Transplantation of mesenchymal stem cells overexpressing interleukin-10 induce autophagy response, promote neuroprotection in a rat model of TBI

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Journal of Cellular and Molecular Medicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>JCMM-01-2019-061.R2</td>
</tr>
<tr>
<td>Wiley - Manuscript type:</td>
<td>Original Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Maiti, Panchanan; Central Michigan University, Psychology and Neuroscience Program; Field Neurosciences Institute, Neurology Peruzzaro, Sarah Kolli, Nivya Andrew, Melissa Al-Gharaibeh, Abeer Rossignol, Julien; College of Medicine, Central Michigan University, Dunbar, Gary; Psychology, Central Michigan University,</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Traumatic brain injury, neuroinflammation, mesenchymal stem cell, autophagy, interleukin-10, mitophagy</td>
</tr>
</tbody>
</table>
Transplantation of mesenchymal stem cells overexpressing interleukin-10 induce autophagy response, promote neuroprotection in a rat model of traumatic brain injury

Authors: Panchanan Maiti¹,²,³,⁴,⁵,⁶*, Sarah Peruzzaro¹,², Nivya Kolli¹,², Melissa Andrews¹,², Abeer Al-Gharaibeh¹,², Julien Rossignol¹,²,⁷, Gary L. Dunbar¹,²,³,⁴*

¹Field Neurosciences Institute of Laboratory for Restorative Neurology, Central Michigan University, Mt. Pleasant, MI 48859, USA
²Program in Neuroscience, Central Michigan University, Mt. Pleasant, MI 48859, USA
³Department of Psychology, Central Michigan University, Mt. Pleasant, MI 48859, USA
⁴Field Neurosciences Institute, St. Mary’s of Michigan, Saginaw, MI 48604, USA
⁵Department of Biology, Saginaw Valley State University, Saginaw, MI 48610, USA
⁶Brain Research Laboratory, Saginaw Valley State University, Saginaw, MI 48610, USA
⁷College of Medicine, Central Michigan University, Mt. Pleasant, MI 48859, USA

*Address correspondence: Panchanan Maiti. Email: maiti1p@cmich.edu, Ph: 9894973026 or Gary L. Dunbar: dunba1g@cmich.edu, Ph: 9894973105
Abstract:

Autophagy, including mitophagy play vital role in neuroprotection in traumatic brain injury (TBI). Transplantation of mesenchymal stem cells (MSCs) provide neuroprotection and induce autophagy by increasing anti-inflammatory cytokines, such as interleukin-10 (IL-10). Due to heterogeneity, it is difficult to measure the levels of IL-10 released by MSCs. We developed genetically engineered MSCs to overexpress IL-10 to deliver greater amount of IL-10. This study investigated the comparative effects of transplantation of MSCs-IL10 and MSCs alone in a rat model of TBI. Adult, male Sprague-Dawley rats were divided into four groups: Sham + vehicle, TBI + vehicle, TBI + MSCs-IL-10 and TBI + MSCs-GFP. First two groups received vehicle, whereas last two groups were transplanted with MSCs-IL-10 or MSCs-GFP, after 36-h of TBI. Following 3 weeks of transplantation, neurodegenerative changes, autophagy, mitophagy, chaperone-mediated autophagy (CMA), cell death/survival markers were investigated. We observed a significant increase number of dead cells in the cortex and hippocampus in TBI rats, whereas transplantation of MSCs-IL-10 significantly reduced their numbers. MSCs-IL-10 group induced autophagy, mitophagy, CMA and restored cell survival markers, along with decreased cell death and neuroinflammatory markers greater than MSCs alone. Transplantation of MSCs-IL10 may be an effective strategy to protect against TBI-induced neuronal damage.

Key words: TBI, neuroinflammation, IL-10 overexpressed MSCs, autophagy markers.
1. Introduction

Traumatic brain injury (TBI) is a serious and debilitating health problem affecting millions of people each year [1, 2]. A sudden impact to the head, such as from a fall, car accident, or a blast are the main events that results in a TBI [2, 3], which cause moderate to severe brain tissue damage, lead to motor, sensory, psychological and cognitive dysfunctions [4]. Importantly, the damage may last for months, years, or even the rest of the person’s life [5, 6].

Although several studies have been conducted to investigate the pathophysiology and molecular events involved in neuronal injury in TBI [7-9], it is still not clearly understood. However, inflammation is the main cause for neuronal damage in TBI, due to activation of brain immune cells [10, 11]. Interestingly, during TBI, M1-subtype of microglia become activated and release significant amount of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), IL-1β, IL-6, IL-12 [12], and reactive oxygen species (ROS), which damage the blood brain barrier (BBB) [13] and triggers neuronal injury [14]. In contrast, M2-subtype of microglia release anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, tumor growth factor (TGF) that promotes wound healing and reduction of neuroinflammation [15]. The IL-10 secreted by M2-microglia, mesenchymal stem cells (MSCs), and by some neurons [16] which regulate immune responses and provide cytoprotection. It directly inhibits production and release of pro-inflammatory cytokines [17], inhibits astrocyte activation and increases expression of excitatory amino acid transporter-2 (EAAT2), thus reducing the glutamate excitotoxicity [18], as noted in animal models of spinal cord injury [19], stroke, and TBI [17].

Several research reports suggested that MSCs transplantation play beneficial roles in the treatment of TBI [20], because they can release anti-inflammatory chemokines and reduce neuronal injury [21]. However, due to its heterogenous population it is difficult to determine to
what extent a population of MSCs may release these anti-inflammatory cytokines [22]. Therefore, making genetic engineered MSCs to overexpress IL-10 may provide greater delivery of IL-10 in the injured brain areas to prevent further neurodegeneration [23, 24]. Genetically engineering MSCs to overexpress IL-10 has been shown to reduce inflammation in collagen-induced model of arthritis, graft-versus-host disease, experimental autoimmune encephalomyelitis, and ischemia-reperfusion injury in the lung [25, 26]. Similarly, recently, we have revealed that the transplantation of MSCs genetically engineered to overexpress interleukin-10 (MSCs-IL-10) promote conversion of M1 to M2 macrophages, thus increased anti-inflammatory cytokines and inflammatory responses in rat model of TBI [24].

In addition, previously, we have also performed Morris water maze (MWM), ladder rung walking, and rotarod tasks to assess cognitive function, stepping and inter-limb coordination, gross locomotor performance, respectively after transplantation of MSCs + IL-10 and MSCs alone in TBI rats. Although we did not find significant improvement in learning in the acquisition phase of the MWM task, however, the mean latency was within the range of uninjured rats in the case of TBI + MSCs + IL-10 transplanted rats during the reversal sessions, suggesting transplantation of MSCs + IL-10 improved learning and memory in TBI rats. Similarly, we also found that transplantation of MSCs + IL-10 or MSCs alone significantly improved in fine motor function on the ladder rung walking test, but not on the rotarod test in rats compared to TBI + vehicle-treated rats [24].

One of the mechanisms for MSCs-transplantation-induced neuroprotection in animal models of neurological diseases could be due to reduction of neurodegeneration and cell death and induction of autophagy mechanism [27]. Autophagy, including mitophagy is the evolutionary conserved cellular active clearance mechanism, which delivers intracellular debris
or constituents to lysosomes for their final degradation [28, 29]. It interacts with apoptotic pathways and helps determine cell death and survival [30-32]. It has been shown that transplantation of MSCs significantly enhances autophagolysosome formation in animal models of AD, which increased neuronal survival against toxic amyloid proteins [33-35].

In the present study, we have investigated the neurodegenerative changes, autophagy, mitophagy, CMA markers, levels of molecular chaperones, cell death and cell survival, neuroinflammatory, as well as synaptic markers by comparing the treatment effects of MSCs-IL-10 and MSCs alone, using the rat model of contusion to the medial frontal cortex (MFC) as the TBI model. We have observed that transplantation of MSCs-IL10 improved neuronal morphology, reduced neurodegeneration, as well as diminished number of DNA-fragmented cells greater than MSCs alone. Furthermore, transplantation of MSCs-IL10 also induced autophagy and mitophagy markers, restored cell survival markers, reduced cell death markers as well as pre- and postsynaptic markers effectively than MSCs alone, in a rat model of TBI.

2. Material and Methods

2.1. Chemicals.

Cresyl violet, polybrene, puromycin, Hank’s balanced salt solution (HBSS) and other accessory chemicals were procured from Sigma (St. Louis, MO). Fluoro-Jade B (FJB) stain was purchased from Millipore, (Burlington, MA, AG310). In situ BrdU-Red DNA fragmentation assay kit (TUNEL staining kit) was from Abcam (Cambridge, MA, ab66110). Polyvinylidene difluoride (PVDF) membrane was from Molecular Probe (Grand Island, NY, IPVH00010). Hoechst 33342 solution (20 mM) was purchased from ThermoFisher Scientific (Grand Island, NY, 62249). pGEM-T Easy Vector was from Promega (Fitchburg, WI, A137A). pLenti-CMV-GFP-2A-Puro Vector was from ABM Inc. (Richmond, BC, Canada). The control plasmid, pLenti-CMV-GFP-
2A-Puro vector, was purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The 293FT cell line was purchased from ThermoFisher Scientific (Grand Island, NY, R70007). The information for different antibodies used for this study is provided in Table 1.

2.2. Animals.

Thirty-nine males, Sprague-Dawley (SD) rats (Charles River) approximately 90 days old were used in this study. Rats were paired housed in a 12h/12h reverse light cycle with food and water ad libitum. Rats were randomly divided into four groups: Sham + HBSS (n=10), TBI + HBSS (n=10), TBI + MSCs-IL-10 (CMV-IL-10-GFP, n=9) and TBI + MSCs (CMV-GFP, n=10). All procedures were approved by the Institutional Animal Care and Use Committee at Central Michigan University.

2.3. Rat model of TBI using controlled Cortical Impactor. The method for TBI rat model was developed as described previously [24, 36]. Briefly, the rats were anesthetized using a mixture of 1-3% isoflurane (Henry Schein Co.) and 500 mL-1L/min oxygen and maintained throughout the surgery. Body temperature was maintained at 37ºC during surgeries using a physiotemp machine (Physitemp Instruments Inc. Clifton, NJ). Rats were placed on a stereotaxic instrument (Kopf Instruments, Tujunga, CA) and a midline incision was made to expose bregma. Sham rats were then be closed and allowed to recover. Rats in the injured groups then underwent a 6-mm craniotomy at 3 mm anterior to bregma (AP +3.0, ML 0.0 mm). The impactor tip was placed over the exposed brain and compress the cortex at a depth -2.5 mm at a velocity of 2.25 m/s with a duration of 0.5 seconds [36]. The upper skin of the head was stiched and allowed to recover. After surgery, the rats were monitored for any signs of pain or discomfort.

2.4. Lentivirus construction for IL-10.
The detail method for lentivirus construction for IL-10 was described by Peruzzaro and colleagues [24].

2.5. Isolation of mesenchymal stem cells.

The MSCs were isolated and cultured as described previously [24, 37]. Viral production and their expression was confirmed from puromycin (10 µg/mL) selected colonies. Flow cytometry and immunocytochemistry (ICC) were performed to confirm MSCs surface markers and viral transfection. Prior to transplantation surgery, all MSCs were labeled with Hoechst 33342 (20 µg/mL) [24].

2.6. Stem cell transplantation.

Transplantation surgery was performed 36-h after injury. Sham + HBSS and TBI + HBSS rats were injected with HBSS, whereas TBI + MSCs-IL-10 rats were injected with MSCs-IL-10 and TBI + MSCs-GFP rats were transplanted with MSCs-GFP. Briefly, the previous incision was reopened, and a burr hole was then drilled on either side of the skull, directly and the injection were made bilaterally at two depths (AP+3.0, ML ±3.5, DV-3.0 and -1.5 mm) from bregma in the area adjacent to the injury site (200,000 cells/2 µL/hemisphere at a rate of 0.33 µL/min). The injection was completed first at the deepest depth and the skull was closed and allowed to recover.

![Schematic diagram showing experimental time scale.](image)

2.7. Tissue processing.
Three weeks after transplantation, all rats were deeply anaesthetized with an overdose of sodium pentobarbital (intraperitoneally), and transcardially perfused with 0.1 M cold PBS, followed by 4% paraformaldehyde (diluted in 0.1 M PBS at pH 7.4) to fix the brains. The brains were then removed, suspended in 4% paraformaldehyde for 24 hours at 4°C, and then transferred to the graded sucrose solutions (10%, 20% and 30%), dissolved in 0.1 M PBS, and then frozen using 2-methylbutane and stored in the -80°C freezer until they were sectioned coronally (30 µm) at a cryostat (Vibratome UltraPro 5000) set at -20°C. Brains from rats used for Western blot analysis were directly removed without perfusion and the fresh tissue were flash-frozen using 2-methylbutane (Sigma) and stored at -80°C until further use.

