create localized immunosuppression near the MSC transplantation site, we used flow cytometry to characterize the host T cell response (Fig 3.9 and 3.10)(n =6 per experimental group). The gating strategy was similar to the previously used in Figure 3.4 and the FMOs (fluorescence minus one) control stainings used to establish the gates are available in appendix A.5. The Agarose-MSC (34.1%±10.59) and Agarose-MSC-FasL (33.27%±7.317) showed a significant increase in the CD3+ T cell population in comparison to the groups injected with Agarose (16.49%±3.82) or Agarose-FasL (15.46%±2.28) according to an ANOVA ($p < 0.0001$) and

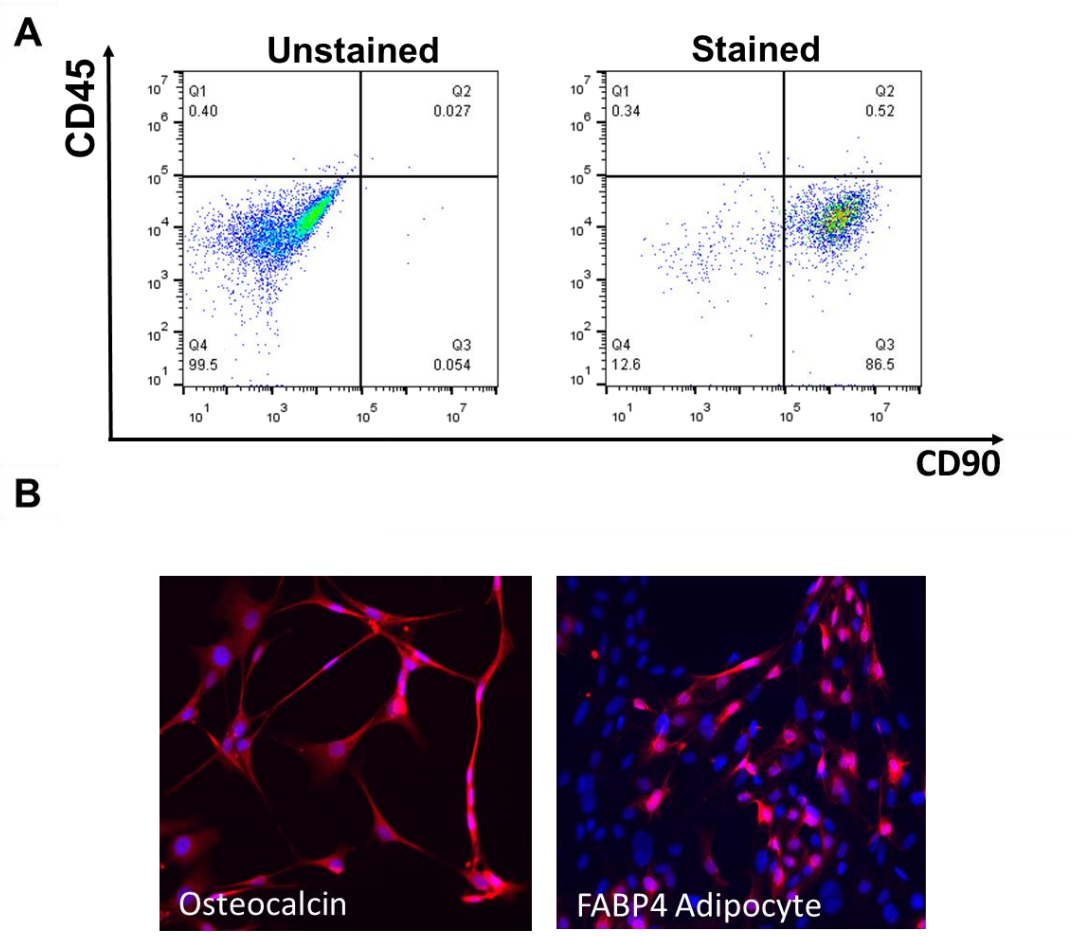


Figure 3.6: GFP+MSC validation *in vitro*. A. Representative flow cytometry data showing the MSC expression of the CD90 surface marker. As expected, MSC did not express the CD45 surface marker. B: Representatives images showing the GFP^{+}MSC differentiation potential into osteoblast and adipocytes. GFP^{+}MSC expressed the markers osteocalcin and FABP4 (adipocytes) after exposure to the respective differentiation media for osteoblast or adipocytes differentiation.

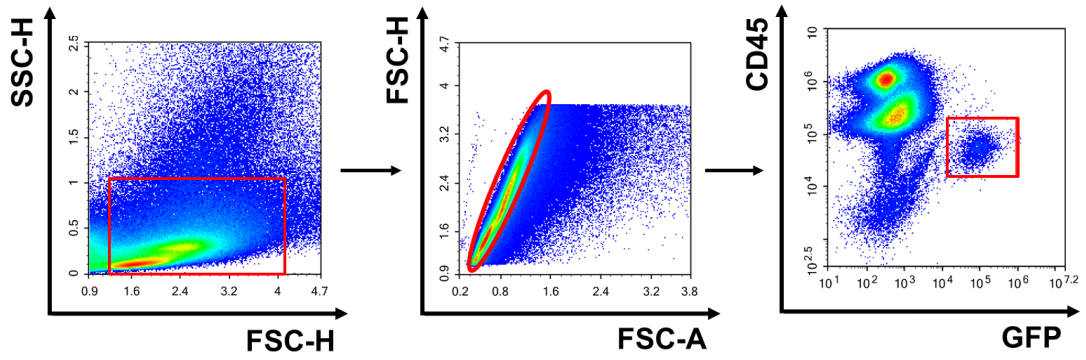


Figure 3.7: Flow cytometry gating to detect CD45$^-$GFP$^+$MSC near the transplantation site. An initial gating using a SSC-H versus FSC-H graph was used to removed cell debris followed by a second gating using a FSC-H versus FSC-A graph to remove cell duplets. Then a CD45 versus GFP graph was used to gate the CD45$^-$GFP$^+$ MSC cell population.

Tukey's multiple comparison test (p values reported in graph B, Figure 3.9). The experimental group injected with Agarose-MSC showed a significantly greater percentage of CD8+ cytotoxic T cells (46.17%±4.64) compared to the Agarose (26.32%±10.27) and Agarose-FasL group (24.00%±10.90) according to an ANOVA (p = .001) and Tukey's multiple comparison test (Agarose-MSC versus Agarose: p =.004; Agarose-MSC versus Agarose-FasL: p =.001). The group injected with Agarose-MSC-FasL (31.31%±6.576) showed a significant decrease in the CD8$^+$ cytotoxic T cells in comparison to the Agarose-MSC group according to an ANOVA (p = .001) and a Tukey's multiple comparison test (p = .042). In addition, the Agarose-MSC-FasL showed a significant increase in the percentage of (42.79%± 7.94) CD4$^+$ T cells in comparison to the Agarose (25.76%±7.83) and Agarose-FasL (23.87%±7.91) groups but not the Agarose-MSC group (34.85%±4.04) according to an ANOVA (p = .0006) and a Tukey's multiple comparison test (Agarose-

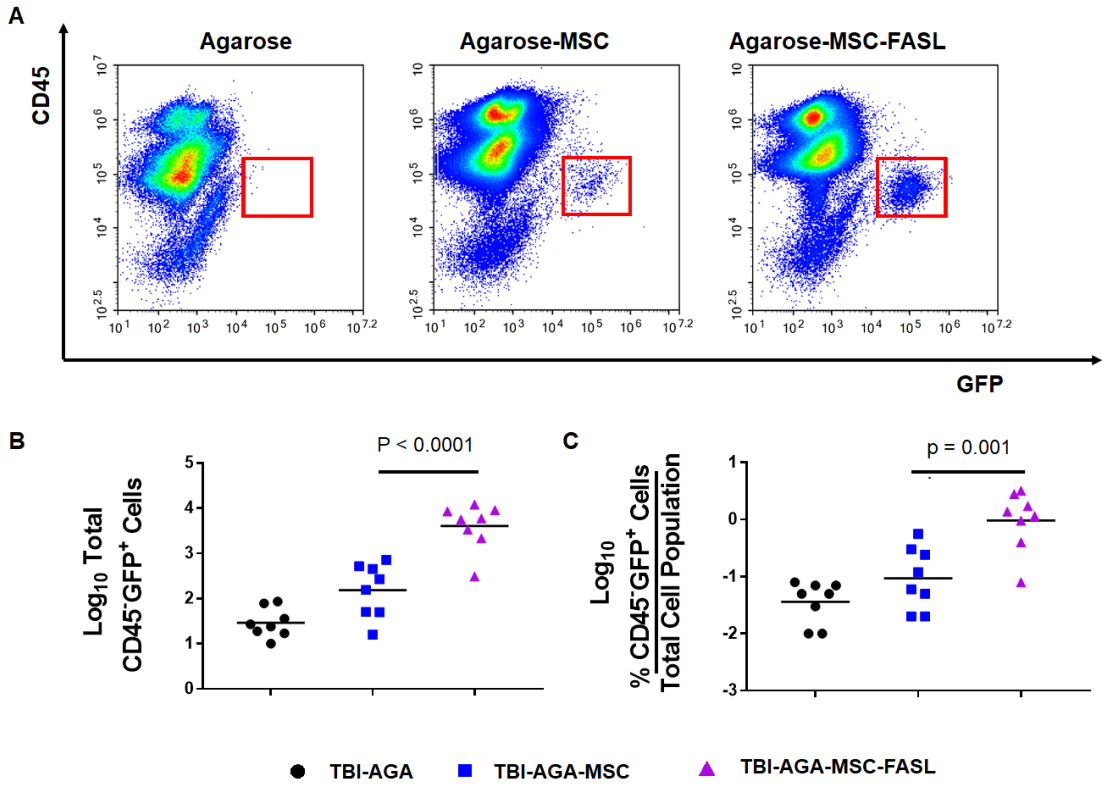


Figure 3.8: FasL hydrogels increase the presence of GFP+MSCs after transplantation into the injured brain. A. Representative flow cytometry data showing the effect of FasL-hydrogels on the survival of the transplanted GFP+ MSC (red square) 6 days post transplantation B: Quantification of Log_{10} Total $CD45^{-}$ GFP^{+} MSC cell population. A general ANOVA showed a significant difference between the experimental groups ($p < 0.0001$) and a Tukey's multiple comparison test showe a significant difference between the Agarose-MSC and Agarose-MSC-FasL groups ($p < 0.0001$). C: Quantification of the normalized percentage of $CD45^{-}GFP^{+}$ MSC cell population with respect to the total number of cells analyzed. A general ANOVA showed a significant difference between the experimental groups ($p < 0.0001$) and a Tukey's multiple comparison test showed a significant difference between the Agarose-MSC and Agarose-MSC-FasL groups ($p < 0.001$). The original non-transformed data is available in the appendix A.3.

MSC-FasL versus Agarose: p = .003; Agarose-MSC-FasL versus Agarose-FasL: p = .0009; Agarose-MSC-FasL versus Agarose-MSC: p = .25) (Fig 3.11, Graph C).

Our original hypothesis is that using FasL hydrogels we could create localized immunosuppression near the MSC transplantation site by inducing the apoptosis of cytotoxic CD8+ T cells that express the Fas receptor (also known as CD95). We tested if using FasL hydrogel can alter the T cell population that specifically express the Fas receptor using flow cytometry (Figures 3.12 and 3.13). To achieve this, the gating strategy after flow cytometry included the use of a CD95 antibody to determine which percent of the general T cell population was positive for the Fas receptor (Figure 3.10). The Agarose-MSC (15.71%± 4.32) and the Agarose-MSC-FasL (18.00%± 9.12) groups showed a lower percentage of T cells expressing the CD95 receptor in comparison to the Agarose (45.03%± 12.23) and Agarose-FasL (45.40%± 16.20) groups according to an ANOVA (p = .0001) and a Tukey's multiple comparison test (Agarose-MSC-FasL versus Agarose: p = .004; Agarose-MSC versus Agarose: p = .002; Agarose-MSC-FasL versus Agarose-FasL: p = .003; Agarose-MSC versus Agarose-FasL: p= .001) (Figure 3.12) . We observed that there was a significant increase in the percentage of $CD95^{+}CD8^{+}$ cytotoxic T cells in the Agarose-MSC group (22.24%±8.88) in comparison to the Agarose (8.17%±7.41) and Agarose-MSC (5.08%±3.56) groups according to an ANOVA (p = .002) and a Tukey's multiple comparison test (Agarose-MSC versus Agarose: p = .01; Agarose-MSC versus Agarose-FasL: p = .002). The use of FasL for MSC delivery (Agarose-MSC-FasL) did not reduce the percentage of $CD95^{+}CD8^{+}$ cytotoxic T cells in comparison to the Agarose-MSC group (p=.12). Also, we did not find any difference between the $CD95^{+}CD4^{+}$ T cell population of all the experimental groups according to an ANOVA (p = .35).

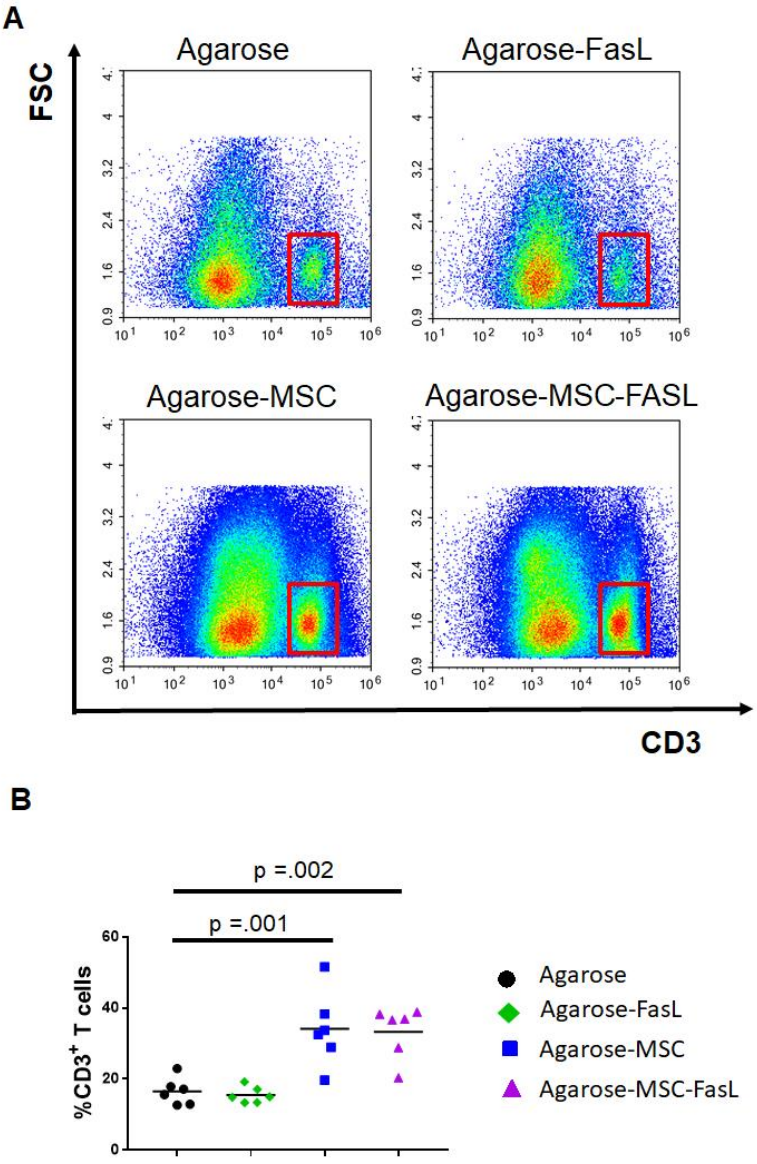


Figure 3.9: MSC transplantation increases T cell infiltration into the injured brain at 6 days post-transplantation. A: Representative cytometry data for the CD3+ general T cell population (red square) near the injury site at 6 days post-transplantation. B: Quantification of the percentage of CD3+ T cells with respect to the total analyzed cells from the brain. An ANOVA showed a significant difference between the experimental groups ($p < 0.0001$) and a Tukey's multiple comparison test showed the significant differences reported in the graph.

