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## Abstract

Stroke is the second leading cause of death worldwide and the third leading cause of adult disability in adults. Ischemic stroke triggers an inflammatory response in the brain that is cytotoxic. In response to ischemic stroke, T-cells from mobilize to the brain and modulate both cytotoxic and protective inflammation. Regulatory T ( $T_{reg}$ )-cells exert a neuroprotective effect after ischemic stroke by inhibiting both inflammation and cytotoxic T-cell activation. Transplantation of bone marrow-derived stem cells (BMSCs) after ischemic stroke has a neuroprotective effect. One way that BMSCs protect neurons from apoptosis is by attenuating innate inflammation, but response of the adaptive immune system has not been well-studied. Our lab has found that implanted stem cells accumulate in locations with known importance to the adaptive immune system like the spleen. In this study, regulatory T-cells and BMSCs were shown to be neuroprotective following ischemic treatment of primary rat neurons.

## Introduction

The timeline following ischemic occlusion of brain tissue is characterized by a phased response of neuronal cell death, inflammation, and injury resolution. Neurons in the infarct zone undergo apoptosis and necrosis, releasing the cell contents into the parenchyma. Excitatory neurotransmitters are released and depolarize neurons in the peri-infarct region, which triggers cellular cascades that eventually leads to further cell death via apoptosis. New treatments aim to rescue the cells in the peri-infarct region.

Stem cells as therapy for stroke has been recently studied as an adjunct to current treatments. Stem cells exert their beneficial effect by attenuating inflammation and promoting neurogenesis<sup>4</sup>. One of the putative mechanisms by which stem cells confer neuroprotection after stroke is by modulating the endogenous immune system<sup>5</sup>. It is not known, however, exactly how regulatory T-cells are affected by the presence of stem cells. This study aims to examine the interplay between regulatory T-cells and BMSCs in neuroprotection of primary rat neuron cells (PRNCs) following oxygen-glucose deprivation and re-perfusion (OGD/R) *in vitro*. It is hypothesized that regulatory T-cells and BMSCs will be neuroprotective in an *in vitro* ischemic stroke model, and their neuroprotective effects will be complementary.

## Methods and Materials

### Regulatory T-Cell Isolation

Regulatory T-cells were harvested from spleens donated from healthy, wild-type mice. Regulatory T-cells were isolated by magnetic sorting as described in previous publication<sup>6</sup>. Briefly, splenic tissue was dissociated manually and a single cell suspension was filtered out. Anti-CD4 and CD25 antibodies were used to label regulatory T-cells and then they were conjugated with magnetic microbeads. Magnetically labeled cells were isolated by passing the cell suspension through a column containing magnetic metal substrate.

### Cell Culture

PRNCs were cultured as described previously<sup>7</sup>. Briefly, PRNCs were suspended in 400  $\mu$ L Neural Medium without antibiotic in poly-L-lysine coated 8-well plates. After three days cell culture, the cells were subjected to an oxygen-glucose deprivation and reperfusion condition for 90 minutes<sup>7</sup>. The cells were re-perfused and co-cultured with T-cells and BMSCs for three days.

### Cell Viability Assessment

Cells were fixed in paraformaldehyde and immediately labeled with live-cell nuclear stain (Hoechst) and imaged under a fluorescent microscope and counted.

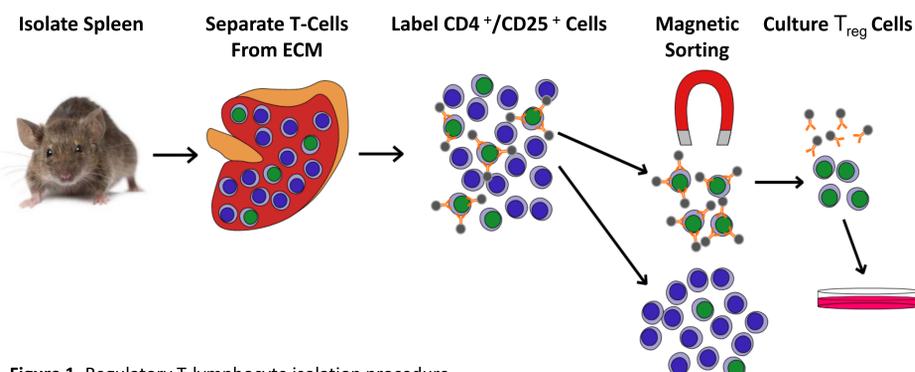


Figure 1. Regulatory T-lymphocyte isolation procedure.

## Results

Regulatory T-lymphocytes were successfully isolated from whole mouse spleens and co-cultured with PRNCs exposed to ischemic conditions. No sign of contamination was appreciated at any stage of cell culture. Neurons prior to OGD/R grew axons and dendrites, which were visualized under bright-field microscope. After OGD/R and three days co-culture, the neurons appeared more sparse and with fewer cellular extensions.