2.8. Neuronal morphology by Cresyl violet staining.

One of the aims and objectives of this study was to investigate whether treatment of MSCs with overexpression of IL-10 could improve neuronal morphology, especially in the cortex, in the CA1 and CA3 subfields of hippocampus. The rat brains from all four groups were sectioned coronally on a cryostat (Leica, Germany) and then they were stained with 0.1% Cresyl violet (CV) as described previously [41]. The sections were then washed, dehydrated, cleared and mounted by cover slipped using DePex mounting media (BDH, Batavia, IL). The slides were dried, and photomicrographs were taken by compound light microscope (Olympus, Japan) using 100x objectives (total magnification of 1000x). Dark, large dot stained cells were considered as pyknotic or tangle-like cells. The number of pyknotic or tangle-like cells were counted manually using Image-J software (http://imagej.nih.gov/ij) and expressed as number of pyknotic cells per 1mm² area. A minimum of 10 serial sections, with 30 different fields were used to count the number of pyknotic cells in each group (n=6). Two researchers counted the cells separately and an average value were reported.
2.9. Neurodegeneration study by Fluoro-Jade B (FJB) staining.

To detect the neurodegeneration, ten coronal sections (at equal interval from Bregma +2.20 mm to 0.70 mm for cortex and Bregma -2.20 mm to -3.60 mm for hippocampus) from control and treatment tissues were cut on a cryostat (20 μm). The sections were collected in 0.1 M neutral phosphate buffer, mounted on gelatin coated (2%) slides and then air dried on a slide warmer at 50°C for at least half an hour. The detail protocol for FJB staining was followed as described previously [38]. The total number of FJB-positive cells were counted using Image-J software (http://imagej.nih.gov/ij), manually and expressed per 1mm² area. A minimum of 10 serial sections, with 20 different fields were used to count the number of pyknotic cells in each group (n=6). All experimenters were blinded to the group identity of the specimens analyzed.

2.10. Terminal deoxyribonucleic acid nick end labeling (TUNEL).

Coronal brain sections (20 μm) from each of the group of rats were taken in poly-lysine-coated glass slide. Then terminal deoxyribonucleic acid nick end labeling (TUNEL) was performed as described previously [39, 40]. Finally, the sections were counter-stained with Hoechst-33342 (20 mM) for 5 min at room temperature in the dark and washed thoroughly with distilled water, after which they were mounted on a glass slide with anti-fading medium (Sigma). The cells were observed under a fluorescent microscope (Leica, Germany), using appropriate filters (ex/em: 488/576). The red fluorescent signal indicated TUNEL-positive cells. The number of TUNEL-positive cells were counted manually using image J software as reference frame (http://imagej.nih.gov/ij) from three experiments to obtain a mean value and they were expressed per 1mm² area.

2.11. Immunohistochemistry of Atg5, Atg7. Immunoperoxidase technique was used for the
levels of Atg5 and Atg7. Briefly, cryosections (40-μm thick) were rinsed with PBS (0.1 M, at pH 7.4) three times and then incubated with 0.5% Triton-X100 (Fisher Scientific, Pittsburgh, PA), along with 3% H₂O₂ solution (for Atg5 and Atg7), for 30 min at room temperature, followed by three washes in PBS, for 10 min each. The unmasking was done by treating the sections with 10% normal goat serum (Santa Cruz Biotech, Dallas, Texas) for 1 h at room temperature. Then sections were incubated with rabbit monoclonal anti-Atg5 anti-Atg7 antibodies (1:200), which were dissolved in PBS, along with 10% goat serum and placed on the plate on a shaker at low speed and kept at 4°C overnight. On the next day, the sections were thoroughly washed with PBS, three times for 10 min each. The sections were incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratory, CA; 1:200) for 4 h at 37°C. After this incubation, the sections were washed three times with PBS, 10 min each, and then treated with ABC reagent for 30 min at room temperature. This was followed by three washes in PBS of 10 min each. Finally, the sections were incubated with peroxidase substrate solution, supplied with the ABC kit (Vector Laboratory, CA), and the signal was developed using diaminobenzidine (DAB) until the desired staining intensity emerged. The tissue was then washed, dehydrated, cleared, mounted on slides and visualized using a compound light microscope (Olympus, Japan).

2.12. Confocal imaging of GFAP, Iba-1, Beclin-1 and LC-3A/B. Immunofluorescent technique was used for detecting Beclin-1 and LC-3A/B levels. Briefly, after blocking with normal goat serum (10%), the sections were incubated with Beclin-1 and LC-3A/B antibodies (1:200, Table 1) for overnight and following day, they were incubated with anti-rabbit secondary antibody (1:500), tagged with FITC (for Beclin-1, Molecular Probes, OR) or Alexa-594 (for LC-3A/B) for 30 min at room temperature. Then the sections were washed thoroughly with distilled water, dehydrated, cleared and mounted on slides using anti-fading fluoro-mount aqueous mounting
media (Sigma), and visualized using table-top Fluoview confocal laser scanning microscope (FV1oi, Olympus) with appropriate filters to optimize excitation and emission.

2.13. Immunohistochemistry and confocal imaging of neuroinflammatory markers

(i) GFAP-immunohistochemistry. Activated astrocytes were detected by immunolabeling with GFAP, as described previously [39]. Briefly, the perfused brains were cryopreserved and coronal sections (40 µm thickness) were obtained using a cryostat (Leica, Germany). The sections were rinsed with 0.1 mM PBS (pH 7.4), blocked with 10% normal goat serum for 1h and incubated with GFAP (rabbit polyclonal, 1:1000) antibody, overnight on a shaker at 4°C. On the next day, the sections were thoroughly washed with PBS, three times, for 10 min each, incubated with anti-rabbit secondary antibody (1:1000), tagged with Alexa-488 (Molecular Probes, OR) for 30 min at room temperature. Then the sections were washed thoroughly with distilled water, dehydrated, cleared and mounted on slides using anti-fading Fluoro-mount aqueous mounting media (Sigma), and visualized using table-top Fluoview confocal laser scanning microscope (FV1oi, Olympus) using appropriate filters for excitation and emission.

(ii) Iba-1 immunohistochemistry. For Iba-1 immunoreactivity, the coronal sections (40 µm) were blocked in 10% normal goat serum in 0.1% Triton X-100 and 0.1M PBS for 1 hour at room temperature. Then the sections were incubated with mouse primary Iba-1 antibody (1:4000, Wako) for 4 hours at room temperature and additional overnight incubation for at 4°C. Following day, tissue was washed 3 times 5 min each in PBS. A secondary antibody conjugated with Alexa fluorophore 546 was applied for 1 hour at room temperature. Both GFAP-IR and Iba-1-IR images were taken using tabletop Fluoview confocal laser scanning microscope (FV1oi, Olympus) using appropriate filters for excitation and emission. Number of GFAP-IR and Iba-1-IR cells were counted around the lesion site, manually, using Image-J software from 10 sections...
in the cortex, CA1 and CA3 subfield of hippocampus and expressed as number of GFAP-IR or Iba-1-IR/1 mm² area.

2.14. Western blot.

About 100 mg of flash-frozen mixed cortex was lysed with cold radio-immuno precipitation assay (RIPA) buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, pH 7.4] with protease and phosphatase inhibitors (Sigma). The tissue was homogenized with tissue homogenizer (ThermoFisher Scientific, Grand Island, NY) and the tissue lysate was centrifuged at 16,000x g for 15 min at 4°C. Following centrifugation, the supernatant was collected and aliquoted in PCR tubes and stored at -80°C until use. Total protein concentrations for individual samples were determined using the Pierce BCA protein assay (ThermoFisher Scientific). Samples were added with equal amounts of 2x SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol and 0.5% bromophenol blue) and boiled for 2 min. Approximately 100 µg of protein, per lane was loaded and electrophoresed on 10% Tris-glycine gel and transferred to PVDF membrane (Millipore, Bedford, MA). After probing with respective primary and secondary antibodies (Table 1), the blots were developed with ImmobilonTM Western Chemiluminescent HRP-substrate (Millipore, Billeria, MA). The relative optical density was measured using Image-J software (https://imagej.nih.gov/ij/). To ensure equal protein loading in each lane, the blots were re-probed for β-tubulin.

2.13. Statistical Analysis. All the data were expressed as Mean ± SEM. All statistics were analyzed using one-way analysis of variance (ANOVA), followed by posthoc Tukey’s Honestly Significant Difference (HSD) test. The probability value, p≤0.05, was considered significant.

3. Results.
Using IHC, RT-PCR and Western blot techniques, we previously confirmed the overexpression of IL-10 levels *in vitro* and *in vivo* in MSCs which was engineered with Il-10 gene [24]. In addition, we have also confirmed an increase levels of IL-10 in TBI rats transplanted with MSCs overexpressed with IL-10 in comparison to other three groups [24] (S1).

3.1. Transplantation of MSCs-IL10 protected cortical and hippocampal neuronal damage better than transplantation of MSCs alone in TBI rats. One of the major objectives of the present study was to characterize the morphological changes in cortical and subcortical areas after TBI and determine whether MSCs-IL-10 can improve their morphology. Therefore, we stained coronal sections with 0.1% Cresyl violet. We observed that the number of pyknotic or tangle-like cells were significantly increased (p<0.01) in the cortex (B), in CA1 (C) (p<0.01), and CA3 (D) (p<0.01) subfields of hippocampus of TBI rats (Fig 1A-D) and that these pyknotic cells were significantly decreased (p<0.01) in rats which received MSCs-IL-10 cells. Greater reduction of pyknotic or tangle like cells were observed in these areas in the case of MSCs-IL10 treated rats in comparison of MSCs alone (p<0.01).

3.2. TBI-induced neurodegeneration was better protected by transplantation of MSCs-IL-10 than MSCs alone. A distinct phenomenon observed in TBI is the degeneration of neurons. To characterize the neuronal injury in TBI model, the coronal sections were stained with FJB (Fig 2A), a marker for neurodegeneration. The number of FJB-positive cells were significantly increased in the cortex (B), in the CA1 (C), and CA3 (D) subfields of hippocampus in TBI group (p<0.01), whereas transplantation of both MSCs-IL-10 and MSCs-GFP significantly (p<0.05) decreased the number of FJB-positive cells (B-D), however, MSCs-IL-10 decreased more degenerated cells than MSCs-GFP alone (p<0.05).
3.3. **Number of DNA fragmented cells were reduced more by transplantation of MSCs-IL-10 than MSCs alone.** To examine the mode of cell death in TBI after transplantation with MSCs-IL-10 or MSCs-GFP, we performed TUNEL staining of the tissue from the cortex, CA1, and CA3 areas of hippocampus (Fig 3). We observed that TBI group significantly increased number of TUNEL-positive cells in the cortex (Fig 3A-B), in the CA1 (Fig 3A and C) and CA3 areas (Fig 3A and D) of hippocampus in comparison to Sham + HBSS (p<0.01). Whereas transplantation of MSCs-IL-10 and MSCs alone significantly decreased (p<0.05) their levels, however, transplantation of MSCs-IL-10 decreased more TUNEL-positive cells than the TBI rats which received MSCs alone (p<0.05).

3.4. **Transplantation of MSCs-IL-10 modulated autophagy markers and PI3K/Akt/mTOR pathway greater than MSCs alone in TBI rats.** One of the aims of the present study was to investigate the autophagic responses after TBI and determine whether transplantation of MSCs-GFP and MSCs-IL-10 cells have any differential regulatory role on autophagy and PI3K/Akt/mTOR pathway. We have observed that autophagy markers Atg5 (Fig 4A-B), Atg7 (Fig 4A, C), LC3A/B-II (4A, D) and p62 (Fig 4A, F) were increased greater in TBI rats which received MSCs-IL10, than by MSCs alone. Whereas, there was a significant decrease levels of PI3K (p85) and p-Akt which were restored by transplantation of MSCs-IL-10, not by MSCs (Fig 4A, G, H, I). Although p-PI3K (p85) was unchanged in TBI rats, however, it was increased by MSCs-IL-10, not by MSCs alone (Fig 4A, H). In contrast, p-mTOR (ser2448) was significantly increased by TBI, and it was restored after transplantation of MSCs-IL-10, but not by MSCs alone (4A, K), whereas there was no meaningful change of total Akt and mTOR levels (Fig 4A, L) in all the four groups.
3.5. Transplantation of MSCs-IL-10 showed greater immunoreactivity of autophagy markers in TBI rats than MSCs alone. Although immunointensity of Atg5 (Fig 5A) and Atg7 (Fig 5B) were appeared to increase in TBI with MSCs-IL-10 and MSCs-GFP groups in the cortex, in the CA1 and CA3 areas of hippocampus in comparison to TBI + HBSS and Sham + HBSS groups, but we did not observe any meaningful changes between Sham + HBSS and TBI + HBSS rats in these areas. Whereas, we observed a decrease levels of immunofluorescent signal of Beclin-1 in TBI rats, and transplantation of MSCs-IL-10, but not by MSCs alone, appeared to increase its level (5C). In addition, immunofluorescent signal for LC3A/B appeared to show more in TBI with MSCs-IL-10 in the cortex, in the CA1 and CA3 areas of hippocampus in comparison to Sham + HBSS, TBI + HBSS and TBI + MSCs (Fig 5D).

3.6. Transplantation of MSCs-IL-10 induced mitophagy markers greater than MSCs alone in TBI rat.

Mitophagy is important for removal of damaged mitochondria, which help in cell survival. Although we did not observe much alteration of NIX, FUNDC1, and BNIP3 in TBI rats in comparison to Sham control, however, transplantation of MSCs-IL-10, but not MSCs alone significantly increased (p<0.05) their levels (Fig 6A-D). In contrast, PINK-1 and HIF-1α levels were significantly down-regulated by TBI rats (p<0.05) and levels of both these proteins were restored by MSCs-IL10, whereas transplantation of MSCs restored HIF-1α levels (Fig 6A, F), but not the PINK-1 levels (Fig 6A, E).