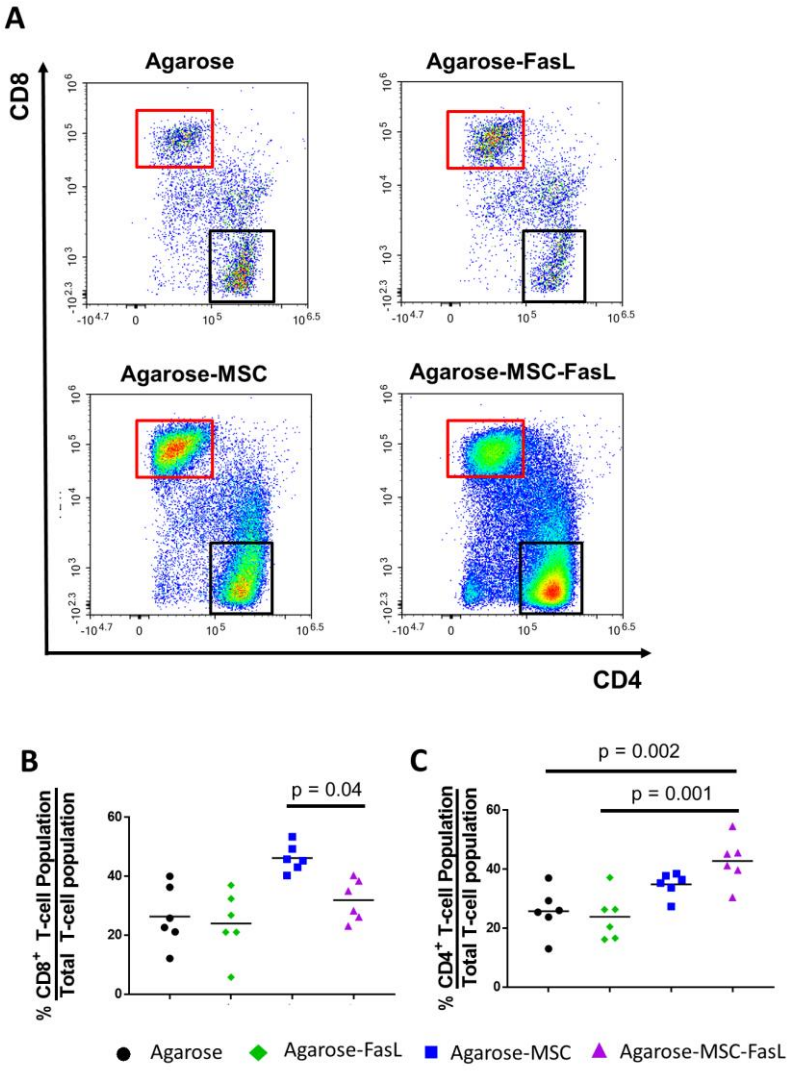


Figure 3.10: MSC delivery using FasL hydrogels decrease the CD8+ cytotoxic T cell population within the injured brain at 6 days post-transplantation. A: Representative flow cytometry data for the T cell phenotypes (red square represents the CD8+ cytotoxic T cell population, black square represents the $CD4^+$ T cell population). B: Quantification of the percentage of $CD8^+$ cytotoxic T cell population within the general T cell population. A general ANOVA showed a significant difference between the experimental groups ($p = 0.001$) and a Tukey's multiple comparison test showed the significant difference reported in the graph. C: Quantification of the percentage of $CD4^+$ T cell population within the general T cell population. A general ANOVA showed a significant difference between the experimental groups ($p = 0.0006$) and a Tukey's multiple comparison test showed the significant differences reported in the graph.

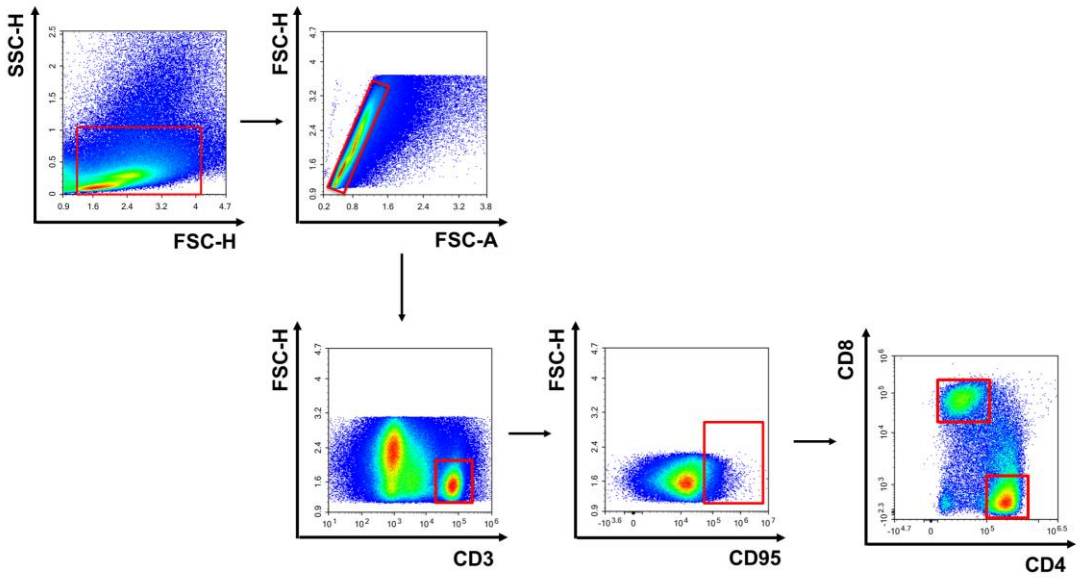


Figure 3.11: Flow cytometry gating to detect the effect of FasL-hydrogels in the host T Cell Response. The cells were gated using the CD3+ biomarker, then the CD95 marker was used to determine the FasL target population. Finally, the CD8+ and CD4+ markers were used to identify the T cell subtypes within the CD3+CD95+ population.

3.3.3 MSC-FasL agarose hydrogel modulate pro-inflammatory and anti-inflammatory cytokines in vivo 4 days after TBI and 2 days after transplantation

We tested if MSC transplantation or the use of FasL hydrogels could alter the expression of pro-inflammatory and anti-inflammatory markers. Using RT-PCR, we estimated the relative fold change in the mRNA levels of the following cytokines: IFNγ, IL12β, TNFα, IL-1β, IL-4, IL-10, TGFβ, and IL-1βRA (Figures 3.14 and 3.15). Results are expressed as the relative mRNA expression compared to a Naïve group (no TBI) baseline. Among the pro-inflammatory cytokines tested, the Agarose-MSC-FasL group showed a significant

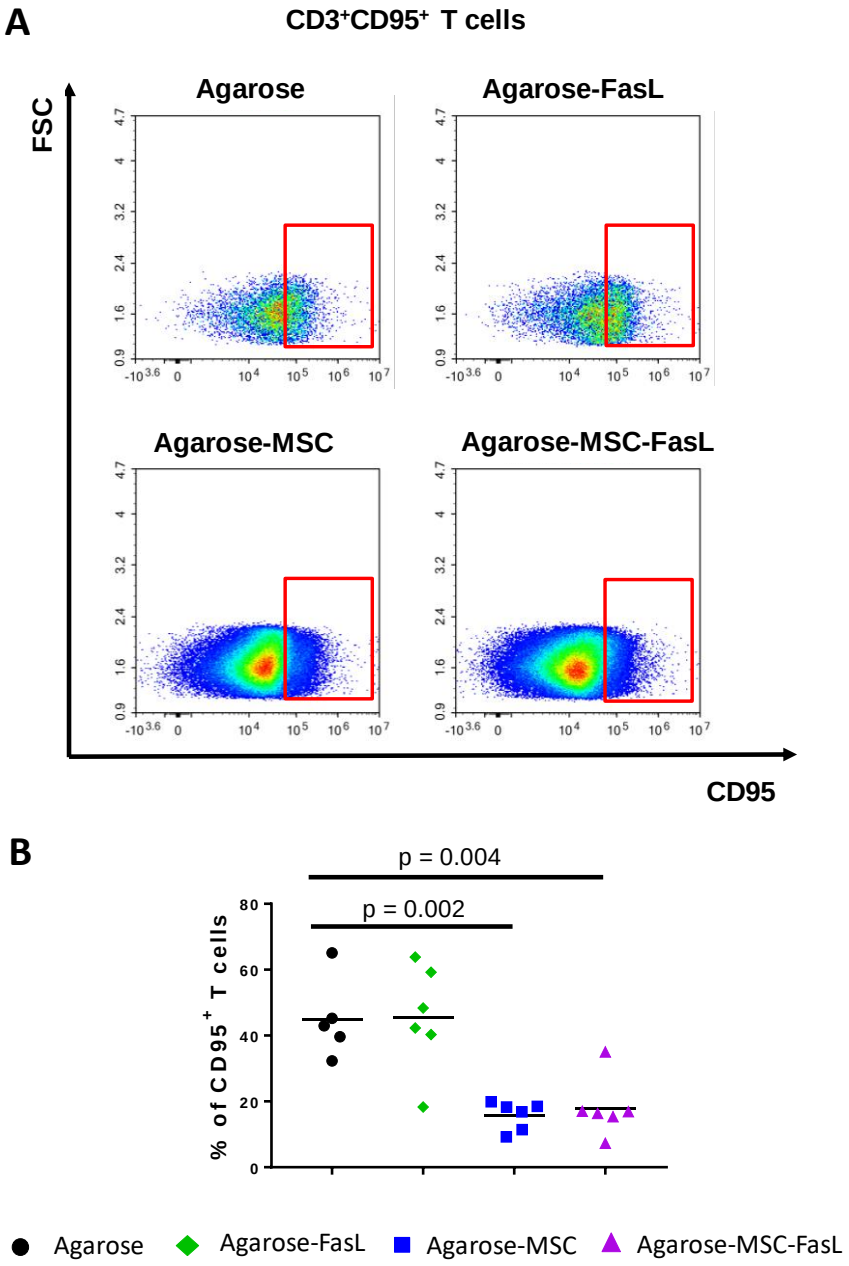


Figure 3.12: MSC transplantation shifts the CD3$^+$CD95$^+$ T cell population in the injured brain. A: Representative cytometry data for the CD3$^+$CD95$^+$ T cell population (red box) near the injury site at 6 days post-transplantation. B: Quantification of the percentage of CD3$^+$CD95$^+$ T cells within the general CD3+ T cell population. A general ANOVA showed a significant difference between the experimental groups (p = 0.0001) and a Tukey's multiple comparison test showed the significant differences reported in the graph.

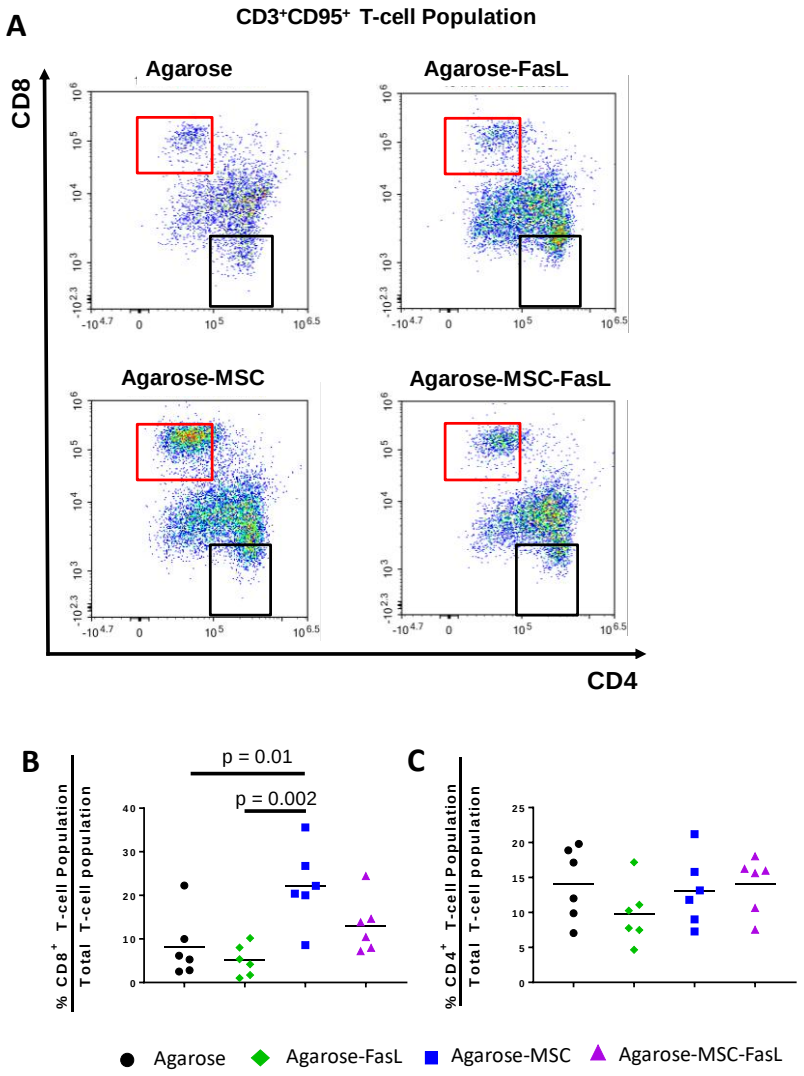


Figure 3.13: FasL hydrogels did not alter the $CD3^+CD95^+$ T cell population phenotypes in the injured brain after MSC transplantation. A: Representative flow cytometry data for the $CD3^+CD95^+$T cell phenotypes (red square represents the $CD3^+CD95^+CD8^+$ cytotoxic T cell population, black square represents the $CD3^+CD95^+CD4^+$ T cell population) at 6 days post-transplantation. B: Quantification of the percentage of $CD3^+CD95^+CD8^+$ cytotoxic T cell population within the $CD3^+CD95^+$T cell population. A general ANOVA showed a significant difference between the experimental groups ($p = 0.002$) and a Tukey's multiple comparison test showed the significant differences reported in the graph. C: Quantification of the percentage of $CD3^+CD95^+CD4^+$ T cell population within the $CD3^+CD95^+$T cell population. An ANOVA did not shown any significant difference between the groups ($p = .35$).

increase in the mRNA expression for IL-1β, TNFα, and IL-12β in comparison to the Agarose and Agarose-MSC groups (Figure 3.14 statistical analysis: TNF-α was analyzed using an ANOVA ($p < .0001$) and a Tukey's Multiple Comparison Test (p values reported in graph); IL-12β and IL- β were analyzed using a non-parametric Kruskal-Wallis Test (IL-12β: $p < .0001$; IL-1β: $p = .0001$) and a Dunn's Multiple Comparison Test(p values reported in graphs)). The TNF-α genetic expression of the Agarose-MSC was also significantly higher than the Agarose group according to an ANOVA ($p < .0001$) and a Tukey's Multiple Comparison Test ($p = .04$). Among the anti-inflammatory cytokines tested, the Agarose-MSC-FasL group showed a significant increase in the genetic expression of IL-1βRA in comparison to the Agarose group according to using a Kruskal-Wallis Test ($p < .0001$) and a Dunn's Multiple Comparison Test ($p < .0001$) (Figure 3.15). The IL-1βRA genetic expression of the Agarose-MSC group was not significantly different from the Agarose group a Kruskal-Wallis Test ($p < .0001$) and a Dunn's Multiple Comparison Test ($p=.12$). In addition, IL-10 also showed significantly higher gene expression in the Agarose-MSC-FasL group compared to the Agarose group according to an ANOVA ($p = .003$) and a Tukey's Multiple Comparison Test ($p = .002$).