An average of 42% of PRNCs survived the OGD/R treatment, a significant ( $p < 0.005$ ) reduction from PRNCs in the normoxic condition. In comparison, 84% of PRNCs cultured with BMSCs after OGD/R survived, a significant ( $p < 0.005$ ) improvement to the OGD/R control. Similarly, 66% of PRNCs survived OGD/R when cultured with regulatory T-cells, a significant ( $P < 0.05$ ) improvement compared to the OGD/R control. PRNCs cultured with both BMSCs and regulatory cells survived OGD/R at a rate of 56%.

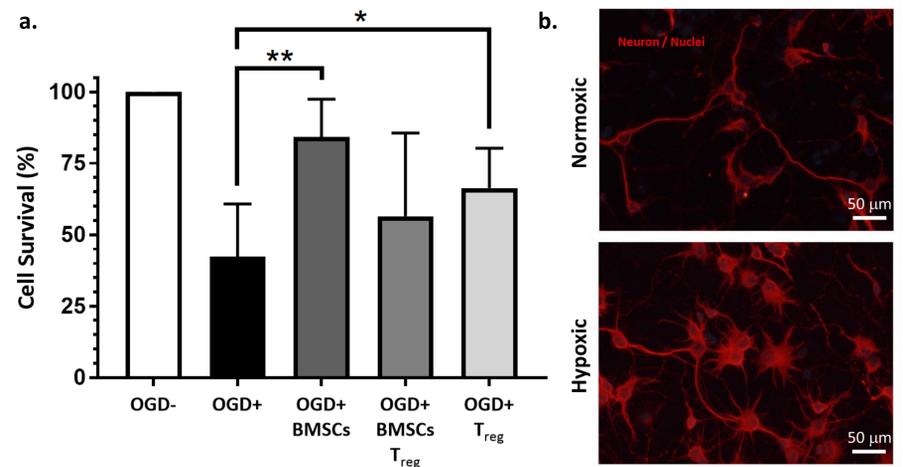


Figure 2. a) Cell viability after OGD/R and co-culture with BMSCs and/or regulatory T-cells. b) Immunocytochemistry showing morphological differences between neurons exposed to OGD and healthy neurons. (\*  $p < 0.05$ , \*\*  $p < 0.005$ )

## Discussion

Primary rat cortical cells were protected from ischemic conditions in co-culture with regulatory T-cells. These data suggest a neuroprotective role for regulatory T-cells, which is likely due to immunomodulation mediated by astrocytes. However, the double co-culture of regulatory T-cells and BMSCs did not produce an augmentation of neuroprotection.

It is possible that maximal effect of regulatory T-cells will be time-dependent, so future studies will examine the relationship of  $T_{reg}$ -cell inoculation time and degree of neuroprotection. Or, it may be that the necessary cell-types were not present in culture. Regulatory T-cells are known to directly and indirectly modulate proliferation and activation of B and T lymphocytes. Future studies will examine the interplay between different T-cell populations including cytotoxic T ( $CD8^+$ ), helper T ( $CD4^+$ ), and B lymphocytes.

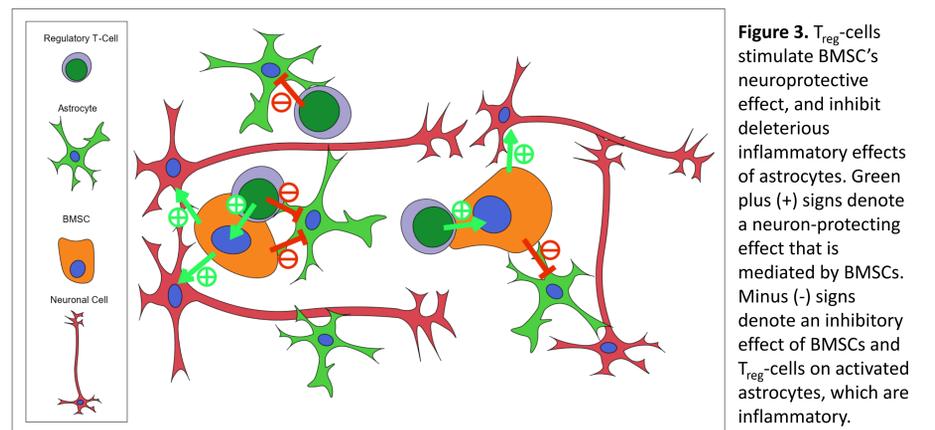


Figure 3.  $T_{reg}$ -cells stimulate BMSC's neuroprotective effect, and inhibit deleterious inflammatory effects of astrocytes. Green plus (+) signs denote a neuron-protecting effect that is mediated by BMSCs. Minus (-) signs denote an inhibitory effect of BMSCs and  $T_{reg}$ -cells on activated astrocytes, which are inflammatory.

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