3.7: Transplantation of MSCs-IL-10, but not MSCs alone induced cell survival markers and reduced cell death markers in TBI rats. The levels of PSD95 (Fig 7A-B) and synaptophysin (Fig 7A, C) were significantly decreased (p<0.01) in TBI rats and transplantation of MSCs-IL-10 restored both the PSD95 and synaptophysin levels, not by MSCs alone. In
addition, cell death markers, such as Bax, cytochrome-C, caspase-3 and p53 levels were significantly increased (p<0.05) in TBI rats and transplantation of MSCs-IL10 decreased their levels, however, transplantation of MSCs alone was not able to downregulate Bax and cytochrome-C (Fig 7A, E-F). In contrast, Bcl2 levels was significantly increased by MSCs-Il-10 and MSCs alone in comparison to Sham + HBSS and TBI +HBSS rats, the induction of Bcl2 levels was significantly higher (p<0.05) in the case of MSCs-IL10 in comparison to MSCs alone (Fig 7A, and D).

Supplementary figures

S1: Levels of IL-10 was increased more in MSCs-IL10 than MSCs alone in vitro and in vivo. A: Representative immunocytochemistry images of MSCs with and without IL-10 overexpression. MSCs + IL10-GFP group appeared to have higher IL-10 immunofluorescent signal than MSCs + GFP group. B-C: Western blot data revealed that IL-10 levels was more in the case of MSCs + IL-10 than MSCs + GFP (p < 0.05) in vitro. D-F: Similarly, Western blot from rat cortical tissue showed that there was a significant decrease levels of IL-10 in TBI rats and IL-10 levels was increased after transplantation of MSCs-IL-10, but not by MSCs alone (p < 0.05).

S2: Transplantation of MSCs-IL-10 cells decreased neuroinflammatory markers greater than MSCs alone in TBI rat. Neuroinflammation is one of the principal causes for neuronal injury and death in TBI. Therefore, we investigated the levels of GFAP and Iba-1, the markers for activated astrocytes and microglia. TBI rats showed a significant increase in GFAP-IR in the cortex (A-B), as well as in the CA1 (A, C) and CA3 (A, D) areas of hippocampus, whereas rats transplanted with MSCs-IL-10 and MSCs-GFP had decreased levels. Although we did not find any meaningful changes in the number of Iba-1-IR cells in CA1 (F) and CA3 (G) areas of
hippocampus among the groups, but Iba-1-IR was increased in TBI cortex and it was
downregulated by MSCs-IL10 (E), but not by MSCs alone. In addition, both TBI with
transplanted groups showed more hyper-ramified or bushy or amoeboid like morphology of
microglia (data not shown). Our Western blot data for GFAP from cortical tissue and their
densitometric analysis revealed that there was a significant increase level of GFAP in the cortex
of TBI rats, which was reduced by transplantation of both MSCs-IL-10 and MSCs-GFP (H-I).

S3: Molecular chaperones and chaperone-mediated autophagy responses in TBI rat after
transplantation of MSCs-IL-10 and or MSCs-GFP. Our Western blot data indicate that
HSP90 (A-B) become upregulated and HSP40 (A, E) was downregulated by TBI rats and
transplantation of MSCs-IL-10 restored their levels, but not by MSCs alone. Whereas, HSP60
was unaltered in all the groups (A, D), but HSP70 (A, C), and CHIP (A, E) levels were increased
in TBI with MSCs-IL-10 and MSCs-GFP transplanted rats. Furthermore, chaperone-mediated
autophagy (CMA) markers, such as HSC70 (A, F) and LAMP2A (A, G) were decreased in TBI
rats and they were upregulated by TBI with transplantation of MSCs-IL-10 and MSCs-GFP in
comparison to TBI + HBSS and Sham + HBSS rats.

4. Discussion
Traumatic brain injury (TBI) is one of the leading causes of motor, sensory, psychological, and
cognitive dysfunction, and is largely a consequence of increases in neuroinflammation and
neurodegeneration [41]. Cell death in TBI is associated with dysregulation of autophagy
mechanisms, including mitophagy dysfunction [42, 43]. In the present study, we found a mild to
moderate neuroprotective effects, including induction of autophagy, including mitophagy
markers, decreased cell death and neuroinflammatory markers, increased cell survival markers,
as well as restoration of pre and post-synaptic markers after transplantation of MSCs-IL-10
and/or MSCs alone in a rat model of TBI. Furthermore, MSCs-IL10 transplanted rats displayed greater neuroprotective effects than rats which received MSCs alone.

Neuroinflammation is one of the key mechanisms associated with neuronal injury in TBI. Pro-inflammatory cytokines, such as IL-10 become down-regulated in TBI, which triggers neuronal death [17, 44]. Transplantation of MSCs have been shown beneficial therapeutic effects in different brain injury models [45], because they secret many neurotropic factors, including many cytokines, such as IL-4, IL-6, IL-10, IL-11, and IL-13 [23, 46, 47]. Among them, IL-10 is the most important because it exerts neuroprotective effects via suppressing the expression of various pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-2, IL-6, and TNF-α, as observed in stroke [23, 48] and in TBI [46, 47, 49-51]. Although many researchers demonstrated that MSCs have immunomodulatory effects which can secrete many cytokines, including IL-10, whereas some researchers have also showed contradictory results [52]. MSCs may secrete IL-10 under specific conditions, such as inflammatory environment (in presence of IFN-γ, IL-1β and TNF-α), as noted in brain injuries [52]. Although it is unknow in what ideal conditions under which MSCs could secrete IL-10, but it is speculated that transplantation of MSCs may stimulate the cells surrounding the injury and trigger secretion of IL-10 and other neurotrophic factors [52]. Therefore, we genetically modified MSCs to secrete abundant IL-10 and hypothesized that they may improve MSCs-based cell therapy for TBI-induced neuronal injury [24]. We observed and reported an improve levels of IL-10 by MSC-IL-10 than by MSCs alone [24](S1).

We have used the rat controlled cortical impactor injury model and characterized the cell death in the cortex and in the hippocampal subfields, using multiple staining methods. A significant increase immunoreactivity of GFAP and Iba-1 in the cortex after 3 weeks of TBI (S2), were decreased after transplantation of both MSCs-IL-10 and MSCs alone, suggesting
reduction of neuroinflammation [24]. Whereas, transplantation of MSCs alone, was unable to decrease number of GFAP-IR cells in CA1 and CA3 areas of hippocampus. Similarly, transplantation of MSCs-IL-10 significantly decreased the Iba-I-IR cells in the frontal cortex but not by MSCs alone (S2)[24], suggesting MSCs-IL-10 exert greater anti-inflammatory effects than MSCs alone. This observation was also supported by Nakajima and colleagues in mouse model of ischemic stroke after MSCs-IL-10 transplantation [23].

We further investigated the autophagy mechanisms, which are the chief routes for bulk degradation of aberrant organelles and cytoplasmic components of damage cells and thus, provide cytoprotection [53-59], as seen in animal models of TBI, and other neurological diseases [43]. IL-10 may induce autophagy or autophagy can enhance IL-10 production, as reported previously [60]. Therefore, this study aimed to achieve neuroprotective effects through induction of autophagy after transplantation of MSCs [23]. We have investigated several autophagy markers, such as Atg5, Atg7, Beclin-1, LC3A/B, mTOR, p-mTOR levels. We did observe a significant increase in Atg5 and Atg7 levels after transplantation of MSCs-IL-10 and MSCs-GFP, indicating that MSCs-IL-10 or MSCs-GFP can induce autophagosome formation (Fig 4). These findings were supported by other investigators in animal models of AD [31], acute ischemic stroke [23] and in TBI [61]. We have investigated levels of Beclin-1, a Bcl2 interacting partner, which are involved in autophagic cell death and apoptosis [62]. We observed that Beclin-1 level was less in TBI rats (Fig 4), which was also reported by Au and colleagues in a mouse model of closed head injury produced by dropping a weight onto the intact skull [56]. Interestingly, transplantation of MSCs-IL-10 and/or MSCs-GFP restored Beclin-1 levels, indicating that the transplants exerted cytoprotective effects [59]. Similarly, conversion of microtubule-associated protein light chain-3A/B-I (LC-3A/B-I) to LC3A/B-II, is a reliable
biomarker for autophagy [63]. LC3A/B-II was significantly increased by MSCs-IL-10, but not by MSCs-GFP (Fig 4), indicating MSCs-IL-10 is a greater autophagy inducer. However, Klionsky and colleagues indicated that to study autophagy, it is essential to investigate autophagy flux, along with LC3A/B levels [57]. Therefore, to monitor autophagic activity we investigated p62 (also called sequestosome1, SQSTM1) levels. It directly binds to LC3 and its degradation can indicate decreased level of LC-II [59]. We have observed a significant increased level of p62 levels in TBI-MSCs-IL10 rats, suggesting autophagy mechanisms were induced by MSCs-IL-10, not by MSCs alone (Fig 4F). Although increase p62 levels is an indicator of decrease autophagy, whereas we found an increased level of p62 in MSCs-IL10 rats, which may be due to the blocking of fusion of autophagy vacuoles with lysosome or by the inhibition of a later maturation step of autophagosome degradation, which needs further experiment to confirm the findings.

Then we have investigated the key proteins which regulate autophagy mechanisms, such as phosphoinositol 3-kinase (PI3K), Akt (protein kinase B) and mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR pathway) [64]. These protein have been reported to be involved in the neuroprotection in cerebral injury [61]. The levels of mTOR and p-mTOR have been reported to increase in the cortex and hippocampus of mice at 24 hours after TBI [64]. Increased levels of p-mTOR are the indicators of decreased autophagic responses [65-67]. Using rapamycin Zhang and colleagues observed that there was an increase in autophagic responses, including enhancement of Beclin-1 levels, along with improve neurobehavioral function, increase neuronal survival, reduce inflammation and gliosis in TBI [61]. In the present study, we observed that the levels of PI3K (p85) and p-Akt were decreased [p-PI3K (p85) was unchanged] and p-mTOR was up-regulated by TBI, whereas transplantation of MSCs-IL-10, but
not MSCs alone increased their levels (Fig 4G-I), suggesting autophagy was inhibited by TBI and transplantation of MSCs-IL10 activated this pathway. Decreased levels of p-mTOR correlated with increased levels of LC-3A/B-II (Fig 4E) and induction of autophagy mechanisms by MSCs-IL-10 may be regulated by PI3K/Akt/mTOR pathway in TBI rats, which require further experiment to confirm the findings.

In addition, mitochondria dysfunction, including reduction of mitochondrial respiration, increase production of ROS have been observed in TBI, which triggers apoptotic cell death [72]. We have investigated whether transplantation of MSCs or MSC-IL-10 have any role in selective degradation of damaged mitochondria by mitophagy. Therefore, we have investigated the most important mitophagy markers, such as NIX, BNIP3, FUNDC1, PINK1 and HIF-1α. Interestingly, we found a significant increase in mitophagy markers, such as NIX, BNIP3, FUNDC1, PINK-1 and HIF-1α levels following transplantation of MSCs-IL10 (Fig 6), but not by MSCs alone, suggesting that the damaged mitochondria were selectively degraded via mitophagy, and provided neuroprotection greater by MSCs-IL-10 than MSCs alone.

Molecular chaperones, or heat shock proteins (HSPs), are involved in cell death and survival by degrading small, misfolded proteins [68, 69]. We observed that HSP90 was significantly upregulated (S3) by TBI rats. Increase HSP90 have been reported to be involved in brain injury [70]. Decrease levels of HSP90 by MSC-IL-10 was greater than MSCs alone, suggesting MSC-II-10 has greater cytoprotective roles by downregulating its levels. In contrast, loss of HSP40 and HSP70 increase brain injury and death of neurons [71], whereas they can induce and arrest inflammation and improved neurological outcome [72]. We found a decreased of HSP40 and HSP70 in TBI rats and their levels were restored by MSCs-IL.10, but not by MSCs
(S3), suggesting MSCs-IL-10 may induce immunomodulatory and neuroprotective roles perhaps through HSPs. In addition, CMA markers, such as HSC70 and LAMP2A, were only modestly decreased in the TBI rats (S3), with respect to control rats, but their levels were increased by both the transplanted groups, suggesting CMA was activated to remove some of the debris generated by the transplanted cells.