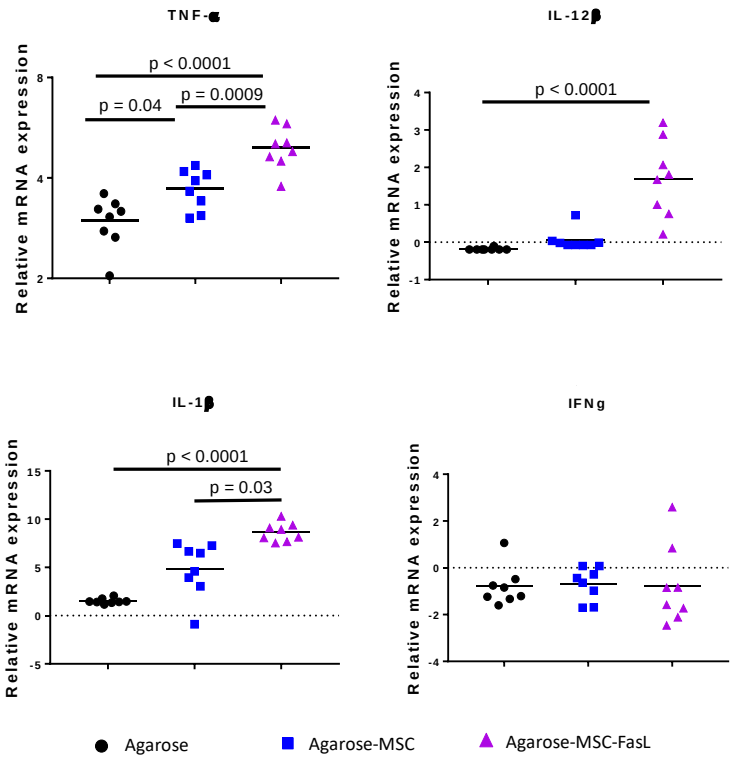
In order to corroborate the gene expression data, the protein expression of IL-10, TNF-α, IL-12β, IL-1β, IL-1βRA, and TGF-β was determined using ELISA assays according to the manufacturer's instructions (Figure 3.16). The protein concentrations of the anti-inflammatory cytokine IL-10 was lower in all the experimental groups with a brain injury according to an ANOVA ($p<.0001$)and Tukey's multiple comparison test (all p values were lower than .0001). The pro-inflammatory cytokines TNF-α was significantly lower in all experimental groups with a brain injury compared to the Naïve group according to an

ANOVA ($p<.0001$)and Tukey's multiple comparison test (all p values were equal or lower than .0001). The protein expression of the pro-inflammatory cytokine IL-1β was significantly lower in the treatment groups Agarose-FasL, Agarose-MSC, and Agarose-MSC-FasL in comparison to both the Naïve group(ANOVA $p<.0001$, Tukey's multiple comparison test p equal or lower than .007) and the Agarose group (ANOVA $p<.0001$, Tukey's multiple comparison test p equal or lower than .003). Interestingly, the protein expression of the anti-inflammatory protein IL-1β RA was significantly higher in the Agarose-MSC-FasL experimental group in comparison to the Naïve (ANOVA $p<.0001$, Tukey's multiple comparison test $p<.0001$), Agarose group (ANOVA $p<.0001$, Tukey's multiple comparison test $p<.0001$), and Agarose-MSC group (ANOVA $p<.0001$, Tukey's multiple comparison test $p<.004$).

1.9 Discussion

The disruption of the BBB following a brain injury results in the infiltration of peripheral immune cells into the brain which contribute to the development of an inflammatory response and the propagation of the secondary injury [19, 37, 38, 179]. MSCs transplanted during the acute phase of a TBI will be exposed to a tremendously harsh environment that could potentially limit the stem cells' survival or alter its functional plasticity diminishing the cell's therapeutic effect. MSC has been found to have a limited survival after transplantation in the non-injured brain, in which MSC grafts were highly infiltrated by microglia and astrocytes along with few $CD8^+$ cytotoxic T cells [16-18]. The infiltration of peripheral immune cells caused by a brain injury might contribute to the survival of transplanted MSCs as it has been showed that allogeneic MSCs are eliminated by infiltrated

A

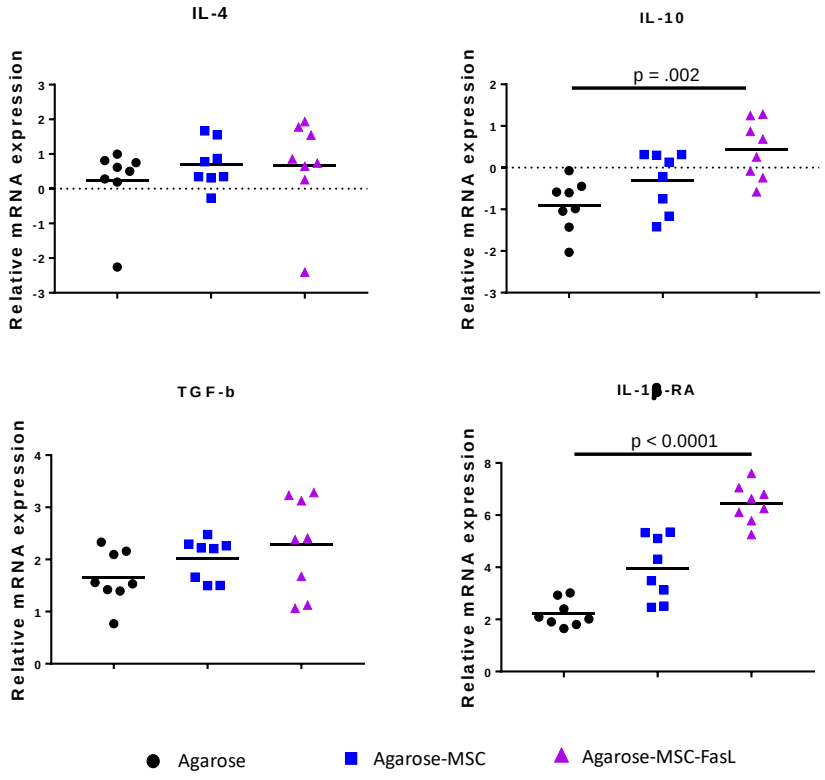


B

Experimental Group	IFN-Ɣ	IL-12β	TNF-α	IL-1β
Agarose	-0.79±0.83	-0.18±0.03	2.98±0.49	1.51±0.28
Agarose-MSC	-0.69±0.71	0.06±0.27	3.71±0.50	4.82±2.82
Agarose-MSC-FasL	-0.76±1.69	1.69±1.03	4.95±0.71	8.63±0.95

Figure 3.14. MSC delivered along with FasL significantly increases the genetic expression of pro-inflammatory 4 days after TBI (2 days after transplantation). A:The injection of MSC using FasL hydrogels significantly increased the genetic expression of TNF-α, IL-12β, and IL-1β. Statistical Analsysis: TNF-α was analyzed using ANOVA ($p < .0001$) and a Tukey's Multiple Comparison Test for the p values reported in the graph; IL-12β was analyzed using a non-parametric Kruskal-Wallis Test ($p < .0001$) and a Dunn's Multiple Comparison Test for the p values reported in the graph; IL-1β was analyzed using a non-parametric Kruskal-Wallis Test ($p = .0001$) and a Dunn's Multiple Comparison Test for the p values reported in the graph; IFN-Ɣ was analyzed using ANOVA ($p = .98$). B: Summary of the fold changes in the gene expression data.

A



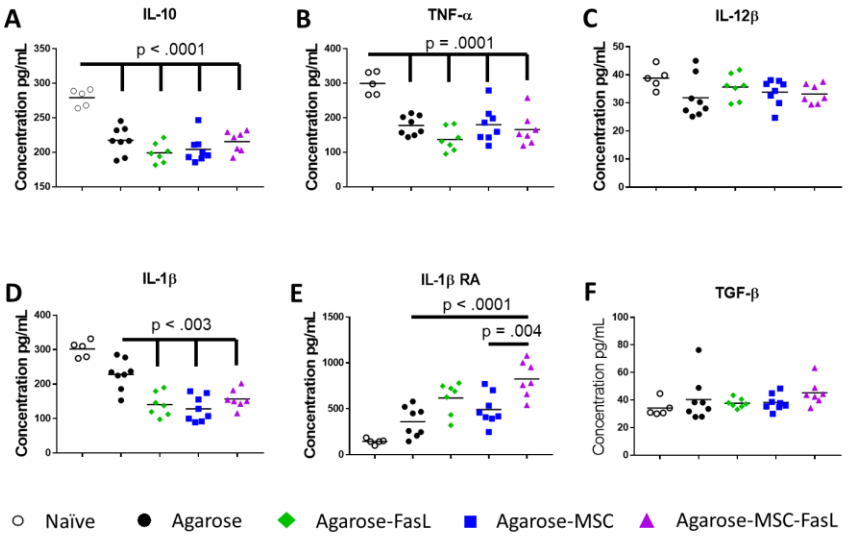
B

Experimental Group	IL-4	IL-10	TGF-β	IL-1β RA
Agarose	0.24±1.04	-0.89±0.61	1.66±0.51	2.22±0.51
Agarose-MSC	0.70±0.66	-0.31±0.71	2.02±0.39	3.96±1.22
Agarose-MSC-FasL	0.67±1.38	0.43±0.69	2.29±0.91	6.43±0.74

Figure 3.15. MSC delivered along with FasL significantly increases the genetic expression of the anti-inflammatory cytokines IL-1βRA 2 days after transplantation. A: The injection of MSC using FasL hydrogels significantly increased the expression of IL-10 and IL-1β-RA. Statistical Analysis: IL-10 was analyzed using ANOVA ($p = .003$) and a Tukey's Multiple Comparison Test for the p values reported in the graph; IL-1β-RA was analyzed using a non-parametric Kruskal-Wallis Test ($p < .0001$) and a Dunn's Multiple Comparison Test for the p values reported in the graph. B. Summary of the fold changes in the gene expression data.

CD8+ T cells, Natural killer T (NKT) and NK after transplantation within the skin [100]. In this chapter, we explored if targeting the $CD8^+$ cytotoxic T-cell population could enhance the survival of transplanted MSC in the injured brain. In order to achieve this, we designed FasL releasing agarose hydrogels that could potentially induce the apoptosis of Fas-receptor expressing $CD8^+$ cytotoxic T cells near the site of stem cell transplantation and thereby, alleviate the active rejection of the transplanted MSC by the host immune system. In order to increase the probability of FasL inducing apoptosis on $CD8^+$ cytotoxic T cells, we used lipid microtubes to prolong the release of FasL from the agarose hydrogels. This lipid microtubes/agarose hydrogel protein delivery system has been previously developed in our lab and successfully used to deliver chondroitinase ABC, Rho GTPases, and BDNF within the spinal cord after an injury [123, 178]. *In vitro*, we were able to prolong the release of FasL on lipid microtubes/agarose hydrogels in comparison to FasL loaded agarose hydrogels without lipid microtubes. A potential limitation of this delivery system is the extremely high concentrations of FasL used to load the lipid microtubes right before transplantation. MSCs express the Fas receptor but have shown resistance to FasL mediated cell death [180-182]. We tested the effect of FasL on the MSC survival and did not find a significant decrease in MSC viability but it did inhibit MSC proliferation (Appendix A.2). *In vitro*, we tested up to 400ng/mL while the initial concentration used right before *in vivo* transplantation is 200ug/mL. Although according to our FasL release experiments *in vitro* we expect an initial burst release of FasL in the first 24 hours, more testing is needed to verify the effect of extreme FasL concentrations on the MSC behavior. Hydrogel delivery systems that reduce FasL diffusion more efficiently than lipid

microtubes and therefore, require lower initial FasL concentrations could be beneficial to circumvent this



E

Experimental Group	IL-10	TNF-α	IL-12β	IL-1β	IL-1β RA	TGF-β
Naive	279.3±12.54	299.7±32.82	38.83±3.96	302±24.12	144.8±30.34	34.18±6.08
Agarose	217.5±19.99	177.8±28.04	31.85±7.36	228.3±43.69	359.9±163.5	40.35±16.05
Agarose-FasL	199.3±14.19	137.2±33.84	35.71±4.62	140.4±34.26	618±174.3	37.71±3.39
Agarose-MSC	204.2±19.39	180.2±50.66	33.86±4.64	128.2±36.92	492.1±172.1	38.27±5.84
Agarose-MSC-FasL	215.5±15.36	166±46.81	33.19±3.427	156.8±28.04	825.7±194.8	45.26±9.145

Figure 3.16. MSC delivery using FasL hydrogels increased the expression of IL-1β RA at 2 days post-transplantation. A, B and D: Effect of MSC transplantation using FasL hydrogels on the protein expression of inflammatory/anti-inflammatory proteins. Brain injury significantly decreased the expression of IL-10, TNF-α and IL-1β in comparison to the Naïve group. Statistics: All proteins were analyzed using ANOVA (IL-10 p <.0001; , TNF-α p <.0001; IL-1β p<.0001). A Tukey's multiple comparison test was used to obtain the p values reported in the graph. E: MSC delivering using FasL hydrogels significantly increased the protein expression of IL-1β RA. Statistics: IL-β RA was analyzed using ANOVA (p <.0001). A Tukey's multiple comparison test was used to obtain the p values reported in the graph. C and F: No significant differences were found in the protein expression of IL-12β (p = .19) and TGF-β (p = .36).

limitation. Furthermore, the observed MSC resistance to FasL mediated apoptosis *in vitro* could be decreased *in vivo* due to the harsh environment near the transplantation site which could expose the stem cells to multiple stressors (oxidative stress, hypoxia) simultaneously [183]. It would be interesting to perform similar experiments *in vivo* to test the survival of MSCs with a knockout of the Fas receptor in order to determine any potential toxicity of FasL under *in vivo* conditions.

Using flow cytometry, we studied the survival of transplanted GFP^+MSC delivered into the injured brain using Agarose-FasL hydrogels. GFP^+MSC were transplanted into the injured brain of immunocompetent rats 2 days-post-injury and the stem cell presence near the injury site was determined 6 days post-transplantation. We found that MSC delivery using Agarose-FasL hydrogels (Agarose-MSC-FasL group) significantly increased the presence of transplanted stem cells near the injury site in comparison to MSC transplanted using Agarose hydrogels (Agarose-MSC group), which suggest a potential increase in the survival of the transplanted stem cells (Figure 3.8). The use of FasL-hydrogel could have potentially delayed the death and/or clearance of the transplanted MSCs suggesting that inducing localized immunosuppression at the site of transplantation could enhance stem cell therapy after brain injury. It is important to emphasize that although we observe an increased presence of GFP^+MSCs after delivery using FasL-hydrogels, we do not have knowledge about the functional status of the transplanted stem cells. MSC has been found to have a phenotype paradigm, similar to macrophages, in which environmental signals could induce a pro-inflammatory (MSC1) or anti-inflammatory (MSC2) phenotype [184, 185]. Interestingly, Naftali-Shani et al found that the environmental signals found in the infarcted myocardium switched MSC toward an inflammatory phenotype and exacerbated

the damage to the heart [186]. They found that knocking out the Toll-Like Receptor 4 (TLR4) favored the MSC anti-inflammatory phenotype and enhanced the stem cell therapeutic effect [186]. Commonly, stem cell transplantation research focuses on the effect of the transplanted stem cell on the injured brain milieu with some emphasis on stem cell survival. However, more research is needed to understand the effect of the injured brain milieu on the MSC functional phenotype and therapeutic potential in order to move forward the development of cell therapies for TBI.

An important limitation in this study is the use of GFP as a fluorescence label in order to track the transplanted rat MSCs *in vivo*. GFP labeling could elicit an immunogenic response depending on multiple factors such as GFP variant, labeled-cell transplantation route, and mice/rat strain[187-190]. Various studies found that transplantation of GFP-labeled cells was correlated with an increased T cell infiltration, specifically with a generation of a cytotoxic T lymphocyte (CTL) response [188, 189]. However, Moloney et al compared the immunogenicity of GFP^{+}MSCs obtained from the "green rat" (SD-Tg [CAG-EGFP] CZ-004Osb) and non-labeled MSC after transplantation in the intact adult brain of rats, and found no significant difference in the volume of the transplanted grafts (with or without GFP labeling) and the activation of the brain neuroglia (astrocytes and microglia) [18]. Nevertheless, there still the possibility that the altered brain's immune environment after a brain injury could lead to an exacerbated immune response against GFP labeled MSCs. An alternative to the use of GFP-MSCs is to perform similar studies using rat females as immunocompetent hosts and male rats as donors in order to use antibodies against the Y chromosome to identify the transplanted stem cells. However, the female hormone progesterone has been found to be neuroprotective after brain injury and

therefore, the female estrous cycle needs to be taken into consideration during the study of TBI using female rats [191].

Multiple studies exploring the survival of allogeneic MSC in the brain have used a non-injured brain model and focused on the infiltration of microglia and astrocytes within the transplanted grafts [17, 18, 163]. We found that GFP^+MSC transplantation after in the injured brain of immunocompetent rats leads to an increase in the general $CD3^+$ T cell population near the injury site which suggests a delay in the usual resolution of host immune response after TBI [19]. We also found that the transplantation of MSC significantly increases the percentage of $CD3^+CD8^+$ cytotoxic T cells, but the use of FasL hydrogels as MSC carriers reversed this effect. As mentioned previously, the use of GFP as a label to track MSC could elicit an independent immune response exacerbating the host allogeneic response towards the transplanted stem cells [187-190]. Therefore, further studies using non-labeled MSCs are needed to determine if the increased infiltration of $CD3^+$ T cells, including the increase in the $CD8^+$ T cell population, could be due to a host response against GFP. However, even with the possibility of a stronger immune response caused by the GFP^+MSCs, the FasL hydrogels were able to prolong the presence of the transplanted stem cells near the injury site and to reduce the increase in the $CD8^+$ T cell population. These results suggest that creating localized immune-privileged zones near the stem cell transplantation site could be a strategy to enhance stem cell survival after transplantation in the injured brain.

We also observed that the animal injected with MSCs using FasL hydrogels showed an increased percentage of $CD3^+CD4^+$ T cell population in comparison to the groups injected without MSCs (Agarose and Agarose-FasL) but not in comparison to the Agarose-MSC.

Previous studies have shown that an increase in regulatory T cells can reduce neural damage after brain injury [7, 8]. Some of the population of the increased $CD3^+CD4^+$ T cells observed in our study might be comprised of regulatory T cells and could potentially help in the post-injury repair processes. Therefore, it is important to emphasize that using FasL as our immunosuppressive agent allows us to selectively eliminate an undesired component of the immune response such as the $CD8^+$ cytotoxic T cells population without compromising other desirable components such as the $CD4^+$ regulatory T cell population.

The pathophysiological changes caused due to the secondary injury and the MSC transplantation to the injury site with or without FasL could alter the host inflammatory response. MSC have been shown to possess immunomodulatory properties that alter the inflammatory response in the acute phase of a brain injury [162]. We have shown in our study that MSC transplantation in agarose –FasL hydrogels altered the genetic and protein expression of inflammatory cytokines after TBI. The Agarose-MSC-FasL group showed greater mRNA levels for IL-1β, TNF-α, IL-1β-RA compared to the Agarose group. Surprisingly, although the mRNA levels of IL-1β and TNF-α were significantly increased, the protein expression of both was significantly lower in the animal subjected to a brain injury in comparison to Naïve levels. Moreover, the Il-1β protein expression on the rats treated with FasL, MSC or both was significantly lower than the injured animals without treatment. Interestingly, the transplantation of Agarose-MSC-FasL significantly increased the protein concentration of the IL-1β RA compared to the Naïve, Agarose and Agarose-MSC groups. IL-1β RA has been shown to be an important regulator of the IL-1β pro-inflammatory cytokine and has been shown to reduce neuronal damage in various models of brain injury including TBI and Ischemic stroke [39-42].

As a final comment, in this project, we specifically targeted the host cytotoxic T cell response in order to enhance the survival of transplanted MSCs. However, the survival of transplanted stem cells can be affected by other factors non-related to the host immune response such as hypoxia and limited nutrients among others [137]. A strategy to enhance the survival of transplanted stem cells using hydrogels is the incorporation of ECM cues into the hydrogel matrix, which can provide signals to enhance the survival, proliferation, of the transplanted stem cells or provide resistance to detrimental signals present in the injured milieu [138, 139]. A disadvantage of the agarose hydrogel system used in this study is the lack of ECM contact sites, which could limit stem cell survival. Further studies could design bifunctional hydrogels that provide the stem cells a direct survival stimulus using ECM cues while simultaneously suppressing the host immune system. Hyaluronan or chondroitin sulfate hydrogels could be modified to sustain the release of FasL or other immunomodulatory proteins in order to enhance stem cell survival using a multi-targeted approach [119, 146].

1.10 Conclusion

The initial insult during TBI leads to the creation of a complex environment that includes the infiltration of multiple immune cells, which could jeopardize the survival of transplanted allogeneic MSC. Our goal in this chapter was to explore the role of the T cell response on the MSC survival after transplantation in the injured brain and to determine if T cell suppression could lead to enhance MSC survival. To achieve this, we used FasL releasing hydrogel that induces the apoptosis of cytotoxic $CD8^+$ T cells near the site of MSC transplantation. We showed that the injection of MSC into the injured brain leads to an increased T cell population near the injury site, including an increased percentage of

$CD8^+$ cytotoxic T cells. However, MSC injection using FasL hydrogels was able to reduce the percentage of the $CD8^+$ cytotoxic T cells without compromising the $CD4^+$ T cell population. The selective decrease of the $CD8^+$ T cell allows us to protect the MSC without losing the potential beneficial effect of $CD4^+$ regulatory T cells. We were able to show that FasL-agarose hydrogels enhance the presence of MSCs after transplantation in the injured brain whereas the injection of MSC without FasL resulted in the almost complete elimination of the transplanted cells. The altered immune response observed after MSC transplantation using FasL hydrogels could alter the expression of inflammatory cytokines. We found that injecting MSC using FasL hydrogels resulted in an increased protein concentration of IL-1β-RA, a cytokine that has been shown to reduce neuronal damage after brain injury due to its anti-inflammatory properties. Therefore, the combination of MSC transplantation with FasL delivery could be enhancing the MSC therapeutic through the inhibition of the IL-1β pathway. However, further studies are needed in order to characterize the functional status of the MSCs after transplantation in the injured brain in order to improve the design of hydrogel carriers that enhance stem cell therapy after TBI.

EVALUATE THE EFFECT OF MSC TRANSPLANTATION USING FASL-HYDROGELS ON THE INJURED BRAIN ENVIRONMENT.

1.11 Introduction

A unique property of MSC therapy for brain injury is the stem cell's ability to target multiple mechanisms of the secondary injury in order to contain neuronal damage. The release of cytokines and chemokines due to the initial injury attract and stimulate MSCs, which can coordinate the response of multiple cell types such as immune cells, endothelial cells, neuroglia and neural progenitor cells to promote tissue repair [5]. Systemic MSC transplantation by intravenous injections has been shown to alters the host peripheral immune profile increasing the number of Regulatory T cells and indirectly increasing the infiltration of regulatory T cells within the injury site, which has been proved to be beneficial after brain injury [7, 8]. In addition to the immune response, non-neuronal brain resident cells such as microglia and astrocytes play important roles in tissue protection and repair. Microglia, similar to macrophages, is considered to have two opposite polarization, part of a spectrum, known as M1 (classically activated, inflammatory) and M2 (alternatively activated, anti-inflammatory). Zanier et al found that MSC transplantation into the injured brain modulates the microglia response towards an M2 phenotype, which is considered beneficial due to its pro-healing anti-inflammatory properties [10]. Also, In vitro studies confirm the MSC's ability to reduce inflammatory cytokines on LPS activated microglia [9]. MSCs have been shown to reduce astrocyte apoptosis and to upregulate the astrocyte's gene expression of trophic factors after an *in vitro* anaerobic insult [96]. In vivo, the transplantation of MSC after stroke has been correlated to an altered glial scar

composition and increased expression of the glial-derived neurotrophic factor (GDNF) [12, 97]. The MSC's ability to modulate multiple cell types makes MSC therapy a promising multifunctional approach for TBI.

Another mechanism reported in the literature to mediate the MSC therapeutic effect is the increased protein expression of growth factors that promote neuronal survival around the injury site. Studies have reported an increased expression of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF) [11]. In addition, MSC has been shown to increase the expression of neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and GDNF [11, 14]. The neuroprotective effect of NGF and BDNF in multiple brain injury models has been extensively studied in the literature. Exogenous NGF administration has been correlated with reduced neurologic deficits and reduced neuronal cell damage/death [87-91]. Similarly, BDNF delivery has also shown therapeutic effect after brain injury by promoting neuronal survival and neurogenesis [92-94].

Stem cell therapy efficiency depends on multiple factors including cell source, culture conditions and delivery route [43, 44}. Pre-clinical studies testing MSC therapeutic effect after TBI usually used two main routes of cell delivery: intravenous injections and intracerebral injection. Intravenous injection, although minimally invasive limit the amount of cell that reaches the cerebral cortex. [85, 192, 193]. On the other hand, intracerebral injection delivers the MSC to a potentially harsh environment that might hinder the cells' survival and therapeutic effect. In order to optimize MSC transplantation, multiple questions still need to be addressed. Does prolonged MSC survival enhance the

stem cell's therapeutic effect? What is the minimum stem cell dosage needed for a successful treatment? Does the injured brain environment alter the MSC functional phenotype?

In the previous chapter, we demonstrated that MSC transplantation increases the infiltration of T cells and the percentage of $CD8^+$ cytotoxic T cells within the injury site. Also, using FasL-agarose hydrogels as stem cell carriers reversed the increase in the $CD8^+$ cytotoxic T cell population. We also showed that using FasL hydrogels as MSC carriers after TBI results in an increased MSC presence around the injury site. In this chapter, we evaluate if the enhanced MSC presence near the injury site ameliorates the neuronal pathophysiology after TBI. We will determine the effect of MSC transplantation using FasL hydrogels on the protein expression of multiple growth factors previously reported to mediate the MSC therapeutic effect. In addition, we will use MRI and examine various histological markers to determine the neurodegenerative state around the injury site after MSC transplantation using FasL hydrogels.

1.12 Methods

1.12.1 Controlled Cortical Impact (CCI)

All procedures involving animals were performed according to the guidelines set forth in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, Pub no. 85-23, 1985) and was approved by the Georgia Institute of Technology's and Duke University's Institutional Animal Care and Use Committees. Male Sprague-Dawley rats (8 weeks; Charles River) were housed in plastic cages and kept on a 12-h light-dark cycle. Food and water were available ad libitum. Rats were induced into

anesthesia using 5% isoflurane for 3-5 minutes and kept under 2-3% isoflurane for the duration of the surgical procedure. Rats were mounted in a stereotaxic device after shaving the head area. The incision area was cleaned using chlorhexidine and 70% ethanol. A sagittal incision was made in the scalp and the fascia retracted to expose the cranium. A 5-mm craniotomy was made over the left frontoparietal cortex using a 5mm diameter dental drill (center: -3.0 mm AP, +2.0 mm ML from bregma). After removal of the bone, unilateral contusions of the lateral frontoparietal cortex were created using a controlled cortical impact (CCI) device. Briefly. the injury was produced by activating a pneumatic piston (3mm diameter tip) positioned 10 grades from vertical in the coronal plane to a depth of 2 mm (4m/s velocity, 100ms duration). Following the injury, the wound cavity was thoroughly cleaned, and all bleeding stopped before suturing the incision.

1.12.2 MSC transplantation after CCI

Just before transplantation, GFP-MSC (passage 6) were harvested and counted and hemocytometer and Trypan Blue. 5uL aliquots containing approximately 500,000 cells were prepared and kept on ice until transplantation. The rats were randomly separated in the following experimental groups and 5uL of the MSC aliquot was mixed according to the described formulation: 1) Agarose group- 10uL of 2% agarose hydrogel + 5uL plain DMEM media, 2) Agarose-FasL group- 10uL of 2% agarose hydrogel + 5uL of FasL/Lipid microtubes (4ug FasL), 3) Agarose-MSC group- 10uL of 2% agarose hydrogel + 5uL of plain media, and 4) Agarose-MSC-FasL group-10uL of Agarose hydrogel + 5uL FasL/Lipid microtubes (4ug FasL). All the injections were done using a 50uL Hamilton syringe with a 26-gauge needle at a rate of 2 uL per minute and a 3mm depth in the middle

of the injury site. The syringe was cooled using ice for 3 minutes before the injection in order to initiate the agarose gelling process.

1.12.3 Protein Analysis

Brain tissue for protein extraction was obtained at two days post-transplantation after cardiac perfusion using PBS and following the appropriate protocol as laid out by IACUC. The harvested tissue was rapidly frozen in liquid nitrogen. For total protein extraction, we used a modified protocol adapted from methods previously described for the analysis of cytokine/chemokine panels in the brain [179]. The extracted brain tissue was weighed and homogenized with the Tissue Ruptor II (Qiagen) using 10mL/g of tissue of N-PER reagent with a protease inhibitor as an extraction solution. The homogenized solution was centrifuged three times at 3000g for 15 minutes to remove broken cell debris. The final supernatant or homogenous tissue lysate was aliquoted in fresh tubes and stored at -80 until further use. For protein quantification, Bicinchoninic Acid assay was used to calculate the total protein concentration. ELISA was performed after normalizing the amount of protein used for each sample. The following cytokines were estimated: NGF, BDNF, IGF, and VEGF following the manufacturer's protocol.

1.12.4 Tissue processing for Immunocytochemistry

Brain tissue was obtained at 3 weeks post-transplantation, after cardiac perfusion with PBS and 4% Paraformaldehyde following the appropriate protocol as laid out by IACUC. The brains were left in 4% Paraformaldehyde overnight and in a .5% ProHance solution for 14 days. After MRI imaging, the brains were placed in a 30% sucrose solution for 4 days. Then, the brains were placed in OCT cryosectioning media, frozen in liquid nitrogen and

kept in -80C until further use. The brains were placed at -20C the night before sectioning. On the day of sectioning, the brains were cut in 12um sections and kept at -20 until the immunostaining protocol.

1.12.5 Statistics

The statistical analysis used consisted of an initial Brown-Forsythe analysis to test the assumption of equal variances between the experimental groups. If the assumption of equal variances between the experimental groups was confirmed an ANOVA test was performed to identify any potential significant difference in an experimental data set. A Tukey's multiple comparison test was used to identify specific significant differences between experimental groups if the ANOVA result suggested a significant difference in the experimental data set. However, if the assumption of equal variances between the experimental groups was rejected two data analyses options were used. In the first option, a Log10 transformation of the data set was used to eliminate the significantly different variances between the groups and it was confirmed by repeating a Brown-Forsythe test. If the variances between the groups were no longer significant, the data set was analyzed using an ANOVA and Tukey's multiple comparison test as explained above. In the second option, the original data set was analyzed using a non-parametric Kruskal-Wallis test to identify any potential significant difference in the data set. Then, a Dunn's multiple comparisons test was used to identify specific significant differences between experimental groups if the Kruskal-Wallis test result suggested a significant difference in the experimental data set.

1.13 Results

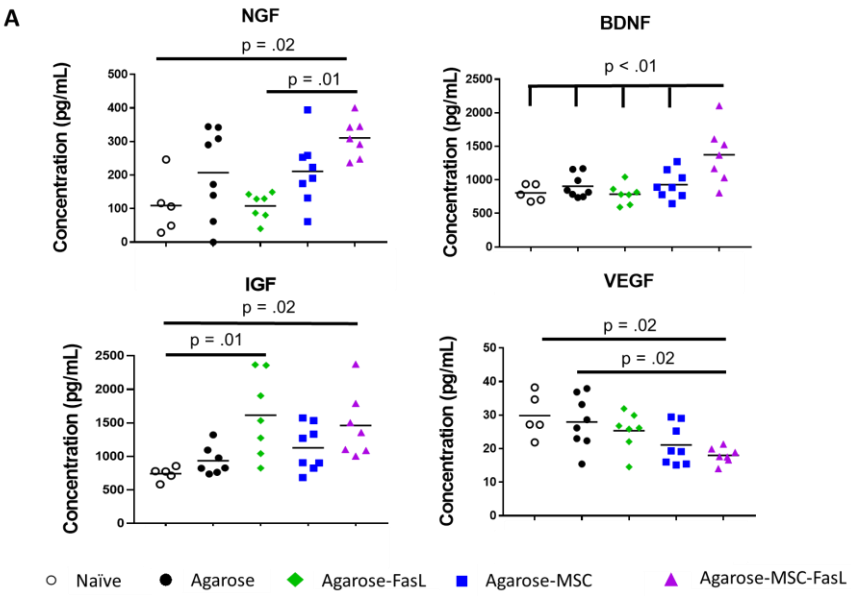
1.13.1 Effect of FasL hydrogels on the growth factors protein expression 2 days post-transplantation.