There were less pyknotic or tangle-like cells as revealed by CV stain (Fig 1), along with decreased neurodegeneration as shown by FJB stain (Fig 2), and reduced number of TUNEL-positive cells (Fig 3) in MSCs-IL-10 transplanted TBI rats, relative to those in TBI + MSCs rats, suggesting the neuroprotective effects might be due to IL-10. We have further investigated the important cell death and cell survival markers, such as Bax, cleaved caspase-3, cytochrome-C, and p53, along with Bcl2, PSD95 and synaptophysin levels. We clearly observed that there were decreased levels of Bax, caspase-3, cytochrome-C after transplantation of MSCs-IL10, but not by MSCs. Similarly, anti-apoptotic markers Bcl2, postsynaptic markers PSD95 and synaptophysin were also restored by MSCs-IL10, but not by MSCs alone. In addition, increase levels of p53 is also involved in TBI-induced cell death [73], which was decreased by MSCs-IL10, but not by MSCs alone, suggesting MSCs-IL10 showed greater neuroprotective effects than MSCs alone. These observations also suggest that the neuroprotective effects may be coming from IL-10, which was supported by our previous finding where we found that MSCs-IL10 increase conversion of M1 to M2 macrophage which secrete anti-inflammatory cytokines to protect further brain injury [24, 74]. Increased levels of these markers may be due to decrease neuroinflammation by increasing anti-inflammatory cytokines (by M2 macrophages which was increased by MSC-IL-10), increased neurotropic supports coming from transplanted cells, as well as increases in the autophagy mechanisms [79, 80]. These findings also indicating that
transplanted cells may secrete many other neurotropic factors [23], along with IL-10, as reported by other investigators in mouse models of TBI [81, 82], which supports our findings. Overall, transplantation of MSCs-IL-10 showed greater neuroprotection than by MSCs alone, by improving neuronal morphology and decreased cell death in the TBI rats, along with increased autophagy and mitophagy mechanism, which may be regulated by PI3K/Akt/mTOR pathway. Additional experiments are required to investigate the detail mechanism of MSC-IL-10-induced neuroprotection in TBI.

Overall, the controlled cortical impact model of TBI in rats produced significant neurodegeneration and cell death in the cortex and in the hippocampus, whereas transplantation of MSCs-IL-10 provided greater neuroprotection than MSCs alone. Transplanted MSCs-IL-10 induced autophagy, mitophagy, chaperone-mediated autophagy, molecular chaperones, regulated PI3K/Akt/mTOR pathway, and influenced cell death and cell survival markers more efficiently than MSCs alone. Therefore, induction of autophagy mechanisms, using MSCs that overexpress IL-10, may be an effective strategy for protecting the brain against TBI-induced cell death.

Acknowledgments: This work was supported by the Field Neurosciences Institute, St. Mary’s of Michigan, and the John G. Kulhavi Professorship in Neurosciences and the Neuroscience Program, College of Medicine at Central Michigan University. We are thankful to Dr. Jeffery Smith, Dr. Michael Hoane, the Brain Research Laboratory, Saginaw Valley State University, for generously lending their contusion devise and proving a supportive infrastructure. Also, we would like to thank Olivia Pupiec, Melissa Resk for their technical help and Dr. Ming Lu for his critical advice on viral construction.

Disclosure of Interest: The authors declare that they have no competing interests to publish this research article.

Funding: This work was supported by the Field Neurosciences Institute, St. Mary’s of Michigan, and the John G. Kulhavi Professorship in Neurosciences and the Neuroscience Program at Central Michigan University.
Author’s contributions: PM and SP designed the study. PM collected, analyzed, and interpreted data and wrote the manuscript. NK was involved in histology and immunohistochemistry. SP and MA were involved in animal surgery, tissue collection. AG was involved in Western blots for mitophagy markers. GD and JR oversaw, edited and contributed to discussion and provided overall supports. All authors approved the final manuscript.

References


36. Peruzzaro ST, Gallagher J, Dunkerson J, Fluharty S, Mudd D, Hoane MR, Smith JS. The impact of enriched environment and transplantation of murine cortical embryonic stem cells on


44. Csuka E, Morganti-Kossmann MC, Lenzlinger PM, Joller H, Trentz O, Kossmann T. IL-10 levels in cerebrospinal fluid and serum of patients with severe traumatic brain injury:


Figures legends

Fig 1: Transplantation of MSCs-IL-10 improved neuronal morphology greater than MSCs alone in the cortex and hippocampus of TBI rats. Rat brains were sectioned and stained with 0.1% Cresyl violet and images were taken by compound light microscope (Olympus) with 100x objectives (total mag 1000x). A: Representative photomicrograph of TBI rats showed increase number of pyknotic or tangle-like cells in the cortex, in the CA1 and CA3 subfields of hippocampus. B-D: Number of pyknotic cells were significantly decreased by transplantation of MSCs-IL-10 in comparison to TBI rats (p<0.01) and with TBI+MSCs (p<0.01). The greater reduction of pyknotic cells was observed in the case of MSCs-IL-10 rats. Arrows indicate pyknotic or tangle-like cells. Scale bar indicates 100 µm and is applicable to other images. **p<0.01 in comparison to TBI + HBSS, TBI + MSCs-IL-10 and TBI + MSCs; *p<0.05 in comparison to TBI + MSCs; ##p<0.01 in comparison to TBI + MSCs.

Fig 2: Transplantation of MSCs-IL-10 reduced more number of degenerated neurons in in the cortex and hippocampus of TBI rats than MSCs alone. Rat brains were sectioned (20 µm) and stained with Fluoro-Jade B (FJB) solution (0.0004%) and images were taken using fluorescent microscope (Leica, Germany). A: Representative images of FJB-stained sections from the cortex, in the CA1 and CA3 area of hippocampus. B-D: The number of FJB-positive cells were significantly increased (**p<0.01) in TBI rats, whereas transplantation of both MSCs-IL-10 and MSCs alone, significantly decreased the number of FJB-positive cells. The number of FJB cells were significantly less (#p<0.05) in the case of TBI + MSCs-IL-10 in comparison to TBI + MSCs. Green signals (red arrows) indicate FJB-positive cells and blue signal is for DAPI (nuclear) stain. Scale bar indicates 100 µm and is applicable to other images. **p<0.01 in
comparison to TBI + HBSS, TBI + MSCs-IL-10 and TBI + MSCs; *p<0.05 in comparison to TBI+MSCs; #p<0.05 in comparison to TBI+MSCs.

**Fig 3:** Transplantation of MSCs-IL-10 reduced greater number of DNA fragmented cells in the cortex and hippocampus of TBI rats than MSCs alone. Terminal deoxyribonucleic acid nick end labeling (TUNEL) was performed in coronal sections from cortex, CA1 and CA3 subfields of hippocampus. A: Representative photomicrographs showed an increase number of TUNEL-positive cells in the cortex, as well as in the CA1 and CA3 areas of the hippocampus. B-D: The number of TUNEL-positive cells were significantly increased (**p<0.01) in TBI rats, whereas transplantation of both MSCs-IL-10 and MSCs alone, significantly decreased their numbers. The number of TUNEL positive cells were significantly less (#p<0.05) in the case of TBI + MSCs-IL10 in the cortex and CA3 area of hippocampus in comparison to TBI + MSCs. Red signals (white arrows) indicate TUNEL-positive cells and blue signal is for DAPI (nuclear) stain. Scale bar indicates 100 µm and is applicable to other images. **p<0.01 in comparison to TBI+HBSS, TBI+MSCs-IL-10 and TBI+MSCs; *p<0.05 in comparison to TBI+HBSS; #p<0.05 in comparison to TBI+MSCs.

**Fig 4:** Transplantation of MSCs-IL-10 modulated autophagy markers and PI3K/Akt/mTOR pathway greater than MSCs alone in the TBI rats.

Equal amount of protein from cortical tissue homogenates were electrophoresed, blotted on PVDF membrane and different autophagy markers were studied. A: Representative Western blots of Atg5, Atg7, Beclin-1, LC3A/B, p62, PI3K, p-Akt, Akt, p-mTOR, mTOR from mixed cortical tissue from different animal groups. B-C: Densitometric data indicating that TBI with MSCs-IL-10 and MSCs groups of rats showed an increase in Atg5 (B) and Atg7 (C) in comparison to TBI + HBSS or Sham + HBSS rats and greater increase was noted in the case of
MSCs-IL-10 rats in comparison to MSCs alone. **D-E:** Western blot data showed that there was a decrease in Beclin-1 levels in the TBI rats, which was restored by transplantation of MSCs-IL-10, but not by MSCs alone. **E-F:** Western blot data showed that there was an increase levels of in LC-3A/B-II and p62 in TBI rats transplanted with MSCs-IL-10, but not by MSCs alone. **G-I:** PI3K (p85) and p-PI3K (p85) and p-Akt levels were less in TBI rats and they were restored by MSCs-IL-10, not by MSCs alone. **K:** p-mTOR levels was increased by TBI rats and restored by MSCs-IL-10, not by MSCs alone. *p<0.05 and **p<0.01 in comparison to other groups; #p<0.05, ##p<0.01 in comparison to MSCs alone.

**Fig 5:** Immunohistochemistry of autophagy markers in TBI rats after transplantation of MSCs-IL-10, and MSCs-GFP cells. Coronal sections from each group were immunolabeled with Atg5, Atg7, Beclin-1 and LC3A/B antibodies. The images were taken by either light microscope (Olympus) or by tabletop Fluoview confocal laser scanning microscope (FV1oi, Olympus). Atg5 (**A**) and Atg7 (**B**) appeared to increase their levels in TBI rats after transplantation of MSCs-IL-10 and MSCs-GFP when compared to TBI and sham controls. Whereas, TBI section appeared to contain less beclin-1 immunofluorescent signal in the TBI rats when compared to sham control or the other transplanted groups (**C**). Furthermore, TBI rats showed relatively less immunofluorescent puncta of LC3A/B (**D**) in comparison to TBI rats, whereas its level was increased after transplantation of MSCs-IL-10 cells and by transplants of MSCs-GFP cells in comparison to sham control and TBI rats. Arrows indicate LC-3A/B immunoreactivity. Blue color: Hoechst-3442 and green color: secondary antibody tagged with Alexa fluoro-488. Scale bar indicates 50 µm and applicable to other images.

**Fig 6:** Transplantation of MSCs-IL-10, but not MSCs alone increased mitophagy markers in TBI rats. Western blot analyses showed that mitophagy markers, such as NIX, FUNDC1, and
BNIP3 were unaltered, whereas PINK-1 and HIF-1α were downregulated by TBI rats, and transplantation of MSCs-IL-10, but not transplantation of MSCs alone improved their levels. *p<0.05 and **p<0.01 in comparison to TBI + HBSS and Sham + HBSS and TBI + MSCs-GFP rats; #p<0.05 and ##p<0.01 in comparison to TBI + MSCs.

Fig 7: Transplantation of MSCs-IL-10, but not MSCs-GFP improved synaptic and cell survival markers and decreased cell death markers in TBI rats. A-C: Pre-synaptic and post-synaptic markers, such as synaptophysin and PSD95 were down-regulated by TBI and transplantation of MSCs-IL-10 and MSCs alone improved both, but MSCs-IL-10 improved greater than MSCs alone. D: Anti-apoptotic markers Bcl2 was increased greater by MSCs-IL10, than MSCs alone. E-H: Whereas cell death markers, such as Bax, cytochrome-C, caspase-3 and p53 levels were increased by TBI and transplantation of MSCs-IL10, decreased their levels more effectively than by MSCs alone. *p<0.05 and **p<0.01 in comparison to Sham + HBSS rats, #p<0.05 and ##p<0.01 in comparison to TBI + MSCs.

Supplemental figures

S1: Levels of IL-10 in MSCs in vitro and rat cortical tissue after transplantation of MSCs-IL-10 and MSCs. A: Representative immunocytochemistry images of MSCs with and without IL-10 level. MSCs + IL10-GFP group appeared to have higher IL-10 immunofluorescent signal than MSCs-GFP cells. B-C: Western blot from in vitro studies showed that there was a significantly higher levels (*p<0.05) of IL-10 production in MSCs-IL10 group in comparison to MSCs alone. D-E: Similarly, in vivo studied showed that there was a significantly higher amount of IL-10 (*p<0.05) production in TBI + MSCs-IL-10 rats in comparison to MSCs alone. Scale bar = 50 μm. Error bars represent standard error of the mean (± SEM).
S2: Transplantation of MSCs-IL-10 cells decreased GFAP levels, but not Iba-1 in TBI rats.

Coronal section (40-µm) from cortex and hippocampus were immuno-labeled with GFAP and Iba-1 antibodies. A-D: Both the transplanted groups significantly decreased the mean number of GFAP-IR cells in the cortex (B), whereas in the hippocampus, the mean number of GFAP-IR cells were significantly decreased by MSCs-IL-10, but not by MSCs alone (C-D). *p<0.05 and **p<0.01 in comparison to Sham + HBSS, TBI + MSCs-IL-10 and MSCs-GFP rats, #p<0.5 in comparison to MSCs-GFP. E-G: Iba-1-IR was significantly decrease by MSCs-IL-10, but not by MSCs alone in the cortex, whereas there were no significant differences in Iba-1-IR cells in hippocampus among the groups. *p<0.05 in comparison to Sham + HBSS and TBI + MSCs-IL-10 rats, #p<0.5 in comparison to MSCs-GFP. Scale bar indicates 50 µm and applicable to other images. Green: GFAP and Red: Iba-1. H-I: Western blot data (H) and their densitometric analysis (I) showed that there was a significant increase level of GFAP in cortical tissue in TBI rats and both transplantation of MSCs-IL-10 and MSCs alone reduced its levels. ** p<0.01, compared to TBI + HBSS.