In order to determine the effect of using FasL-agarose hydrogels as MSC carriers for TBI, we determined the protein expression of the following growth factors: NGF, BDNF, IGF, and VEGF using ELISA (Figure 4.1)(n=8 per experimental group). Agarose-MSC-FasL was the only experimental group in which the NGF protein expression was significantly higher than the Naïve and Agarose-FasL groups according to a Kruskal-Wallis test ($p = .005$) and a Dunn's multiple comparisons test (p values reported in Figure 4.1). In addition, the BDNF protein expression was significantly higher in the Agarose-MSC-FasL group in comparison to all other experimental groups (Naïve, Agarose, Agarose-FasL, Agarose-MSC) according to an ANOVA ($p = 0.0007$) and a Tukey's multiple comparison test (all p values were equal or less than .01). The injection of FasL increased the IGF protein expression as the Agarose-FasL and Agarose-MSC-FasL had significantly higher protein expression than the Naïve group according to a Kruskal-Wallis test ($p = 0.004$) and a Dunn's multiple comparisons test (p values reported in Figure 4.1). The protein expression of VEGF in the Agarose-MSC-FasL was significantly lower than the Naïve and Agarose groups according to an ANOVA ($p = 0.007$) and a Tukey's multiple comparison test (p values reported in Figure 4.1).

1.13.2 Effect of FasL hydrogels on the injury volume and the host neural milieu three weeks post-transplantation.

The effect of MSC transplantation using FasL hydrogels on the volume of the injured region and neuronal degeneration was studied three weeks post-transplantation. MRI

A

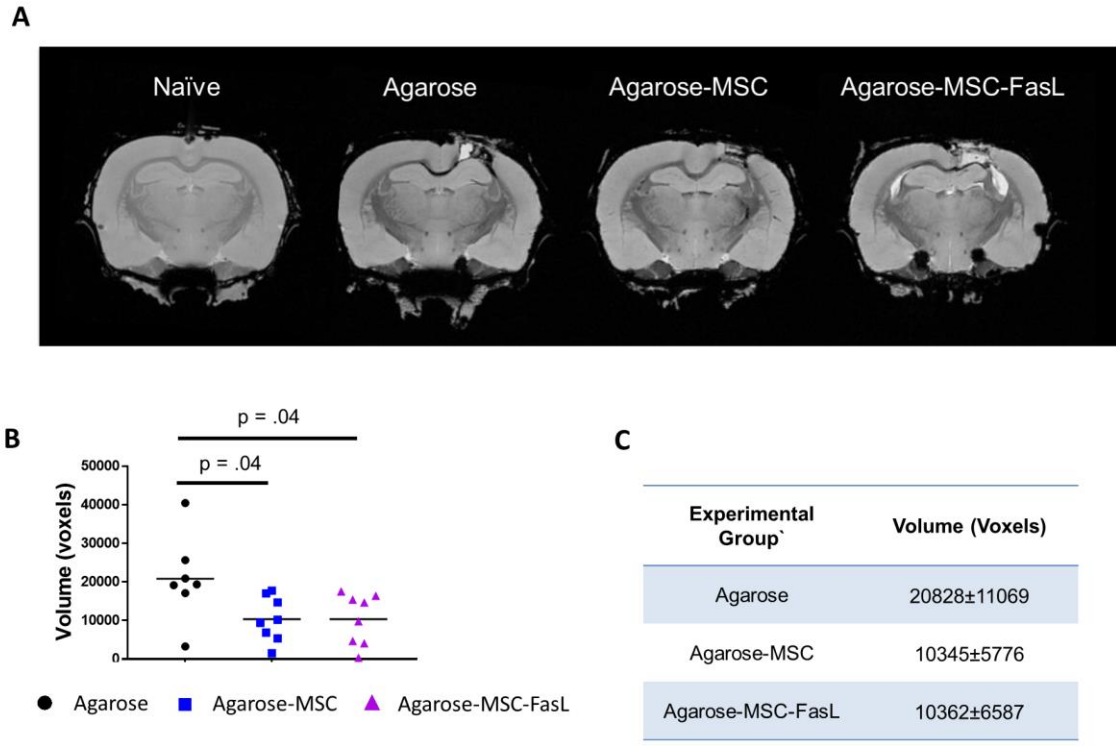


B

Experimental Group	NGF	BDNF	IGF	VEGF
Naive	109.4±85.1	805.3±125	742.3±102.3	29.85±6.55
Agarose	207.4±133.1	905.7±176.9	935.4±211.7	27.96±7.78
Agarose-FasL	108.3±40.0	785.8±149.6	1616±616.4	25.35±5.69
Agarose-MSC	211±98.7	927.5±210.4	1129±341.1	21.11±5.97
Agarose-MSC-FasL	310.5±57.9	1373±427.1	1461±488.1	18±2.36

Figure 4.1: MSC injection using FasL-hydrogels increase the protein expression of NGF and BDNF. A) NGF protein expression in the Agarose-MSC-FasL was significantly higher than the Naïve and Agarose-FasL groups according to a Kruskal-Wallis test (p = .005) and a Dunn's multiple comparisons test (p values reported in the graph). BDNF protein expression was also significantly increased in the Agarose-MSC-FasL in comparison to all other experimental groups according to an ANOVA (p = 0.0007) and a Tukey's multiple comparison test (all p values were equal or less than .01). IGF protein expression was significantly increased in the Agarose-FasL and Agarose-MSC-FasL groups in comparison to the Naïve group according to a Kruskal-Wallis test (p = 0.004) and a Dunn's multiple comparisons test (p values reported in graph). B: Summary of the protein concentration results. VEGF protein expression was significantly reduced in the Agarose-MSC-FasL group in comparison to the Naïve and Agarose groups according to an ANOVA (p = 0.007) and a Tukey's multiple comparison test (p values reported in graph).

imaging was used to determine the volume of the injured region (n=7 for Agarose group, n=8 for Agarose-MSC and Agarose-MSC-FasL groups). MSC transplantation with or without FasL hydrogels (Agarose-MSC and Agarose-MSC-FasL groups) significantly decreased the injury volume in comparison to the control Agarose group according to an ANOVA ($p = .03$) and a Tukey's multiple comparison test (p values reported in Figure 4.2). In addition, the neuronal milieu was examined using multiple cellular markers and histological examination (n=5 for Agarose and Agarose-MSC groups, n = 4 for Agarose-MSC-FasL). The neuronal density within the scar tissue in the injury site and surrounding limits was indirectly determined by calculating the percentage area expressing the neuronal nuclei marker NEUN. The experimental groups injected with MSCs (Agarose-MSC and Agarose-MSC-FasL) did not show a significant increase in the percentage area of $NEUN^+$ tissue in comparison to the Agarose control group according to an ANOVA ($p = .13$). We also analyzed the tissue degeneration around the injury site using an antibody against cleaved poly (ADP-ribose) polymerase (cleaved PARP) as an apoptosis marker. None of the experimental groups injected with MSCs (Agarose-MSC and Agarose-MSC-FasL) showed a significant decrease in the percentage area expressing cleaved PARP in comparison to the Agarose group according to an ANOVA ($p = .3$). We also analyzed the potential effect of MSC transplantation using FasL hydrogels on the recruitment of neural progenitor cells to the injury site by using nestin as a neural stem cell marker. The Agarose-MSC and Agarose-MSC-FasL groups did not show a significant difference in the percentage area of $nestin^+$ tissue according to an ANOVA ($p = .37$). In addition, we did not find any difference in the expression of neuroglial markers GFAP for astrocytes and IBA1 for microglia.



Experimental Group`	Volume (Voxels)
Agarose	20828±11069
Agarose-MSC	10345±5776
Agarose-MSC-FasL	10362±6587

Figure 4.2: MSC injection decreased the size of the injury cavity at 3 weeks post-transplantation. A) Representative MRI images of each experimental group. B) Quantification of the volume of the injured tissue. The injection of MSC with or without FasL significantly decreased the volume of the injured tissue in comparison to the Agarose group according to an ANOVA ($p = .03$) and Tukey's multiple comparisons test (p values reported in graph). C) Summary of the injured tissue volume results.

1.14 Discussion

MSC transplantation after brain injury has been shown to reduce neuronal damage by targeting multiple mechanisms of the secondary injury [13, 85, 86, 89, 194-197]. Multiple factors such as the cell preparation and delivery process, time of transplantation, and dosage could affect the stem cell's survival and therapeutic effect [198-200]. Bone marrow stromal cells and neural stem cells have shown a dose-dependent functional recovery in

stroke models of brain injury [198, 199]. This dosage-dependent therapeutic effect could be due to a higher probability of stem cell engraftment at higher dosages in comparison to lower dosages. In the previous chapter, we found an increased presence of $CD45^{-}$ GFP^{+} MSC after transplantation in the injured brain using FasL-hydrogels. In this chapter, we aimed to determine if the increased presence of MSCs near the transplantation site can result in an enhanced therapeutic effect as measure by protein and histological analysis.

MSCs have been reported to increase the protein expression of neurotrophic and growth factors that reduce cell apoptosis, promote neurogenesis, and alleviate neurological deficits [13, 14, 195, 201, 202]. Thus, we used ELISA assays to determine the protein expression of NGF, BDNF, VEGF, and IGF after MSC transplantation in the injured brain. We found that MSC transplantation using Agarose-FasL hydrogels increased the protein expression of the neurotrophic factors NGF and BDNF while MSC transplantation without FasL failed to do so. Also, delivering FasL alone to the injured brain did not increase NGF or BDNF protein expression suggesting that the increased expression of neurotrophic factors is specifically MSC mediated. The increased expression of NGF and BDNF could lead to increased neuronal survival around the injury site due to the activation of the PI3K-AKT pathway which is involved in cell survival and proliferation [203]. As a result, we could suggest that using FasL hydrogels as MSC carriers enhances the stem cell ability to induce the protein expression of neurotrophic factors, which could reduce neuronal damage around the injury site.

In addition, we found that FasL delivery with or without MSC increased the protein expression of IGF, a polypeptide hormone which delivery to the injured brain has been correlated with reduced neuronal loss and infarct volume, enhanced neurogenesis, and

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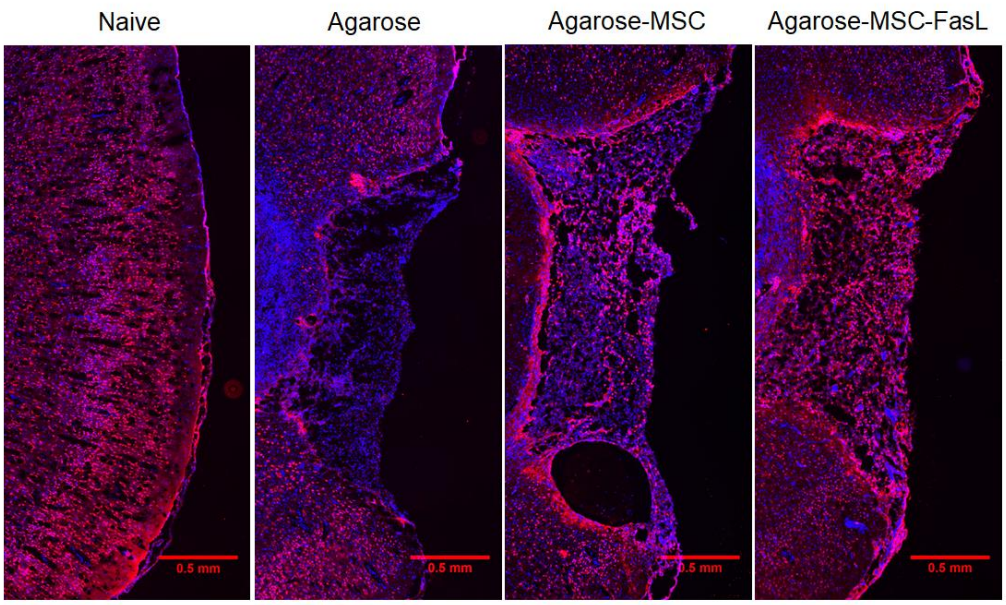

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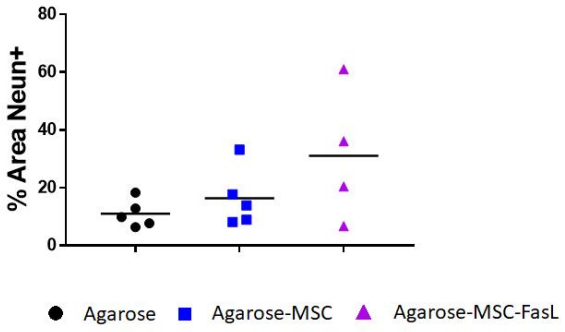

C

Experimental Group	% Area Neun +
Agarose	11.01±4.76
Agarose-MSC	16.36±10.18
Agarose-MSC-FasL	31.04±23.31
Significance	No

Figure 4.3: MSC injection using FasL-hydrogels did not significantly increased NEUN expression. A; Representative image of Neun+ staining at the injury site 3 weeks post-transplantation. B: Quantification of the Area percentage expressing Neun. C: Summary of the quantification results.

improved functional recovery [201, 204]. The FasL mediated increase of the IGF protein expression brings up the question of how FasL delivery into the injured brain is affecting other Fas receptor-expressing host cells in addition to the targeted cytotoxic $CD8^{+}$T cells. Although FasL is commonly associated with cell apoptosis, it has been found to be involved in other cellular processes such as cell differentiation [182, 205, 206]. FasL has been found to have different effects on Fas receptor-expressing neuroglial cells such as astrocytes and microglia [206, 207]. Microglia up-regulates the expression of the Fas receptor under the presence of the cytokines TNF-α and IFN-ϒ and is susceptible to FasL mediated apoptosis [206]. On the contrary, astrocytes express high constitutive levels of the Fas receptor and its activation induce the expression of the chemokine macrophage inflammatory protein-1b (MIP-1) but do not induce cell apoptosis [206]. Interestingly, neural progenitor cells also express the Fas receptor and FasL activation enhances the stem cell's survival [208]. Moreover, Corsini et al found that mice with mutations in the Fas receptor (loss of function) showed reduced neurogenesis and deficits in the working memory [209]. Therefore, the delivery of FasL into the injured brain could be modulating the cellular behavior of unintended targets. It would be interesting to perform an in-depth characterization *in vitro* of the FasL effect on neuronal cells, glial cells, and neural stem cells in the presence of cell stressors such as hypoxia, oxidative stress, or inflammatory cytokines that mimic the injured brain. These studies could help us to determine both the beneficial and detrimental effects of FasL if used as an agent to create localized immunosuppression.

Lastly, Chen et al reported that intravenous administration of MSC induced angiogenesis after transplantation in a stroke model of brain injury [202]. In this project, we did not

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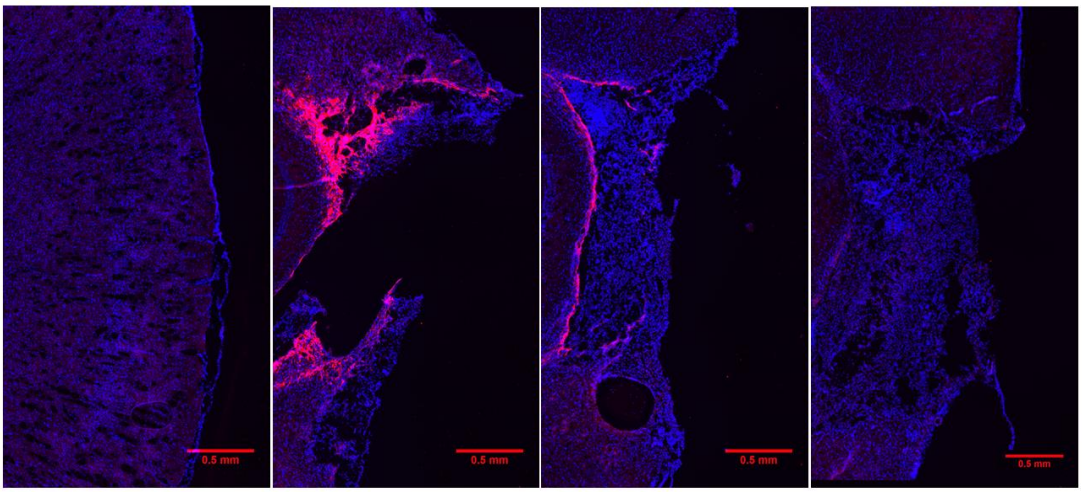

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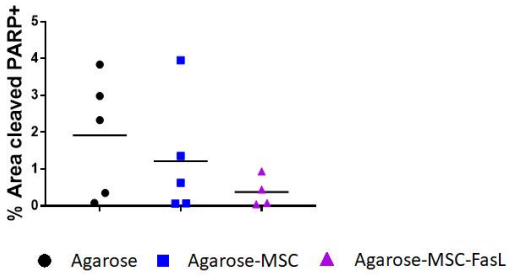

C

Experimental Group`	% Area PARP +
Agarose	1.91±1.64
Agarose-MSC	1.21±1.62
Agarose-MSC-FasL	0.38±0.42
Significance	No

Figure 4.4: MSC injection using FasL-hydrogels did not significantly reduce the expression of the apoptotic marker cleaved PARP. A; Representative image of cleaved PARP+ staining at the injury site 3 weeks post-transplantation. B: Quantification of the Area percentage expressing cleaved PARP. C: Summary of the quantification results.

observe any change in the protein expression of the angiogenic factor VEGF in the animal transplanted with MSC without FasL. This could be due to the different transplantation route (intracerebral) used in our project that could alter the MSC therapeutic mechanism. Injection of FasL alone also did not change the VEGF protein expression in the injured brain. However, VEGF protein expression was unexpectedly reduced in the Agarose-MSC-FasL group in comparison to the Naïve and Agarose groups. Therefore, an unknown interaction between FasL, the transplanted MSC, and/or the transplantation environment is limiting the expression if this angiogenic factor.

In order to determine if the increased NGF and BDNF protein expression translated into a healthier brain milieu after an injury, we examined the injury volume using MRI and explored the expression of multiple cellular markers using histological examination three weeks post-injury. MSC transplantation with and without FasL significantly decreased the volume of the injury cavity at three weeks post-injury suggesting the potential neuroprotective effect of the MSC. No difference was observed between the Agarose-MSC and Agarose-MSC-FasL groups suggesting that the increased MSC survival in the Agarose-MSC-FasL could not induce a greater rescue of the tissue surrounding the injury site. However, it should be noted that in this project we are using a severe injury model and that any neuronal and glial cell destruction caused by the initial impact (primary injury) cannot be reversed. MSC transplantation can only rescue the tissue damage caused by the secondary injury, which could limit the stem cell's maximum potential effect on the injury volume and difficult the analysis of the effectiveness of MSC delivery using FasL-Agarose hydrogels.

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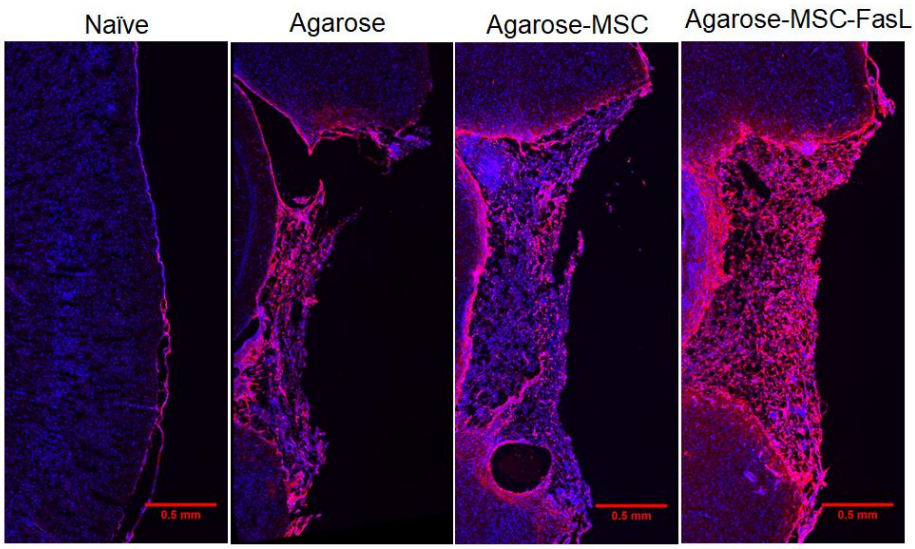

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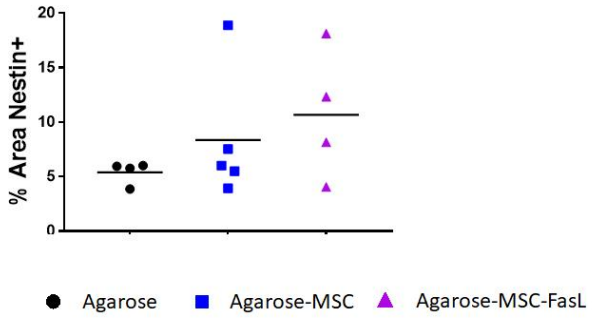


C

Experimental Group	% Area Nestin +
Agarose	5.389±1.03
Agarose-MSC	8.365±6.01
Agarose-MSC-FasL	10.66±6.01
Significance	No

Figure 4.5: MSC injection using FasL-hydrogels did not significantly increase the expression of the neuronal stem cell marker nestin. A; Representative image of nesitn+ staining at the injury site 3 weeks post-transplantation. B: Quantification of the Area percentage expressing nestin+. C: Summary of the quantification results.

We also studied the neuronal degeneration near the injury site by using the neuronal marker Neun and the apoptotic marker cleaved poly(ADP-ribose) polymerase (cleaved PARP). Although the Agarose-MSC-FasL has a higher average of Neun+ tissue within the injury site it was not significantly different. Similarly, the average expression of cleaved PARP in the Agarose-MSC-FasL was not significantly lower than the Agarose group. It has to be noted that in this study we used a 3 weeks post-injury endpoint in order to calculate the injury size using MRI. However, neuronal degeneration and apoptosis markers have been found to have higher expression around 1-7 days post-injury [210, 211]. Therefore, an earlier characterization of neuronal degeneration after TBI might be needed to determine the effect of using FasL hydrogels as stem cell carriers.

In addition, we determined the effect of MSC transplantation using FasL-Agarose hydrogels on the expression of the neural progenitor cell marker nestin, as MSC has been reported to increase neural stem cell survival in a stroke model [194]. However, we did not found a significant difference in the expression of the nestin marker between the experimental groups at 3 weeks post-injury. An important limitation found during the histological examination was the high variability within the groups' variance, which difficulted the statistical analysis. This variability could be related to the controlled cortical injury model, specifically to minor changes in the positioning and baseline of the pneumatic impactor tip during the set up to perform a brain injury. Minor changes in the positioning of the tip could cause large variability due to the potential diverse damage of the blood vessels near the injury site and the potential variability in the injury depth, which in consequence can alter the severity of the molecular response after TBI. Another source of variability in this project is the MSC injection process in which small leakages of the

hydrogel matrix out of the brain could lead to a lower cell and/or FasL concentration within the site of injury and therefore, a variable therapeutic effect. Although the regenerative signals (neuronal death and neural stem cell recruitment) in the histological analysis were not significant, the observed increase in the protein expression of the neurotrophic factors NGF and BDNF, and the reduced volume of the injured region suggest that improving MSC survival after transplantation could enhance its therapeutic effect. However, more histological analysis at earlier endpoints is needed to corroborate this hypothesis. In addition, this project did not address if using FasL hydrogel as MSC carriers could translate to decreased neurological deficits, which is the ultimate functional outcome goal during the design of hydrogels to enhance stem cell survival. Therefore, future experiments should focus on the neurobehavioral characterization of the use of immune-suppressive hydrogel as stem cell carriers.

1.15 Conclusion

In this chapter, we explored if enhancing MSC survival using Fasl-Agarose hydrogels could enhance the MSC therapeutic effect. We found that MSC transplantation using FasL-Agarose hydrogels significantly increased the expression of the neurotrophic factors NGF and BDNF, which suggests a potential enhancement of the therapeutic effect of the MSC. In addition, we found that MSC transplantation with or without FasL reduced the volume size of the injured region which might be related to the higher expression of neurotrophic factors that can enhance the survival of neurons around the injury site [11, 14]. However, we did not found any significant difference in the neuronal or survival according to histological examination using the neuronal marker Neun and the apoptosis marker cleaved PARP at 3 weeks post-injury. We also did not find any significant difference in the

expression of the neural stem cell marker nestin. Therefore, more studies, including an earlier(1-7 days post-injury) histological examination and behavioral testing, should be done to verify if the MSC improved survival found using FasL hydrogels as stem cell carriers can significantly enhance the stem cell therapeutic effect.

CONCLUSIONS AND FUTURE DIRECTIONS

1.16 Key Findings and Conclusions

During TBI, the initial insult triggers multiple cellular mechanisms that create a complex brain environment and propagate the initial damage. Stem cell transplantation, specifically MSC, has been shown to reduce neuronal damage and improve neurological deficits after TBI by modulating multiple aspects of the secondary injury. However, immune cells, infiltrated within the injury site after an injury could potentially limit the MSC survival. Our goal in this project was to explore the role of the T cell response on the MSC survival after transplantation in the injured brain and to determine if T cell suppression could lead to enhance MSC survival. To achieve this, we used FasL releasing hydrogels that induce the apoptosis of CD8+ T cells near the site of MSC transplantation. We showed that FasL-agarose hydrogels enhance the survival of MSC after transplantation in the injured brain whereas the injection of MSC without FasL resulted in the rapid elimination of the transplanted cells. Also, we found that MSC transplantation into the injured brain leads to an infiltration of the general T cell population increase the percentage of the $CD8^+$ cytotoxic T cell population. However, MSC injection using FasL hydrogels was able to reduce the number of $CD8^+$ cytotoxic T cells without compromising the $CD4^+$ T cell population, which could be beneficial to reduce neurological deficits due to the presence of regulatory T cells. MSC transplantation using FasL hydrogels altered the expression of inflammatory cytokines by increasing the protein expression of IL-1β-RA, a cytokine that has been shown to reduce neuronal damage after brain injury due to its anti-inflammatory properties. Therefore, the combination of MSC transplantation with FasL delivery could

be enhancing the MSC therapeutic through the inhibition of the IL-1β pathway. We also explored if enhancing MSC survival using Fasl-Agarose hydrogels could enhance the MSC therapeutic effect. We found that MSC transplantation using FasL-Agarose hydrogels significantly increased the expression of the neurotrophic factor NGF and BDNF, which suggests a potential enhancement of the therapeutic effect of the MSC. In addition, we found that MSC injection with or without FasL reduced the volume size of the cavity injury, suggesting the neuroprotective effect of the MSC. However, we did not found any significant difference in the neuronal survival or density according to histological examination using the neuronal marker Neun and the apoptosis marker cleaved PARP. Therefore, more studies, including behavioral testing, should be done to verify if MSC enhanced survival can significantly enhance the stem cell therapeutic effect.

1.17 Future Directions

1.17.1 Effect of the innate immune response on MSC survival

The MSC healing properties have been explored in multiples scenarios such as cardiac infarction, and brain injury among others. Despite the bidirectional communication between the injured tissue and the transplanted MSC, studies commonly focus on the MSC therapeutic effect on the injured environment without too much emphasis on the environmental effect on the MSC survival or functional phenotype. In this project, we found a rapid clearance of the transplanted MSC as early as 6 days post-transplantation. Although we found that reducing CD8+ cytotoxic T cells near the transplantation site lead to an increased MSC presence, other components of the host immune system could be contributing to the stem cell's survival. Specifically, the host innate immune response might

play an important role in stem cell's survival after early transplantation in the injured brain. The innate immune response is a fast-acting and non-specific response activated immediately after an injury in order to remove foreign agents and pathogens with a limited capacity. The innate immune system includes the activation of the complement cascade and the recruitment of multiple immune cells including cellular effectors such as phagocytes (e.g., neutrophils, macrophages, and dendritic cells), granulocytes (e.g., eosinophils, basophils, mast cells, and neutrophils), and innate lymphoid cells (e.g., natural killer cells) [95]. In addition, within the brain neuroglial cell such as microglia and astrocytes can have immune properties and influence MSC survival. In fact, various studies have shown that MSC grafts in the intact brain are surrounded and/or infiltrated by neuroglial cells and have limited survival [10, 84]. However, multiple other components of the innate immune response could be contributing to the limited MSC survival observed in this project.

The component cascade encompasses more than 30 proteins that are usually circulating the blood as inactive precursors (zymogen) that form a membrane attack complex (MAC) after activation due to an injury or pathogen. This MAC complex can insert into the membranes causing cell lysis [96, 97]. It has been found that the complement cascade induces cellular injury on MSC in vitro and that covalent binding on the MSC surface of the complement inhibitor heparin enhances the stem cell survival in vivo [98, 99]. Another innate component that has been found to affect MSC survival is the natural killer cells (NK cells). Studies have found a cross-modulation between MSC and NK cells. In vitro, IL-2 activated NK cells can induce the generation of reactive oxygen species within MSC and induce stem cell lysis [100]. On the other hand, MSC can alter NK cell's behavior by

reducing the proliferation of resting NK cells, increase their release of perforin and granzymes (degranulation process), and up-regulated their secretion of IFN-γ and TNF-α [100, 101]. Therefore, although MSCs have been shown to have a neuroprotective effect after transplantation in the injured brain, its transplantation could trigger the innate immune response potentially causing unintentional damage.

Except for a few studies, most of the interaction between the MSC and innate system has been studied *in vitro* and none of the studies has been done within a brain injury context. Despite the potential benefits of using stem cell therapy after a brain injury, it is important to determine if a potential activation of the host innate and immune response could be counteracting the therapeutic effect of the transplanted stem cells. Transplantation models that hinder the MSC interactions with specific immune cells without strongly affecting other components of the innate response could provide insight into the potential collateral damage (and activated mechanisms) caused by the MSC transplantation. In order to enhance MSC therapeutic effect within the brain, it will be needed to keep the stem cells neuroprotective effect such as the release of NGF and BDNF while reducing the potential collateral damage caused by the MSC derived activation of the host innate response.

1.17.2 MSC healing phenotype

The environmental cues in an injured environment could not only affect MSC survival but also their functional therapeutic phenotype. It has been found that similar to macrophages, MSC can have alternative pro-inflammatory (MSC1) or anti-inflammatory (MSC2) phenotypes that can be induced by signals in the inured environment [184, 185]. MSC express Toll-like receptors (TLRs, specifically TLR3 and TLR4, that recognize danger

signals from the injured environment and the MSC immune-modulatory properties can be affected by the specific engagement of each receptor. Stimulation of the TLR3 receptor leads to the secretion immune-suppressive factors including IL-1RA while stimulation of the TLR4 receptor leads to the secretion of more pro-inflammatory such as IL-8 and IL-6 [185]. The MSC polarization after transplantation into the injured brain has not been determined. It would be interesting to explore if the injured brain environment can preferentially induce an MSC1 or MSC2 phenotype which could influence the stem cell therapeutic potential. The development of biomaterials that induce and/or sustain the MSC2 anti-inflammatory phenotype could potentially prolong the MSC therapeutic effect and counteract potential collateral damages caused by the host response to the transplanted stem cells.

1.17.3 Multifunctional hydrogels to enhance stem cell transplantation

In addition to the host immune response, stem cell survival after transplantation in the injured brain can be affected by multiple factors such as limited oxygen (hypoxia) and nutrients supply or the development of oxidative stress [137]. Current efforts using hydrogels to enhance stem cell delivery have focused on the development of hydrogel matrixes that incorporate cues from the extracellular matrix (ECM), which can provide signals to enhance the survival, proliferation, and migration of the transplanted stem cells or provide resistance to detrimental signals present in the injured milieu [138, 139]. As an example, hydrogels based on hyaluronan or chondroitin sulfate, two glycosaminoglycans highly abundant in the brain ECM, have been found to enhance the survival of neural stem cells transplanted in the injured brain. In this project, we used agarose based hydrogels that lack the binding sites required to promote stem cell-hydrogel interactions that could result

in the activation of survival mechanisms in the transplanted stem cells. Therefore, it would be interesting to explore the design of hyaluronan or chondroitin sulfate hydrogels that deliver FasL or other immune-modulatory cytokines for stem cell delivery. The development of multifunctional hydrogels that can increase stem cell survival by providing survival signals to the transplanted stem cells while simultaneously modulating the host immune response could be the next step to enhance stem cell therapy. In addition, multifunctional hydrogels could be engineered to enhance stem cell efficacy by controlling stem cell differentiation (promoting an undifferentiated state) or directing the stem cells toward specific functional phenotypes associated with wound healing or tissue regeneration [119, 185].

APPENDIX A. SUPPLEMENTARY DATA

A.1 Hydrogels releasing multimeric FasL induced the apoptosis of Jurkat T cells.