S3: Molecular chaperones and chaperone-mediated autophagy markers were greater regulated by MSCs-IL10 than MSCs alone in TBI rats. Three weeks after transplantation, the flash-frozen mixed cortex was homogenized with RIPA buffer, and the supernatant was collected for measuring protein levels. Equal amount of protein was run with SDS-PAGE, blotted on PVDF membrane and probed with different heat shock proteins antibodies and its signal was detected by chemiluminescent reagents. A: Representative Western blot data showed the HSPs levels in mixed cortex tissue from different animal groups. B-F: Densitometric analysis revealed that TBI in the MSCs-IL-10 and MSCs-GFP groups of rats showed an improvement of different HSPs and CHIP levels when compared to TBI rats. G-H: Western blot analysis indicated that the
chaperone-mediated autophagy markers, such as HSC70 (G) and LAMP2A (H) were downregulated in TBI rats and transplantation of MSCs-IL-10 and MSCs-GFP restored their levels. *p<0.05 and **p<0.01 in comparison to Sham + HBSS, TBI + MSCs-IL-10 and TBI + MSCs-GFP, #p<0.05 in comparison to TBI + MSCs-GFP.
Transplantation of mesenchymal stem cells overexpressing interleukin-10 induce autophagy response, promote neuroprotection in a rat model of traumatic brain injury

Authors: Panchanan Maiti¹,²,³,⁴,⁵,⁶*, Sarah Peruzzaro¹,², Nivya Kolli¹,², Melissa Andrews¹,², Abeer Al-Gharaibeh¹,², Julien Rossignol¹,²,⁷, Gary L. Dunbar¹,²,³,⁴*

¹Field Neurosciences Institute of Laboratory for Restorative Neurology, Central Michigan University, Mt. Pleasant, MI 48859, USA
²Program in Neuroscience, Central Michigan University, Mt. Pleasant, MI 48859, USA
³Department of Psychology, Central Michigan University, Mt. Pleasant, MI 48859, USA
⁴Field Neurosciences Institute, St. Mary’s of Michigan, Saginaw, MI 48604, USA
⁵Department of Biology, Saginaw Valley State University, Saginaw, MI 48610, USA
⁶Brain Research Laboratory, Saginaw Valley State University, Saginaw, MI 48610, USA
⁷College of Medicine, Central Michigan University, Mt. Pleasant, MI 48859, USA

*Address correspondence: Panchanan Maiti. Email: maiti1p@cmich.edu, Ph: 9894973026 or Gary L. Dunbar: dunba1g@cmich.edu, Ph: 9894973105
**Abstract:**

Autophagy, including mitophagy play vital role in neuroprotection in traumatic brain injury (TBI). Transplantation of mesenchymal stem cells (MSCs) provide neuroprotection and induce autophagy by increasing anti-inflammatory cytokines, such as interleukin-10 (IL-10). Due to heterogeneity, it is difficult to measure the levels of IL-10 released by MSCs. We developed genetically engineered MSCs to overexpress IL-10 to deliver greater amount of IL-10. This study investigated the comparative effects of transplantation of MSCs-IL10 and MSCs alone in a rat model of TBI. Adult, male Sprague-Dawley rats were divided into four groups: Sham + vehicle, TBI + vehicle, TBI + MSCs-IL-10 and TBI + MSCs-GFP. First two groups received vehicle, whereas last two groups were transplanted with MSCs-IL-10 or MSCs-GFP, after 36-h of TBI. Following 3 weeks of transplantation, neurodegenerative changes, autophagy, mitophagy, chaperone-mediated autophagy (CMA), cell death/survival markers were investigated. We observed a significant increase number of dead cells in the cortex and hippocampus in TBI rats, whereas transplantation of MSCs-IL-10 significantly reduced their numbers. MSCs-IL-10 group induced autophagy, mitophagy, CMA and restored cell survival markers, along with decreased cell death and neuroinflammatory markers greater than MSCs alone. Transplantation of MSCs-IL10 may be an effective strategy to protect against TBI-induced neuronal damage.

**Key words:** TBI, neuroinflammation, IL-10 overexpressed MSCs, autophagy markers.
1. Introduction

Traumatic brain injury (TBI) is a serious and debilitating health problem affecting millions of people each year [1, 2]. A sudden impact to the head, such as from a fall, car accident, or a blast are the main events that results in a TBI [2, 3], which cause moderate to severe brain tissue damage, lead to motor, sensory, psychological and cognitive dysfunctions [4]. Importantly, the damage may last for months, years, or even the rest of the person’s life [5, 6].

Although several studies have been conducted to investigate the pathophysiology and molecular events involved in neuronal injury in TBI [7-9], it is still not clearly understood. However, inflammation is the main cause for neuronal damage in TBI, due to activation of brain immune cells [10, 11]. Interestingly, during TBI, M1-subtype of microglia become activated and release significant amount of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), IL-1β, IL-6, IL-12 [12], and reactive oxygen species (ROS), which damage the blood brain barrier (BBB) [13] and triggers neuronal injury [14]. In contrast, M2-subtype of microglia release anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, tumor growth factor (TGF) that promotes wound healing and reduction of neuroinflammation [15]. The IL-10 secreted by M2-microglia, mesenchymal stem cells (MSCs), and by some neurons [16] which regulate immune responses and provide cytoprotection. It directly inhibits production and release of pro-inflammatory cytokines [17], inhibits astrocyte activation and increases expression of excitatory amino acid transporter-2 (EAAT2), thus reducing the glutamate excitotoxicity [18], as noted in animal models of spinal cord injury [19], stroke, and TBI [17].

Several research reports suggested that MSCs transplantation play beneficial roles in the treatment of TBI [20], because they can release anti-inflammatory chemokines and reduce neuronal injury [21]. However, due to its heterogenous population it is difficult to determine to
what extent a population of MSCs may release these anti-inflammatory cytokines [22]. Therefore, making genetic engineered MSCs to overexpress IL-10 may provide greater delivery of IL-10 in the injured brain areas to prevent further neurodegeneration [23, 24]. Genetically engineering MSCs to overexpress IL-10 has been shown to reduce inflammation in collagen-induced model of arthritis, graft-versus-host disease, experimental autoimmune encephalomyelitis, and ischemia-reperfusion injury in the lung [25, 26]. Similarly, recently, we have revealed that the transplantation of MSCs genetically engineered to overexpress interleukin-10 (MSCs-IL-10) promote conversion of M1 to M2 macrophages, thus increased anti-inflammatory cytokines and inflammatory responses in rat model of TBI [24].

In addition, previously, we have also performed Morris water maze (MWM), ladder rung walking, and rotarod tasks to assess cognitive function, stepping and inter-limb coordination, gross locomotor performance, respectively after transplantation of MSCs + IL-10 and MSCs alone in TBI rats. Although we did not find significant improvement in learning in the acquisition phase of the MWM task, however, the mean latency was within the range of uninjured rats in the case of TBI + MSCs + IL-10 transplanted rats during the reversal sessions, suggesting transplantation of MSCs + IL-10 improved learning and memory in TBI rats. Similarly, we also found that transplantation of MSCs + IL-10 or MSCs alone significantly improved in fine motor function on the ladder rung walking test, but not on the rotarod test in rats compared to TBI + vehicle-treated rats [24].

One of the mechanisms for MSCs-transplantation-induced neuroprotection in animal models of neurological diseases could be due to reduction of neurodegeneration and cell death and induction of autophagy mechanism [27]. Autophagy, including mitophagy is the evolutionary conserved cellular active clearance mechanism, which delivers intracellular debris
or constituents to lysosomes for their final degradation [28, 29]. It interacts with apoptotic pathways and helps determine cell death and survival [30-32]. It has been shown that transplantation of MSCs significantly enhances autophagolysosome formation in animal models of AD, which increased neuronal survival against toxic amyloid proteins [33-35].

In the present study, we have investigated the neurodegenerative changes, autophagy, mitophagy, CMA markers, levels of molecular chaperones, cell death and cell survival, neuroinflammatory, as well as synaptic markers by comparing the treatment effects of MSCs-IL-10 and MSCs alone, using the rat model of contusion to the medial frontal cortex (MFC) as the TBI model. We have observed that transplantation of MSCs-IL10 improved neuronal morphology, reduced neurodegeneration, as well as diminished number of DNA-fragmented cells greater than MSCs alone. Furthermore, transplantation of MSCs-IL10 also induced autophagy and mitophagy markers, restored cell survival markers, reduced cell death markers as well as pre- and postsynaptic markers effectively than MSCs alone, in a rat model of TBI.

2. Material and Methods

2.1. Chemicals.

Cresyl violet, polybrene, puromycin, Hank’s balanced salt solution (HBSS) and other accessory chemicals were procured from Sigma (St. Louis, MO). Fluoro-Jade B (FJB) stain was purchased from Millipore, (Burlington, MA, AG310). In situ BrdU-Red DNA fragmentation assay kit (TUNEL staining kit) was from Abcam (Cambridge, MA, ab66110). Polyvinylidene difluoride (PVDF) membrane was from Molecular Probe (Grand Island, NY, IPVH00010). Hoechst 33342 solution (20 mM) was purchased from ThermoFisher Scientific (Grand Island, NY, 62249). pGEM-T Easy Vector was from Promega (Fitchburg, WI, A137A). pLenti-CMV-GFP-2A-Puro Vector was from ABM Inc. (Richmond, BC, Canada). The control plasmid, pLenti-CMV-GFP-
2A-Puro vector, was purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The 293FT cell line was purchased from ThermoFisher Scientific (Grand Island, NY, R70007). The information for different antibodies used for this study is provided in Table 1.

2.2. Animals.

Thirty-nine males, Sprague-Dawley (SD) rats (Charles River) approximately 90 days old were used in this study. Rats were paired housed in a 12h/12h reverse light cycle with food and water ad libitum. Rats were randomly divided into four groups: Sham + HBSS (n=10), TBI + HBSS (n=10), TBI + MSCs-IL-10 (CMV-IL-10-GFP, n=9) and TBI + MSCs (CMV-GFP, n=10). All procedures were approved by the Institutional Animal Care and Use Committee at Central Michigan University.

2.3. Rat model of TBI using controlled Cortical Impactor. The method for TBI rat model was developed as described previously [24, 36]. Briefly, the rats were anesthetized using a mixture of 1-3% isoflurane (Henry Schein Co.) and 500 mL-1L/min oxygen and maintained throughout the surgery. Body temperature was maintained at 37°C during surgeries using a physiotemp machine (Physitemp Instruments Inc. Clifton, NJ). Rats were placed on a stereotaxic instrument (Kopf Instruments, Tujunga, CA) and a midline incision was made to expose bregma. Sham rats were then be closed and allowed to recover. Rats in the injured groups then underwent a 6-mm craniotomy at 3 mm anterior to bregma (AP +3.0, ML 0.0 mm). The impactor tip was placed over the exposed brain and compress the cortex at a depth -2.5 mm at a velocity of 2.25 m/s with a duration of 0.5 seconds [36]. The upper skin of the head was stiched and allowed to recover. After surgery, the rats were monitored for any signs of pain or discomfort.

2.4. Lentivirus construction for IL-10.
The detail method for lentivirus construction for IL-10 was described by Peruzzaro and colleagues [24].

2.5. Isolation of mesenchymal stem cells.

The MSCs were isolated and cultured as described previously [24, 37]. Viral production and their expression was confirmed from puromycin (10 µg/mL) selected colonies. Flow cytometry and immunocytochemistry (ICC) were performed to confirm MSCs surface markers and viral transfection. Prior to transplantation surgery, all MSCs were labeled with Hoechst 33342 (20 µg/mL) [24].

2.6. Stem cell transplantation.

Transplantation surgery was performed 36-h after injury. Sham + HBSS and TBI + HBSS rats were injected with HBSS, whereas TBI + MSCs-IL-10 rats were injected with MSCs-IL-10 and TBI + MSCs-GFP rats were transplanted with MSCs-GFP. Briefly, the previous incision was reopened, and a burr hole was then drilled on either side of the skull, directly and the injection were made bilaterally at two depths (AP+3.0, ML ±3.5, DV-3.0 and -1.5 mm) from bregma in the area adjacent to the injury site (200,000 cells/2 µL/hemisphere at a rate of 0.33 µL/min). The injection was completed first at the deepest depth and the skull was closed and allowed to recover.

Schematic diagram showing experimental time scale.

2.7. Tissue processing.
Three weeks after transplantation, all rats were deeply anaesthetized with an overdose of sodium pentobarbital (intraperitoneally), and transcardially perfused with 0.1 M cold PBS, followed by 4% paraformaldehyde (diluted in 0.1 M PBS at pH 7.4) to fix the brains. The brains were then removed, suspended in 4% paraformaldehyde for 24 hours at 4°C, and then transferred to the graded sucrose solutions (10%, 20% and 30%), dissolved in 0.1 M PBS, and then frozen using 2-methylbutane and stored in the -80°C freezer until they were sectioned coronally (30 µm) at a cryostat (Vibratome UltraPro 5000) set at -20°C. Brains from rats used for Western blot analysis were directly removed without perfusion and the fresh tissue were flash-frozen using 2-methylbutane (Sigma) and stored at -80°C until further use.