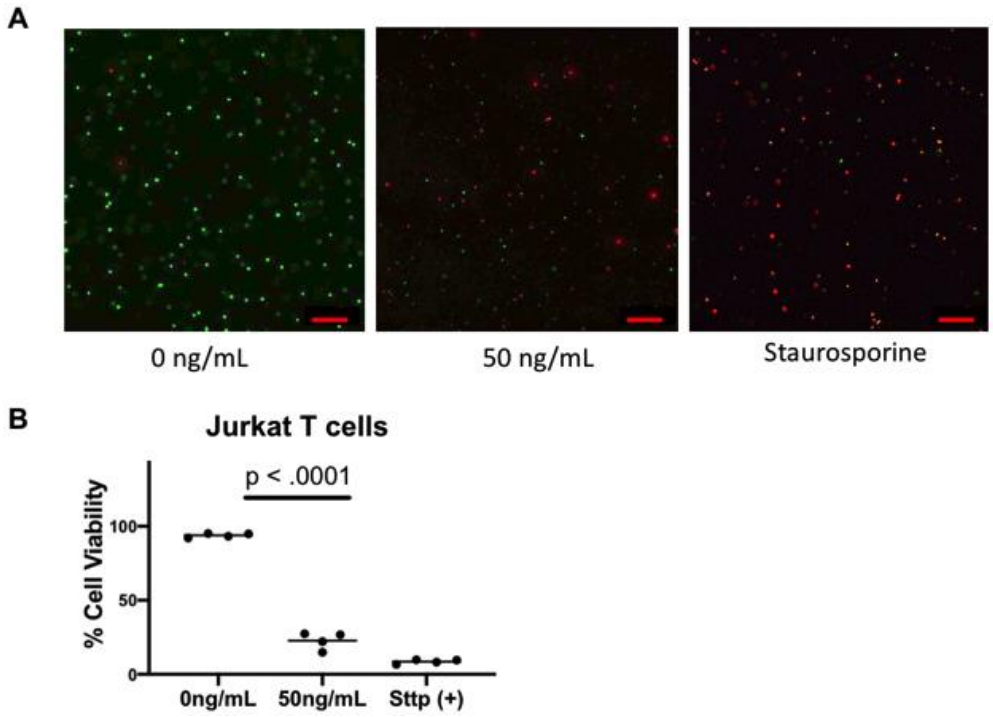


Figure A.1: Multimeric FasL efficiently induce the apoptosis of Jurkat T cell encapsulated in agarose hydrogels. A. Representative images showing the survival of Jurkat T cell encapsulated on hydrogel releasing multimeric FasL. Scale bar = 100um. B Quantification of the of Jurkat T cell viability on FasL releasing hydrogels. A general Anova showed a significant difference between the experimental groups ($p < .0001$) and a Tukey's multiple comparison test was used for the p value reported on the graph.

The ability of solubilized multimeric FasL to induce the apoptosis of Jurkat T cells was tested in vitro using a calcein Am/ ethidium homodimer (live/death cells) staining. 100,000 Jurkat T cells were encapsulated on 300uL of an Agarose hydrogel with or without multimeric FasL (50ng/mL)(n=4 per experimental group). The toxin Staurosporine was used as a positive control. After 24 hours a calcein Am/ethidium homodimer was

performed and the hydrogels were imaged using confocal microscopy. Soluble multimeric FasL significantly reduced the Jurkat T cell viability in comparison to the control group according to an ANOVA ($p < .0001$) and a Tukey's multiple comparison test ($p < .0001$).

A.2. MSC survival *in vitro*

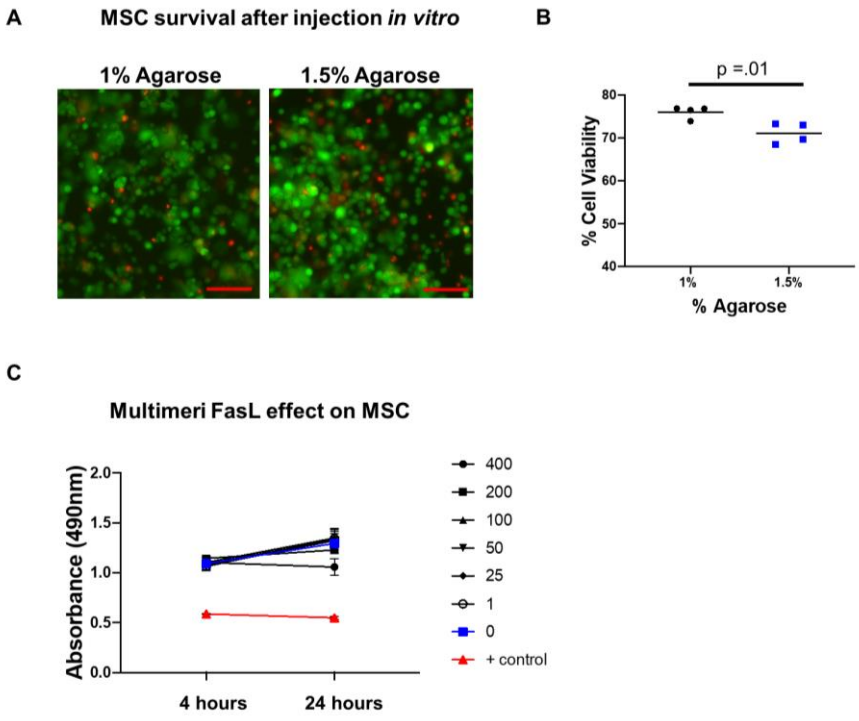


Figure A.2: Multimeric FasL did not reduce MSC viability *in vitro*. A: Representative image of the MSC viability after injection *in vitro* (green represent calcein AM, red represent ethidium homodimer). B: Quantification of the effect of the agarose percentage on the MSC viabililty. MSCs injected using 1.5% agarose had significantly lower viability according to an unpaired t-test ($p= .01$). C: Quatification of the effect of FasL concentration on MSC viability using an MTS assay. Higher concentrations of FasL (400ng/mL) inhibited MSC proliferation in comparison to lower concentrations (1ng/mL-100ng/mL) according to a two-way repeated measure ANOVA ($p<.0001$) (see paragraph below for additional statistical information).

We mimicked the *in vivo* parameters used to inject the MSC into the injured brain to determine the effect of the injection process on the MSC survival *in vitro*. MSCs ($5x10^{\wedge 5}$ total cells) were injected into an agarose hydrogel (to mimic brain tissue) using a 50uL Hamilton syringe with a 26-gauge needle at a rate of 2 uL per minute. The syringe was cooled using ice for 3 minutes before the injection in order to initiate the agarose gelling process. Then, the MSCs were stained using a calcein am/ethidium homodimer cell viability kit and imaged using fluorescence microscopy. We found that that increasing the agarose from 1% to 1.5% significantly decreased the cell viability from 76% to 71% according to an unpaired t-test (p=.01). However, due to the negligible biological difference (5% decrease), we used 1.5% agarose hydrogels for the *in vivo* in order to maximize the probability of prolonging FasL protein release in the injured brain.

The effect of multimeric FasL on the MSC's viability was examined using an MTS assay that measures the cell metabolic activity. MSCs ($4x10^{\wedge 4}$ per well) were seeded in a 96-well plate and exposed to various concentrations of FasL (0, 1, 25, 50, 100, 200, and 400 ng/mL) and the cells metabolic activity was measured at 4 and 24 hours post-exposure. Staurosporine was used as a positive control in order to induce cell apoptosis. At 4 hours we did not find any significant difference in the MSC metabolic activity. At 24 hours the MSC exposed to 400ng/mL of FasL showed significantly lower metabolic activity than the other experimental groups (0, 1, 25, 50, 100, 200 ng/mL) according to a two-way repeated-measures ANOVA (p= .0002) and a Tukey's multiple comparison test (all p values equal or lower than .01). However, the cell's metabolic activity in the 400ng/mL experimental group was similar at 4 and 24hours suggesting than the FasL exposure did not necessarily cause cell apoptosis but inhibited cell proliferation. These results are consistent with the

literature in which has been reported that high FasL concentrations can inhibit MSC proliferation despite the MSC resistance to FasL mediated apoptosis [182].

A.3 Original data of MSC survival after transplantation in the injured brain.

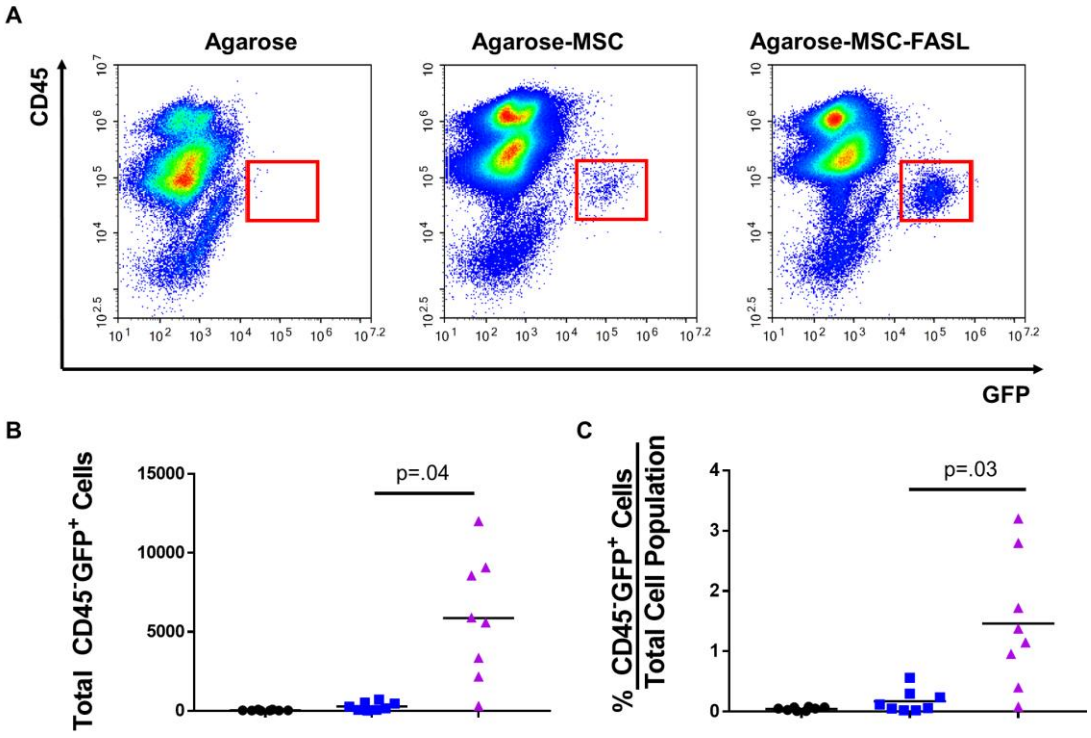


Figure A.3: FasL hydrogels increase MSC presence after transplantation into the injured brain. A. Representative flow cytometry data showing the effect of FasL-hydrogels on the survival of the transplanted GFP^+ MSC (red square) at 6 days post transplantation B: Quantification of the total $CD45^-$ GFP^+ MSC cell population. The Agarose-MSC-FasL group showed significantly higher GFP+MSC presence near the transplantation in comparison to the Agarose-MSC group according to a Kruskal-Wallis test (p= .0003) and a Dunn's multiple comparisons test (p=.04). C: Quantification of the normalized percentage of $CD45^-GFP^+$ MSC cell population with respect to the total number of cells analyzed. The Agarose-MSC-FasL group showed significantly higher of the normalized percentage of GFP+MSC presence near the transplantation in comparison to the Agarose-MSC group according to a Kruskal-Wallis test (p= .0009) and a Dunn's multiple comparisons test (p=.03).

A.4. Effect of Lipid Microtubes on the MSC survival *in vivo*.

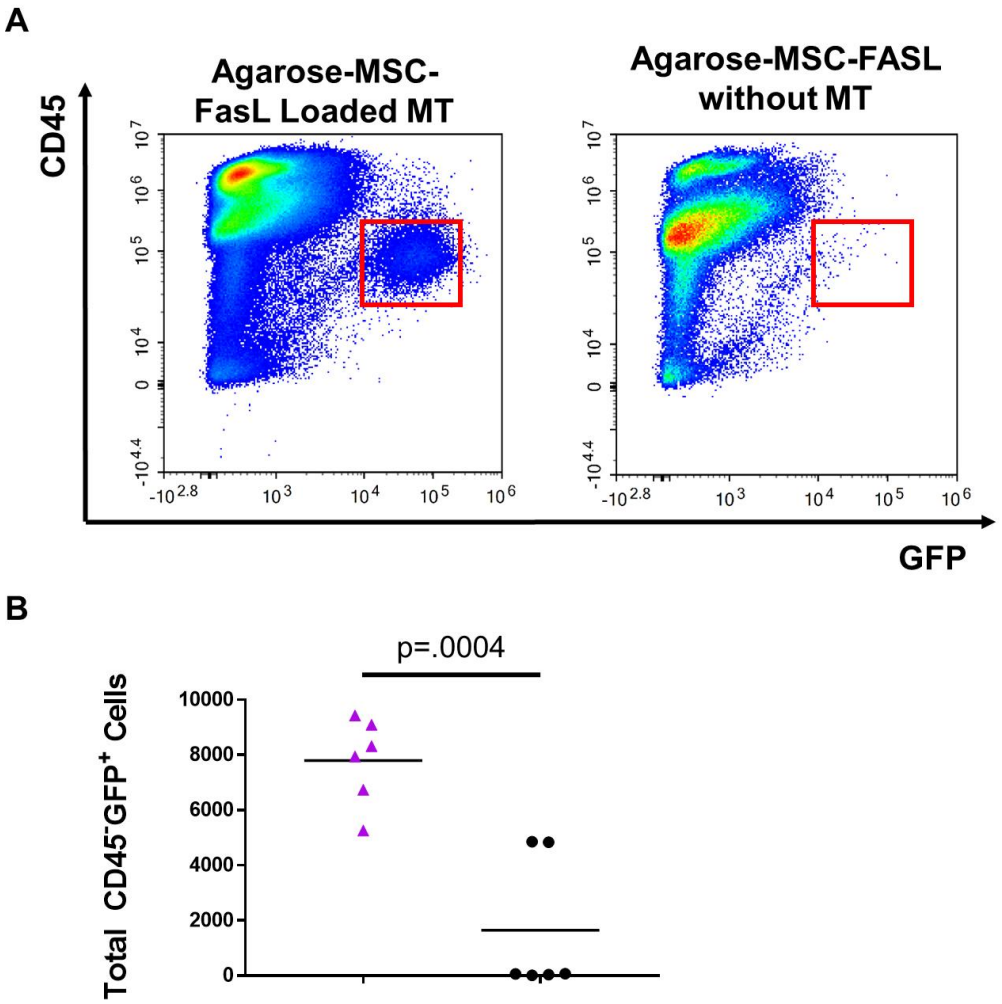


Figure A.4: FasL loaded Lipid microtubes/agarose hydrogels increase the presence of CD45$^-$GFP$^+$ MSC at 6 days post-injury. A. Representative flow cytometry data showing the effect of Lipid microtubes/agarose and agarose hydrogels on the survival of the transplanted GFP+ MSC (red square) at 6 days post transplantation B: Quantification of the total CD45$^-$ GFP$^+$ MSC cell population. FasL loaded lipid microtubes/agarose hydrogels significantly increased the presence of CD45-GFP+ MSC in comparison to FasL agarose hydrogels without lipid microtubes according to a unpaired t-test (p = .0004).

In chapter three, we showed that FasL loaded lipid microtubes/agarose hydrogels increased the presence of GFP^+MSCs near the transplantation site in comparison to lipid microtubes/agarose hydrogels without FasL. However, the effect of the lipid microtubes on the FasL effect *in vivo* was not determined. The use of lipid microtubes should slow FasL release from the agarose hydrogels resulting in potentially longer bioactivity and longer MSC survival. In order to examine if using lipid microtubes alter FasL effectiveness *in vivo*, we determined the survival of GFP^+MSCs transplanted into the brain using FasL loaded lipid microtubes/agarose hydrogels or FasL loaded agarose hydrogels (no lipid microtubes) (n=6 per experimental group). The animals injected with GFP^+MSC using FasL loaded lipid microtubes/agarose showed a higher presence of CD45-GFP+ MSCs at 6 days post-transplantation in comparison to the animals injected with FasL agarose hydrogels (no lipid microtubes) according to an unpaired t-test (p=.0004). This result suggests that using lipid microtubes helps to promote the sustained release of FasL and/or to prolong the protein bioactivity which leads to an increased presence of GFP^+MSC near the transplantation site.

A.5. Effect of Lipid Microtubes on the host T cell response.