2.8. Neuronal morphology by Cresyl violet staining.

One of the aims and objectives of this study was to investigate whether treatment of MSCs with overexpression of IL-10 could improve neuronal morphology, especially in the cortex, in the CA1 and CA3 subfields of hippocampus. The rat brains from all four groups were sectioned coronally on a cryostat (Leica, Germany) and then they were stained with 0.1% Cresyl violet (CV) as described previously [41]. The sections were then washed, dehydrated, cleared and mounted by cover slipped using DePex mounting media (BDH, Batavia, IL). The slides were dried, and photomicrographs were taken by compound light microscope (Olympus, Japan) using 100x objectives (total magnification of 1000x). Dark, large dot stained cells were considered as pyknotic or tangle-like cells. The number of pyknotic or tangle-like cells were counted manually using Image-J software (http://imagej.nih.gov/ij) and expressed as number of pyknotic cells per 1mm² area. A minimum of 10 serial sections, with 30 different fields were used to count the number of pyknotic cells in each group (n=6). Two researchers counted the cells separately and an average value were reported.
2.9. Neurodegeneration study by Fluoro-Jade B (FJB) staining.

To detect the neurodegeneration, ten coronal sections (at equal interval from Bregma +2.20 mm to 0.70 mm for cortex and Bregma -2.20 mm to -3.60 mm for hippocampus) from control and treatment tissues were cut on a cryostat (20 µm). The sections were collected in 0.1 M neutral phosphate buffer, mounted on gelatin coated (2%) slides and then air dried on a slide warmer at 50°C for at least half an hour. The detail protocol for FJB staining was followed as described previously [38]. The total number of FJB-positive cells were counted using Image-J software (http://imagej.nih.gov/ij), manually and expressed per 1mm² area. A minimum of 10 serial sections, with 20 different fields were used to count the number of pyknotic cells in each group (n=6). All experimenters were blinded to the group identity of the specimens analyzed.

2.10. Terminal deoxyribonucleic acid nick end labeling (TUNEL).

Coronal brain sections (20 µm) from each of the group of rats were taken in poly-lysine-coated glass slide. Then terminal deoxyribonucleic acid nick end labeling (TUNEL) was performed as described previously [39, 40]. Finally, the sections were counter-stained with Hoechst-33342 (20 mM) for 5 min at room temperature in the dark and washed thoroughly with distilled water, after which they were mounted on a glass slide with anti-fading medium (Sigma). The cells were observed under a fluorescent microscope (Leica, Germany), using appropriate filters (ex/em: 488/576). The red fluorescent signal indicated TUNEL-positive cells. The number of TUNEL-positive cells were counted manually using image J software as reference frame (http://imagej.nih.gov/ij) from three experiments to obtain a mean value and they were expressed per 1mm² area.

2.11. Immunohistochemistry of Atg5, Atg7. Immunoperoxidase technique was used for the
levels of Atg5 and Atg7. Briefly, cryosections (40-μm thick) were rinsed with PBS (0.1 M, at pH 7.4) three times and then incubated with 0.5% Triton-X100 (Fisher Scientific, Pittsburgh, PA), along with 3% H₂O₂ solution (for Atg5 and Atg7), for 30 min at room temperature, followed by three washes in PBS, for 10 min each. The unmasking was done by treating the sections with 10% normal goat serum (Santa Cruz Biotech, Dallas, Texas) for 1 h at room temperature. Then sections were incubated with rabbit monoclonal anti-Atg5 anti-Atg7 antibodies (1:200), which were dissolved in PBS, along with 10% goat serum and placed on the plate on a shaker at low speed and kept at 4°C overnight. On the next day, the sections were thoroughly washed with PBS, three times for 10 min each. The sections were incubated with biotinylated anti-rabbit secondary antibody (Vector Laboratory, CA; 1:200) for 4h at 37°C. After this incubation, the sections were washed three times with PBS, 10 min each, and then treated with ABC reagent for 30 min at room temperature. This was followed by three washes in PBS of 10 min each. Finally, the sections were incubated with peroxidase substrate solution, supplied with the ABC kit (Vector Laboratory, CA), and the signal was developed using diaminobenzidine (DAB) until the desired staining intensity emerged. The tissue was then washed, dehydrated, cleared, mounted on slides and visualized using a compound light microscope (Olympus, Japan).

2.12. Confocal imaging of GFAP, Iba-1, Beclin-1 and LC-3A/B. Immunofluorescent technique was used for detecting Beclin-1 and LC-3A/B levels. Briefly, after blocking with normal goat serum (10%), the sections were incubated with Beclin-1 and LC-3A/B antibodies (1:200, Table 1) for overnight and following day, they were incubated with anti-rabbit secondary antibody (1:500), tagged with FITC (for Beclin-1, Molecular Probes, OR) or Alexa-594 (for LC-3A/B) for 30 min at room temperature. Then the sections were washed thoroughly with distilled water, dehydrated, cleared and mounted on slides using anti-fading fluoro-mount aqueous mounting
media (Sigma), and visualized using table-top Fluoview confocal laser scanning microscope (FV1oi, Olympus) with appropriate filters to optimize excitation and emission.

2.13. Immunohistochemistry and confocal imaging of neuroinflammatory markers

(i) GFAP-immunohistochemistry. Activated astrocytes were detected by immunolabeling with GFAP, as described previously [39]. Briefly, the perfused brains were cryopreserved and coronal sections (40 μm thickness) were obtained using a cryostat (Leica, Germany). The sections were rinsed with 0.1 mM PBS (pH 7.4), blocked with 10% normal goat serum for 1h and incubated with GFAP (rabbit polyclonal, 1:1000) antibody, overnight on a shaker at 4°C. On the next day, the sections were thoroughly washed with PBS, three times, for 10 min each, incubated with anti-rabbit secondary antibody (1:1000), tagged with Alexa-488 (Molecular Probes, OR) for 30 min at room temperature. Then the sections were washed thoroughly with distilled water, dehydrated, cleared and mounted on slides using anti-fading Fluoro-mount aqueous mounting media (Sigma), and visualized using table-top Fluoview confocal laser scanning microscope (FV1oi, Olympus) using appropriate filters for excitation and emission.

(ii) Iba-1 immunohistochemistry. For Iba-1 immunoreactivity, the coronal sections (40 μm) were blocked in 10% normal goat serum in 0.1% Triton X-100 and 0.1M PBS for 1 hour at room temperature. Then the sections were incubated with mouse primary Iba-1 antibody (1:4000, Wako) for 4 hours at room temperature and additional overnight incubation for at 4°C. Following day, tissue was washed 3 times 5 min each in PBS. A secondary antibody conjugated with Alexa fluorophore 546 was applied for 1 hour at room temperature. Both GFAP-IR and Iba-1-IR images were taken using tabletop Fluoview confocal laser scanning microscope (FV1oi, Olympus) using appropriate filters for excitation and emission. Number of GFAP-IR and Iba-1-IR cells were counted around the lesion site, manually, using Image-J software from 10 sections.
in the cortex, CA1 and CA3 subfield of hippocampus and expressed as number of GFAP-IR or Iba-1-IR/1 mm² area.

2.14. Western blot.

About 100 mg of flash-frozen mixed cortex was lysed with cold radio-immuno precipitation assay (RIPA) buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, pH 7.4] with protease and phosphatase inhibitors (Sigma). The tissue was homogenized with tissue homogenizer (ThermoFisher Scientific, Grand Island, NY) and the tissue lysate was centrifuged at 16,000x g for 15 min at 4°C. Following centrifugation, the supernatant was collected and aliquoted in PCR tubes and stored at -80°C until use. Total protein concentrations for individual samples were determined using the Pierce BCA protein assay (ThermoFisher Scientific). Samples were added with equal amounts of 2x SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol and 0.5% bromophenol blue) and boiled for 2 min. Approximately 100 µg of protein, per lane was loaded and electrophoresed on 10% Tris-glycine gel and transferred to PVDF membrane (Millipore, Bedford, MA). After probing with respective primary and secondary antibodies (Table 1), the blots were developed with ImmobilonTM Western Chemiluminescent HRP-substrate (Millipore, Billeria, MA). The relative optical density was measured using Image-J software (https://imagej.nih.gov/ij/). To ensure equal protein loading in each lane, the blots were re-probed for β-tubulin.

2.13. Statistical Analysis. All the data were expressed as Mean ± SEM. All statistics were analyzed using one-way analysis of variance (ANOVA), followed by posthoc Tukey’s Honestly Significant Difference (HSD) test. The probability value, p≤0.05, was considered significant.

3. Results.
Using IHC, RT-PCR and Western blot techniques, we previously confirmed the overexpression of IL-10 levels *in vitro* and *in vivo* in MSCs which was engineered with Il-10 gene [24]. In addition, we have also confirmed an increase levels of IL-10 in TBI rats transplanted with MSCs overexpressed with IL-10 in comparison to other three groups [24] (S1).

### 3.1. Transplantation of MSCs-IL10 protected cortical and hippocampal neuronal damage better than transplantation of MSCs alone in TBI rats.

One of the major objectives of the present study was to characterize the morphological changes in cortical and subcortical areas after TBI and determine whether MSCs-IL-10 can improve their morphology. Therefore, we stained coronal sections with 0.1% Cresyl violet. We observed that the number of pyknotic or tangle-like cells were significantly increased (p<0.01) in the cortex (B), in CA1 (C) (p<0.01), and CA3 (D) (p<0.01) subfields of hippocampus of TBI rats (**Fig 1A-D**) and that these pyknotic cells were significantly decreased (p<0.01) in rats which received MSCs-IL-10 cells. Greater reduction of pyknotic or tangle like cells were observed in these areas in the case of MSCs-IL10 treated rats in comparison of MSCs alone (p<0.01).

### 3.2. TBI-induced neurodegeneration was better protected by transplantation of MSCs-IL10 than MSCs alone.

A distinct phenomenon observed in TBI is the degeneration of neurons. To characterize the neuronal injury in TBI model, the coronal sections were stained with FJB (**Fig 2A**), a marker for neurodegeneration. The number of FJB-positive cells were significantly increased in the cortex (B), in the CA1 (C), and CA3 (D) subfields of hippocampus in TBI group (p<0.01), whereas transplantation of both MSCs-IL-10 and MSCs-GFP significantly (p<0.05) decreased the number of FJB-positive cells (B-D), however, MSCs-IL-10 decreased more degenerated cells than MSCs-GFP alone (p<0.05).
3.3. **Number of DNA fragmented cells were reduced more by transplantation of MSCs-IL-10 than MSCs alone.** To examine the mode of cell death in TBI after transplantation with MSCs-IL-10 or MSCs-GFP, we performed TUNEL staining of the tissue from the cortex, CA1, and CA3 areas of hippocampus (Fig 3). We observed that TBI group significantly increased number of TUNEL-positive cells in the cortex (Fig 3A-B), in the CA1 (Fig 3A and C) and CA3 areas (Fig 3A and D) of hippocampus in comparison to Sham + HBSS (p<0.01). Whereas transplantation of MSCs-IL-10 and MSCs alone significantly decreased (p<0.05) their levels, however, transplantation of MSCs-IL-10 decreased more TUNEL-positive cells than the TBI rats which received MSCs alone (p<0.05).

3.4. **Transplantation of MSCs-IL-10 modulated autophagy markers and PI3K/Akt/mTOR pathway greater than MSCs alone in TBI rats.** One of the aims of the present study was to investigate the autophagic responses after TBI and determine whether transplantation of MSCs-GFP and MSCs-IL-10 cells have any differential regulatory role on autophagy and PI3K/Akt/mTOR pathway. We have observed that autophagy markers Atg5 (Fig 4A-B), Atg7 (Fig 4A, C), LC3A/B-II (4A, D) and p62 (Fig 4A, F) were increased greater in TBI rats which received MSCs-IL10, than by MSCs alone. Whereas, there was a significant decrease levels of PI3K (p85) and p-Akt which were restored by transplantation of MSCs-IL-10, not by MSCs (Fig 4A, G, H, I). Although p-PI3K (p85) was unchanged in TBI rats, however, it was increased by MSCs-IL-10, not by MSCs alone (Fig 4A, H). In contrast, p-mTOR (ser2448) was significantly increased by TBI, and it was restored after transplantation of MSCs-IL-10, but not by MSCs alone (4A, K), whereas there was no meaningful change of total Akt and mTOR levels (Fig 4A, L) in all the four groups.
3.5. Transplantation of MSCs-IL-10 showed greater immunoreactivity of autophagy markers in TBI rats than MSCs alone. Although immunointensity of Atg5 (Fig 5A) and Atg7 (Fig 5B) were appeared to increase in TBI with MSCs-IL-10 and MSCs-GFP groups in the cortex, in the CA1 and CA3 areas of hippocampus in comparison to TBI + HBSS and Sham + HBSS groups, but we did not observe any meaningful changes between Sham + HBSS and TBI + HBSS rats in these areas. Whereas, we observed a decrease levels of immunofluorescent signal of Beclin-1 in TBI rats, and transplantation of MSCs-IL-10, but not by MSCs alone, appeared to increase its level (5C). In addition, immunofluorescent signal for LC3A/B appeared to show more in TBI with MSCs-IL-10 in the cortex, in the CA1 and CA3 areas of hippocampus in comparison to Sham + HBSS, TBI + HBSS and TBI + MSCs (Fig 5D).

3.6. Transplantation of MSCs-IL-10 induced mitophagy markers greater than MSCs alone in TBI rat.

Mitophagy is important for removal of damaged mitochondria, which help in cell survival. Although we did not observe much alteration of NIX, FUNDC1, and BNIP3 in TBI rats in comparison to Sham control, however, transplantation of MSCs-IL-10, but not MSCs alone significantly increased (p<0.05) their levels (Fig 6A-D). In contrast, PINK-1 and HIF-1α levels were significantly down-regulated by TBI rats (p<0.05) and levels of both these proteins were restored by MSCs-IL10, whereas transplantation of MSCs restored HIF-1α levels (Fig 6A, F), but not the PINK-1 levels (Fig 6A, E).

3.7: Transplantation of MSCs-IL-10, but not MSCs alone induced cell survival markers and reduced cell death markers in TBI rats. The levels of PSD95 (Fig 7A-B) and synaptophysin (Fig 7A, C) were significantly decreased (p<0.01) in TBI rats and transplantation of MSCs-IL-10 restored both the PSD95 and synaptophysin levels, not by MSCs alone. In
addition, cell death markers, such as Bax, cytochrome-C, caspase-3 and p53 levels were significantly increased (p<0.05) in TBI rats and transplantation of MSCs-IL10 decreased their levels, however, transplantation of MSCs alone was not able to downregulate Bax and cytochrome-C (Fig 7A, E-F). In contrast, Bcl2 levels was significantly increased by MSCs-IL-10 and MSCs alone in comparison to Sham + HBSS and TBI +HBSS rats, the induction of Bcl2 levels was significantly higher (p<0.05) in the case of MSCs-IL10 in comparison to MSCs alone (Fig 7A, and D).

Supplementary figures

S1: Levels of IL-10 was increased more in MSCs-IL10 than MSCs alone in vitro and in vivo. A: Representative immunocytochemistry images of MSCs with and without IL-10 overexpression. MSCs + IL10-GFP group appeared to have higher IL-10 immunofluorescent signal than MSCs + GFP group. B-C: Western blot data revealed that IL-10 levels was more in the case of MSCs + IL-10 than MSCs + GFP (p < 0.05) in vitro. D-F: Similarly, Western blot from rat cortical tissue showed that there was a significant decrease levels of IL-10 in TBI rats and IL-10 levels was increased after transplantation of MSCs-IL-10, but not by MSCs alone (p < 0.05).

S2: Transplantation of MSCs-IL-10 cells decreased neuroinflammatory markers greater than MSCs alone in TBI rat. Neuroinflammation is one of the principal causes for neuronal injury and death in TBI. Therefore, we investigated the levels of GFAP and Iba-1, the markers for activated astrocytes and microglia. TBI rats showed a significant increase in GFAP-IR in the cortex (A-B), as well as in the CA1 (A, C) and CA3 (A, D) areas of hippocampus, whereas rats transplanted with MSCs-IL-10 and MSCs-GFP had decreased levels. Although we did not find any meaningful changes in the number of Iba-1-IR cells in CA1 (F) and CA3 (G) areas of
hippocampus among the groups, but Iba-1-IR was increased in TBI cortex and it was downregulated by MSCs-IL10 (E), but not by MSCs alone. In addition, both TBI with transplanted groups showed more hyper-ramified or bushy or amoeboid like morphology of microglia (data not shown). Our Western blot data for GFAP from cortical tissue and their densitometric analysis revealed that there was a significant increase level of GFAP in the cortex of TBI rats, which was reduced by transplantation of both MSCs-IL-10 and MSCs-GFP (H-I).

S3: Molecular chaperones and chaperone-mediated autophagy responses in TBI rat after transplantation of MSCs-IL-10 and or MSCs-GFP. Our Western blot data indicate that HSP90 (A-B) become upregulated and HSP40 (A, E) was downregulated by TBI rats and transplantation of MSCs-IL-10 restored their levels, but not by MSCs alone. Whereas, HSP60 was unaltered in all the groups (A, D), but HSP70 (A, C), and CHIP (A, E) levels were increased in TBI with MSCs-IL-10 and MSCs-GFP transplanted rats. Furthermore, chaperone-mediated autophagy (CMA) markers, such as HSC70 (A, F) and LAMP2A (A, G) were decreased in TBI rats and they were upregulated by TBI with transplantation of MSCs-IL-10 and MSCs-GFP in comparison to TBI + HBSS and Sham + HBSS rats.

4. Discussion

Traumatic brain injury (TBI) is one of the leading causes of motor, sensory, psychological, and cognitive dysfunction, and is largely a consequence of increases in neuroinflammation and neurodegeneration [41]. Cell death in TBI is associated with dysregulation of autophagy mechanisms, including mitophagy dysfunction [42, 43]. In the present study, we found a mild to moderate neuroprotective effects, including induction of autophagy, including mitophagy markers, decreased cell death and neuroinflammatory markers, increased cell survival markers, as well as restoration of pre and post-synaptic markers after transplantation of MSCs-IL-10.
and/or MSCs alone in a rat model of TBI. Furthermore, MSCs-IL10 transplanted rats displayed greater neuroprotective effects than rats which received MSCs alone.

Neuroinflammation is one of the key mechanisms associated with neuronal injury in TBI. Pro-inflammatory cytokines, such as IL-10 become down-regulated in TBI, which triggers neuronal death [17, 44]. Transplantation of MSCs have been shown beneficial therapeutic effects in different brain injury models [45], because they secret many neurotropic factors, including many cytokines, such as IL-4, IL-6, IL-10, IL-11, and IL-13 [23, 46, 47]. Among them, IL-10 is the most important because it exerts neuroprotective effects via suppressing the expression of various pro-inflammatory cytokines, such as IFN-γ, IL-1β, IL-2, IL-6, and TNF-α, as observed in stroke [23, 48] and in TBI [46, 47, 49-51]. Although many researchers demonstrated that MSCs have immunomodulatory effects which can secrete many cytokines, including IL-10, whereas some researchers have also showed contradictory results [52]. MSCs may secrete IL-10 under specific conditions, such as inflammatory environment (in presence of IFN-γ, IL-1β and TNF-α), as noted in brain injuries [52]. Although it is unknow in what ideal conditions under which MSCs could secrete IL-10, but it is speculated that transplantation of MSCs may stimulate the cells surrounding the injury and trigger secretion of IL-10 and other neurotrophic factors [52]. Therefore, we genetically modified MSCs to secrete abundant IL-10 and hypothesized that they may improve MSCs-based cell therapy for TBI-induced neuronal injury [24]. We observed and reported an improve levels of IL-10 by MSC-IL-10 than by MSCs alone [24](S1).

We have used the rat controlled cortical impactor injury model and characterized the cell death in the cortex and in the hippocampal subfields, using multiple staining methods. A significant increase immunoreactivity of GFAP and Iba-1 in the cortex after 3 weeks of TBI (S2), were decreased after transplantation of both MSCs-IL-10 and MSCs alone, suggesting
reduction of neuroinflammation [24]. Whereas, transplantation of MSCs alone, was unable to
decrease number of GFAP-IR cells in CA1 and CA3 areas of hippocampus. Similarly,
transplantation of MSCs-IL-10 significantly decreased the Iba-I-IR cells in the frontal cortex but
not by MSCs alone (S2)[24], suggesting MSCs-IL-10 exert greater anti-inflammatory effects
than MSCs alone. This observation was also supported by Nakajima and colleagues in mouse of
model of ischemic stroke after MSCs-IL-10 transplantation [23].

We further investigated the autophagy mechanisms, which are the chief routes for bulk
degradation of aberrant organelles and cytoplasmic components of damage cells and thus,
provide cytoprotection [53-59], as seen in animal models of TBI, and other neurological diseases
[43]. IL-10 may induce autophagy or autophagy can enhance IL-10 production, as reported
previously [60]. Therefore, this study aimed to achieve neuroprotective effects through induction
of autophagy after transplantation of MSCs [23]. We have investigated several autophagy
markers, such as Atg5, Atg7, Beclin-1, LC3A/B, mTOR, p-mTOR levels. We did observe a
significant increase in Atg5 and Atg7 levels after transplantation of MSCs-IL-10 and MSCs-
GFP, indicating that MSCs-IL-10 or MSCs-GFP can induce autophagosome formation (Fig 4).
These findings were supported by other investigators in animal models of AD [31], acute
ischemic stroke [23] and in TBI [61]. We have investigated levels of Beclin-1, a Bcl2 interacting
partner, which are involved in autophagic cell death and apoptosis [62]. We observed that
Beclin-1 level was less in TBI rats (Fig 4), which was also reported by Au and colleagues in a
mouse model of closed head injury produced by dropping a weight onto the intact skull [56].
Interestingly, transplantation of MSCs-IL-10 and/or MSCs-GFP restored Beclin-1 levels,
indicating that the transplants exerted cytoprotective effects [59]. Similarly, conversion of
microtubule-associated protein light chain-3A/B-I (LC-3A/B-I) to LC3A/B-II, is a reliable
biomarker for autophagy [63]. LC3A/B-II was significantly increased by MSCs-IL-10, but not by MSCs-GFP (Fig 4), indicating MSCs-IL-10 is a greater autophagy inducer. However, Klionsky and colleagues indicated that to study autophagy, it is essential to investigate autophagy flux, along with LC3A/B levels [57]. Therefore, to monitor autophagic activity we investigated p62 (also called sequestosome1, SQSTM1) levels. It directly binds to LC3 and its degradation can indicate decreased level of LC-II [59]. We have observed a significant increased level of p62 levels in TBI-MSCs-IL10 rats, suggesting autophagy mechanisms were induced by MSCs-IL-10, not by MSCs alone (Fig 4F). Although increase p62 levels is an indicator of decrease autophagy, whereas we found an increased level of p62 in MSCs-IL10 rats, which may be due to the blocking of fusion of autophagy vacuoles with lysosome or by the inhibition of a later maturation step of autophagosome degradation, which needs further experiment to confirm the findings.

Then we have investigated the key proteins which regulate autophagy mechanisms, such as phosphoinositol 3-kinase (PI3K), Akt (protein kinase B) and mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR pathway) [64]. These protein have been reported to be involved in the neuroprotection in cerebral injury [61]. The levels of mTOR and p-mTOR have been reported to increase in the cortex and hippocampus of mice at 24 hours after TBI [64]. Increased levels of p-mTOR are the indicators of decreased autophagic responses [65-67]. Using rapamycin Zhang and colleagues observed that there was an increase in autophagic responses, including enhancement of Beclin-1 levels, along with improve neurobehavioral function, increase neuronal survival, reduce inflammation and gliosis in TBI [61]. In the present study, we observed that the levels of PI3K (p85) and p-Akt were decreased [p-PI3K (p85) was unchanged] and p-mTOR was up-regulated by TBI, whereas transplantation of MSCs-IL-10, but
not MSCs alone increased their levels (**Fig 4G-I**), suggesting autophagy was inhibited by TBI and transplantation of MSCs-IL10 activated this pathway. Decreased levels of p-mTOR correlated with increased levels of LC-3A/B-II (**Fig 4E**) and induction of autophagy mechanisms by MSCs-IL-10 may be regulated by PI3K/Akt/mTOR pathway in TBI rats, which require further experiment to confirm the findings.

In addition, mitochondria dysfunction, including reduction of mitochondrial respiration, increase production of ROS have been observed in TBI, which triggers apoptotic cell death [72]. We have investigated whether transplantation of MSCs or MSC-IL-10 have any role in selective degradation of damaged mitochondria by mitophagy. Therefore, we have investigated the most important mitophagy markers, such as NIX, BNIP3, FUNDC1, PINK1 and HIF-1α. Interestingly, we found a significant increase in mitophagy markers, such as NIX, BNIP3, FUNDC1, PINK-1 and HIF-1α levels following transplantation of MSCs-IL10 (**Fig 6**), but not by MSCs alone, suggesting that the damaged mitochondria were selectively degraded via mitophagy, and provided neuroprotection greater by MSCs-IL-10 than MSCs alone.

Molecular chaperones, or heat shock proteins (HSPs), are involved in cell death and survival by degrading small, misfolded proteins [68, 69]. We observed that HSP90 was significantly upregulated (**S3**) by TBI rats. Increase HSP90 have been reported to be involved in brain injury [70]. Decrease levels of HSP90 by MSC-IL-10 was greater than MSCs alone, suggesting MSC-IL-10 has greater cytoprotective roles by downregulating its levels. In contrast, loss of HSP40 and HSP70 increase brain injury and death of neurons [71], whereas they can induce and arrest inflammation and improved neurological outcome [72]. We found a decreased of HSP40 and HSP70 in TBI rats and their levels were restored by MSCs-IL.10, but not by MSCs
(S3), suggesting MSCs-IL-10 may induce immunomodulatory and neuroprotective roles perhaps through HSPs. In addition, CMA markers, such as HSC70 and LAMP2A, were only modestly decreased in the TBI rats (S3), with respect to control rats, but their levels were increased by both the transplanted groups, suggesting CMA was activated to remove some of the debris generated by the transplanted cells.