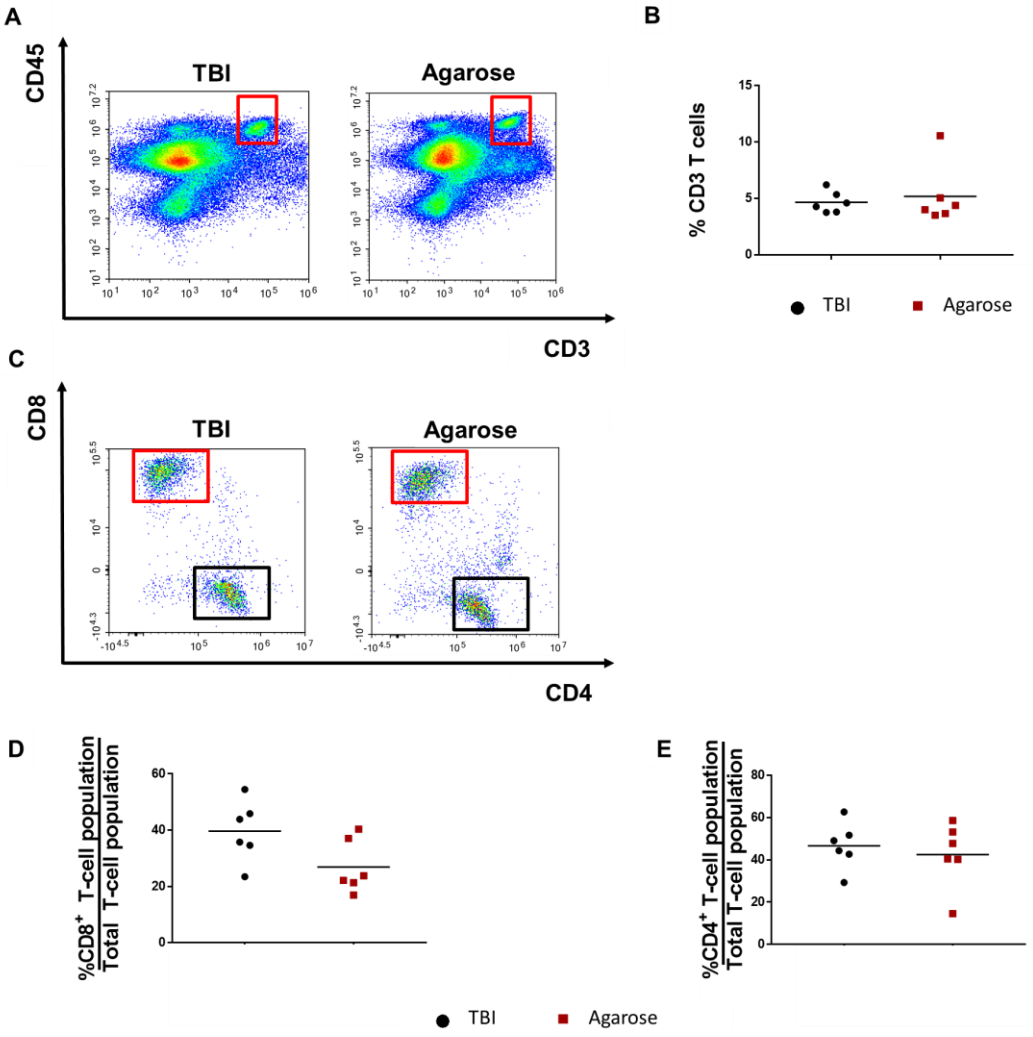


Figure A.5: Lipid microtubes/agarose hydrogels did not alter the host T cell response after transplantation in the injured brain. A. Representative flow cytometry data showing the effect of lipid microtubes/agarose hydrogels on the host general T cell response ($CD3^+$ T cells). B: Quantification of the percentage of $CD3^+$ T cells. No significant difference was found according to an unpaired t-test with Welch's correction ($p = .66$). C: Representative flow cytometry data showing the effect of lipid microtubes/agarose hydrogels on the T cell phenotypes (red square represent the $CD8^+$ cytotoxic T cell population, black square represent the $CD4^+$ T helper/regulatory T cell population). D: Quantification of the percentage of $CD8^+$ cytotoxic T cells. No significant difference was found according to an unpaired t-test ($p = .05$). E: Quantification of the percentage of $CD4^+$ T helper/regulatory T cell population. No significant difference was found according to an unpaired t-test ($p = .60$).

In this project, we used an agarose-based hydrogel matrix to simultaneously deliver rat MSC and FasL in the injured rat brain. The injection of a hydrogel matrix into the brain could potentially induce an immune response due to the hydrogel's inherent properties and/or the small injury caused by the injection procedure. We tested if injecting lipid microtubes/agarose hydrogels into the injured brain elicited an immune response in the injured brain by using flow cytometry at 6 days post-transplantation (Figure A.5) (n=6 per experimental group). We did not find a significant difference in the host T cell response of animals injected with lipid microtubes/agarose hydrogels in comparison to the animals with a brain injury without any injection. The results suggest that the lipid microtubes/agarose hydrogels properties did not elicit an immune response after transplantation making this hydrogel a suitable alternative for the MSC delivery in the injured brain.

A.6. Expression of neuro-inflammatory markers in the injured brain at 3 weeks post-injury.

A.6.1 GFAP expression in the injured brain at 3 weeks post-injury.

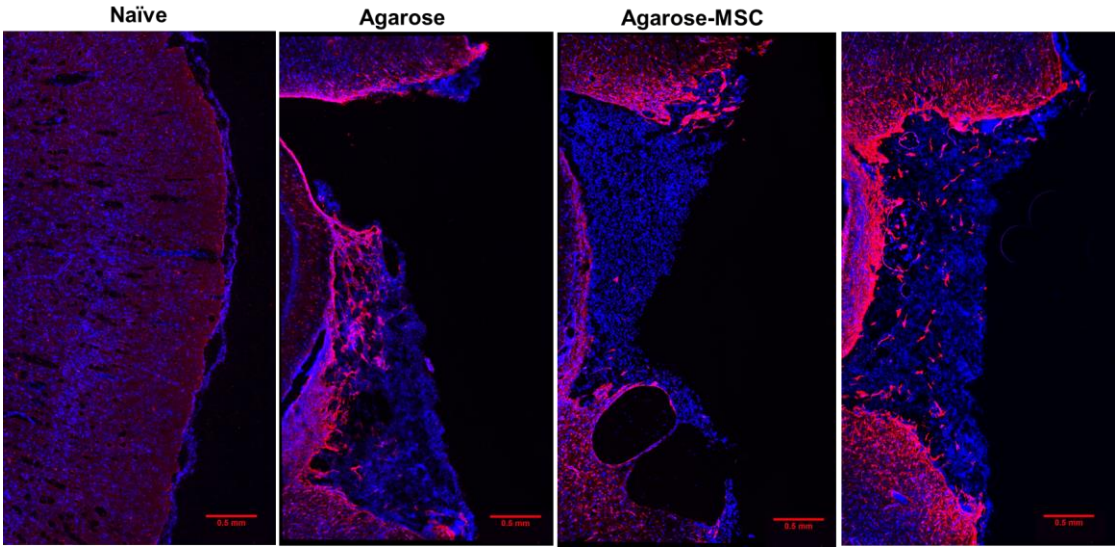


Figure A.6.1: MSC transplantation using FasL hydrogels did not alter GFAP expression in the injured brain at 3 weeks post-injury. A. Representative images of GFAP expression at 3 weeks-post-injury.

The expression of the astrocyte marker GFAP was examined 3 weeks post-injury (n=5 for the Agarose and Agarose-MSC groups, n=4 for the Agarose-MSC-FasL group). A qualitative analysis showed limited staining of the GFAP marker within the injury site and surrounding tissues. Most of the animal subjects in each experimental group with a brain injury (Agarose, Agarose-MSC, Agarose-MSC-FasL) showed similar GFAP expression suggesting a similar astrocyte response.

A.6.2 IBA-1 expression in the injured brain at 3 weeks post-injury.

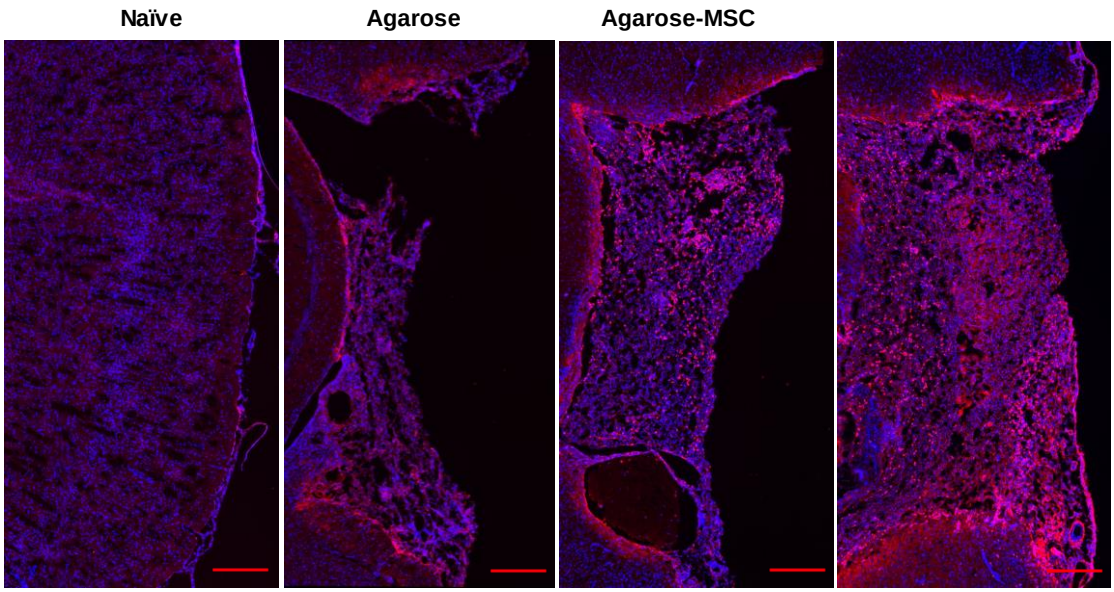
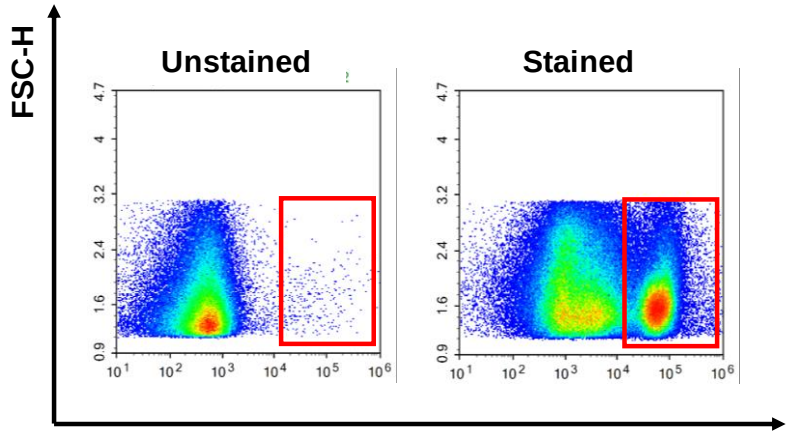


Figure A.6.2: MSC transplantation using FasL hydrogels did not alter IBA-1 expression in the injured brain at 3 weeks post-injury. A. Representative images of GFAP expression at 3 weeks-post-injury.

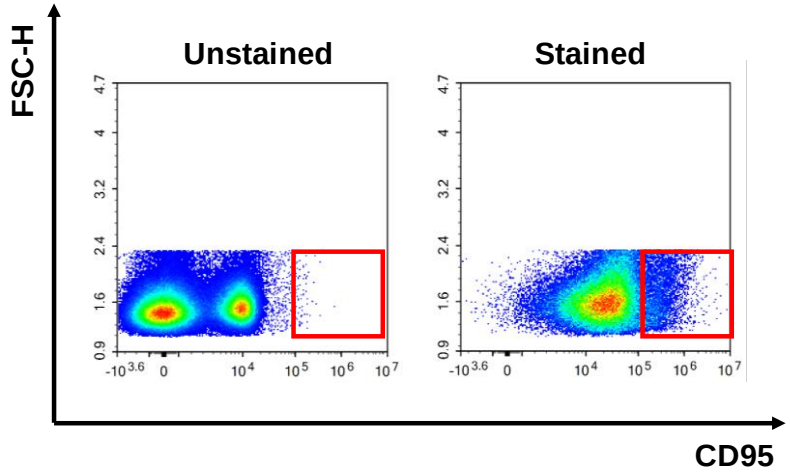
The expression of the microglial marker IBA-1 was analyzed 3 weeks post-injury (n=5 for the Agarose and Agarose-MSC groups, n=4 for the Agarose-MSC-FasL group). A qualitative analysis showed some staining of the IBA-1 marker within the injury site and surrounding tissues. Animal subjects in each experimental group with a brain injury (Agarose, Agarose-MSC, Agarose-MSC-FasL) showed similar expression of the IBA-1 marker suggesting a similar microglial response.

A.7. FMO stainings used to set gates in the T-cells gating strategy

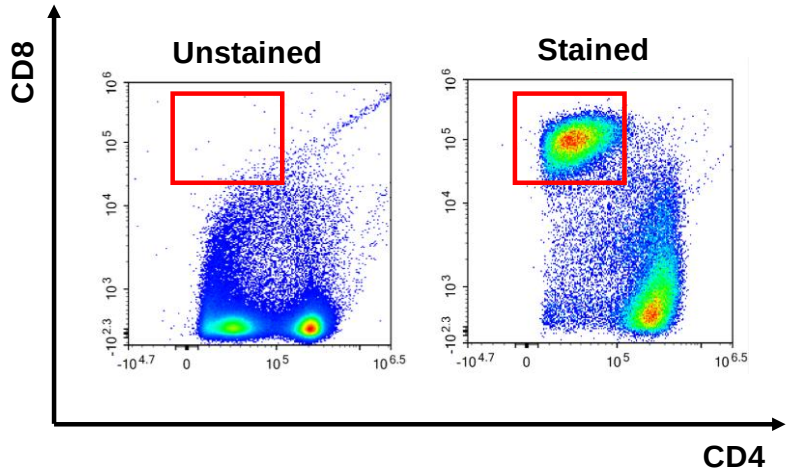
CD3 FMO



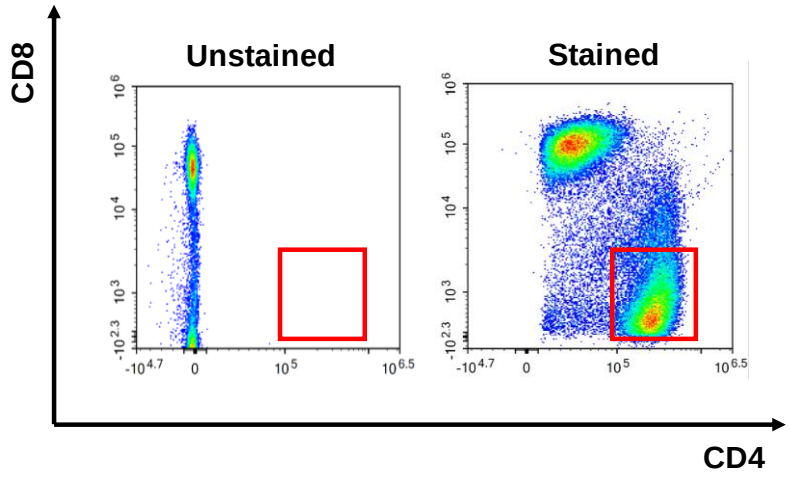
CD95 FMO



CD8 FMO



CD4 FMO



A.8. Primers sequences (qRT-PCR)

Name	**Sequence 5'-3'**
IFNg-F	GATCCAGCACAAAGCTGTCA
IFNg-R	GACTCCTTTTCCGCTTCCTT
IL-4-F	TGTACCTCCGTGCTTGAAGA
IL-4-R	GTGAGTTCAGACCGCTGACA
IL-12-F	AGGTGCGTTCCTCGTAGAGA
IL-12-R	CCATTTGCTGCATGATGAAT
IL-1b--F	AAAGAAGAAGATGGAAAAGCGGTT
IL-1b-R	GGGAACTGTGCAGACTCAAACTC
Il-1RA-F	GAGACAGGCCCTACCACCAG
IL-1RA-R	CGGGATGATCAGCCTCTAGTGT

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