There were less pyknotic or tangle-like cells as revealed by CV stain (Fig 1), along with decreased neurodegeneration as shown by FJB stain (Fig 2), and reduced number of TUNEL-positive cells (Fig 3) in MSCs-IL-10 transplanted TBI rats, relative to those in TBI + MSCs rats, suggesting the neuroprotective effects might be due to IL-10. We have further investigated the important cell death and cell survival markers, such as Bax, cleaved caspase-3, cytochrome-C, and p53, along with Bcl2, PSD95, and synaptophysin levels. We clearly observed that there were decreased levels of Bax, caspase-3, cytochrome-C after transplantation of MSCs-IL10, but not by MSCs. Similarly, anti-apoptotic markers Bcl2, postsynaptic markers PSD95 and synaptophysin were also restored by MSCs-IL10, but not by MSCs alone. In addition, increase levels of p53 is also involved in TBI-induced cell death [73], which was decreased by MSCs-IL10, but not by MSCs alone, suggesting MSCs-IL10 showed greater neuroprotective effects than MSCs alone. These observations also suggest that the neuroprotective effects may be coming from IL-10, which was supported by our previous finding where we found that MSCs-IL10 increase conversion of M1 to M2 macrophage which secrete anti-inflammatory cytokines to protect further brain injury [24, 74]. Increased levels of these markers may be due to decrease neuroinflammation by increasing anti-inflammatory cytokines (by M2 macrophages which was increased by MSC-IL-10), increased neurotropic supports coming from transplanted cells, as well as increases in the autophagy mechanisms [79, 80]. These findings also indicating that
transplanted cells may secrete many other neurotropic factors [23], along with IL-10, as reported by other investigators in mouse models of TBI [81, 82], which supports our findings. Overall, transplantation of MSCs-IL-10 showed greater neuroprotection than by MSCs alone, by improving neuronal morphology and decreased cell death in the TBI rats, along with increased autophagy and mitophagy mechanism, which may be regulated by PI3K/Akt/mTOR pathway. Additional experiments are required to investigate the detail mechanism of MSC-IL-10-induced neuroprotection in TBI.

Overall, the controlled cortical impact model of TBI in rats produced significant neurodegeneration and cell death in the cortex and in the hippocampus, whereas transplantation of MSCs-IL-10 provided greater neuroprotection than MSCs alone. Transplanted MSCs-IL-10 induced autophagy, mitophagy, chaperone-mediated autophagy, molecular chaperones, regulated PI3K/Akt/mTOR pathway, and influenced cell death and cell survival markers more efficiently than MSCs alone. Therefore, induction of autophagy mechanisms, using MSCs that overexpress IL-10, may be an effective strategy for protecting the brain against TBI-induced cell death.

Acknowledgments: This work was supported by the Field Neurosciences Institute, St. Mary’s of Michigan, and the John G. Kulhavi Professorship in Neurosciences and the Neuroscience Program, College of Medicine at Central Michigan University. We are thankful to Dr. Jeffery Smith, Dr. Michael Hoane, the Brain Research Laboratory, Saginaw Valley State University, for generously lending their contusion devise and proving a supportive infrastructure. Also, we would like to thank Olivia Pupiec, Melissa Resk for their technical help and Dr. Ming Lu for his critical advice on viral construction.

Disclosure of Interest: The authors declare that they have no competing interests to publish this research article.

Funding: This work was supported by the Field Neurosciences Institute, St. Mary’s of Michigan, and the John G. Kulhavi Professorship in Neurosciences and the Neuroscience Program at Central Michigan University.
Author’s contributions: PM and SP designed the study. PM collected, analyzed, and interpreted data and wrote the manuscript. NK was involved in histology and immunohistochemistry. SP and MA were involved in animal surgery, tissue collection. AG was involved in Western blots for mitophagy markers. GD and JR oversaw, edited and contributed to discussion and provided overall supports. All authors approved the final manuscript.

References


36. Peruzzaro ST, Gallagher J, Dunkerson J, Fluharty S, Mudd D, Hoane MR, Smith JS. The impact of enriched environment and transplantation of murine cortical embryonic stem cells on...


44. Csuka E, Morganti-Kossmann MC, Lenzlinger PM, Joller H, Trentz O, Kossmann T. IL-10 levels in cerebrospinal fluid and serum of patients with severe traumatic brain injury:


Figures legends

**Fig 1**: Transplantation of MSCs-IL-10 improved neuronal morphology greater than MSCs alone in the cortex and hippocampus of TBI rats. Rat brains were sectioned and stained with 0.1% Cresyl violet and images were taken by compound light microscope (Olympus) with 100x objectives (total mag 1000x). A: Representative photomicrograph of TBI rats showed increase number of pyknotic or tangle-like cells in the cortex, in the CA1 and CA3 subfields of hippocampus. B-D: Number of pyknotic cells were significantly decreased by transplantation of MSCs-IL-10 in comparison to TBI rats (p<0.01) and with TBI+MSCs (p<0.01). The greater reduction of pyknotic cells was observed in the case of MSCs-IL-10 rats. Arrows indicate pyknotic or tangle-like cells. Scale bar indicates 100 µm and is applicable to other images. **p<0.01 in comparison to TBI + HBSS, TBI + MSCs-IL-10 and TBI + MSCs; *p<0.05 in comparison to TBI + MSCs; ##p<0.01 in comparison to TBI + MSCs.

**Fig 2**: Transplantation of MSCs-IL-10 reduced more number of degenerated neurons in in the cortex and hippocampus of TBI rats than MSCs alone. Rat brains were sectioned (20 µm) and stained with Fluoro-Jade B (FJB) solution (0.0004%) and images were taken using fluorescent microscope (Leica, Germany). A: Representative images of FJB-stained sections from the cortex, in the CA1 and CA3 area of hippocampus. B-D: The number of FJB-positive cells were significantly increased (**p<0.01) in TBI rats, whereas transplantation of both MSCs-IL-10 and MSCs alone, significantly decreased the number of FJB-positive cells. The number of FJB cells were significantly less (#p<0.05) in the case of TBI + MSCs-IL-10 in comparison to TBI + MSCs. Green signals (red arrows) indicate FJB-positive cells and blue signal is for DAPI (nuclear) stain. Scale bar indicates 100 µm and is applicable to other images. **p<0.01 in
comparison to TBI + HBSS, TBI + MSCs-IL-10 and TBI + MSCs; *p<0.05 in comparison to TBI+MSCs; #p<0.05 in comparison to TBI+MSCs.

**Fig 3:** Transplantation of MSCs-IL-10 reduced greater number of DNA fragmented cells in the cortex and hippocampus of TBI rats than MSCs alone. Terminal deoxyribonucleic acid nick end labeling (TUNEL) was performed in coronal sections from cortex, CA1 and CA3 subfields of hippocampus. A: Representative photomicrographs showed an increase number of TUNEL-positive cells in the cortex, as well as in the CA1 and CA3 areas of the hippocampus. B-D: The number of TUNEL-positive cells were significantly increased (**p<0.01) in TBI rats, whereas transplantation of both MSCs-IL-10 and MSCs alone, significantly decreased their numbers. The number of TUNEL positive cells were significantly less (#p<0.05) in the case of TBI + MSCs-IL10 in the cortex and CA3 area of hippocampus in comparison to TBI + MSCs. Red signals (white arrows) indicate TUNEL-positive cells and blue signal is for DAPI (nuclear) stain. Scale bar indicates 100 µm and is applicable to other images. **p<0.01 in comparison to TBI+HBSS, TBI+MSCs-IL-10 and TBI+MSCs; *p<0.05 in comparison to TBI+HBSS; #p<0.05 in comparison to TBI+MSCs.

**Fig 4:** Transplantation of MSCs-IL-10 modulated autophagy markers and PI3K/Akt/mTOR pathway greater than MSCs alone in the TBI rats.

Equal amount of protein from cortical tissue homogenates were electrophoresed, blotted on PVDF membrane and different autophagy markers were studied. A: Representative Western blots of Atg5, Atg7, Beclin-1, LC3A/B, p62, PI3K, p-Akt, Akt, p-mTOR, mTOR from mixed cortical tissue from different animal groups. B-C: Densitometric data indicating that TBI with MSCs-IL-10 and MSCs groups of rats showed an increase in Atg5 (B) and Atg7 (C) in comparison to TBI + HBSS or Sham + HBSS rats and greater increase was noted in the case of
MSCs-IL-10 rats in comparison to MSCs alone. **D-E**: Western blot data showed that there was a decrease in Beclin-1 levels in the TBI rats, which was restored by transplantation of MSCs-IL-10, but not by MSCs alone. **E-F**: Western blot data showed that there was an increase levels of in LC-3A/B-II and p62 in TBI rats transplanted with MSCs-IL-10, but not by MSCs alone. **G-I**: PI3K (p85) and p-PI3K (p85) and p-Akt levels were less in TBI rats and they were restored by MSCs-IL-10, not by MSCs alone. **K**: p-mTOR levels was increased by TBI rats and restored by MSCs-IL-10, not by MSCs alone. *p<0.05 and **p<0.01 in comparison to other groups; #p<0.05, ##p<0.01 in comparison to MSCs alone.

**Fig 5**: Immunohistochemistry of autophagy markers in TBI rats after transplantation of MSCs-IL-10, and MSCs-GFP cells. Coronal sections from each group were immunolabeled with Atg5, Atg7, Beclin-1 and LC3A/B antibodies. The images were taken by either light microscope (Olympus) or by tabletop Fluoview confocal laser scanning microscope (FV1oi, Olympus). Atg5 (A) and Atg7 (B) appeared to increase their levels in TBI rats after transplantation of MSCs-IL-10 and MSCs-GFP when compared to TBI and sham controls. Whereas, TBI section appeared to contain less beclin-1 immunofluorescent signal in the TBI rats when compared to sham control or the other transplanted groups (C). Furthermore, TBI rats showed relatively less immunofluorescent puncta of LC3A/B (D) in comparison to TBI rats, whereas its level was increased after transplantation of MSCs-IL-10 cells and by transplants of MSCs-GFP cells in comparison to sham control and TBI rats. Arrows indicate LC-3A/B immunoreactivity. Blue color: Hoechst-3442 and green color: secondary antibody tagged with Alexa fluoro-488. Scale bar indicates 50 µm and applicable to other images.

**Fig 6**: Transplantation of MSCs-IL-10, but not MSCs alone increased mitophagy markers in TBI rats. Western blot analyses showed that mitophagy markers, such as NIX, FUNDC1, and
BNIP3 were unaltered, whereas PINK-1 and HIF-1α were downregulated by TBI rats, and transplantation of MSCs-IL-10, but not transplantation of MSCs alone improved their levels. *p<0.05 and **p<0.01 in comparison to TBI + HBSS and Sham + HBSS and TBI + MSCs-GFP rats; #p<0.05 and ##p<0.01 in comparison to TBI + MSCs.

Fig 7: Transplantation of MSCs-IL-10, but not MSCs-GFP improved synaptic and cell survival markers and decreased cell death markers in TBI rats. A-C: Pre-synaptic and post-synaptic markers, such as synaptophysin and PSD95 were down-regulated by TBI and transplantation of MSCs-IL-10 and MSCs alone improved both, but MSCs-IL-10 improved greater than MSCs alone. D: Anti-apoptotic markers Bcl2 was increased greater by MSCs-IL10, than MSCs alone. E-H: Whereas cell death markers, such as Bax, cytochrome-C, caspase-3 and p53 levels were increased by TBI and transplantation of MSCs-IL10, decreased their levels more effectively than by MSCs alone. *p<0.05 and **p<0.01 in comparison to Sham + HBSS rats, #p<0.05 and ##p<0.01 in comparison to TBI + MSCs.

Supplemental figures

S1: Levels of IL-10 in MSCs in vitro and rat cortical tissue after transplantation of MSCs-IL-10 and MSCs. A: Representative immunocytochemistry images of MSCs with and without IL-10 level. MSCs + IL10-GFP group appeared to have higher IL-10 immunofluorescent signal than MSCs-GFP cells. B-C: Western blot from in vitro studies showed that there was a significantly higher levels (*p<0.05) of IL-10 production in MSCs-IL10 group in comparison to MSCs alone. D-E: Similarly, in vivo studied showed that there was a significantly higher amount of IL-10 (*p<0.05) production in TBI + MSCs-IL-10 rats in comparison to MSCs alone. Scale bar = 50 μm. Error bars represent standard error of the mean (± SEM).
S2: Transplantation of MSCs-IL-10 cells decreased GFAP levels, but not Iba-1 in TBI rats.
Coronal section (40-µm) from cortex and hippocampus were immuno-labeled with GFAP and Iba-1 antibodies. **A-D**: Both the transplanted groups significantly decreased the mean number of GFAP-IR cells in the cortex (**B**), whereas in the hippocampus, the mean number of GFAP-IR cells were significantly decreased by MSCs-IL-10, but not by MSCs alone (**C-D**). *p<0.05 and **p<0.01 in comparison to Sham + HBSS, TBI + MSCs-IL-10 and MSCs-GFP rats, #p<0.5 in comparison to MSCs-GFP. **E-G**: Iba-1-IR was significantly decrease by MSCs-IL-10, but not by MSCs alone in the cortex, whereas there were no significant differences in Iba-1-IR cells in hippocampus among the groups. *p<0.05 in comparison to Sham + HBSS and TBI + MSCs-IL-10 rats, #p<0.5 in comparison to MSCs-GFP. Scale bar indicates 50 µm and applicable to other images. Green: GFAP and Red: Iba-1. **H-I**: Western blot data (**H**) and their densitometric analysis (**I**) showed that there was a significant increase level of GFAP in cortical tissue in TBI rats and both transplantation of MSCs-IL-10 and MSCs alone reduced its levels. **p<0.01, compared to TBI + HBSS.

S3: Molecular chaperones and chaperone-mediated autophagy markers were greater regulated by MSCs-IL10 than MSCs alone in TBI rats. Three weeks after transplantation, the flash-frozen mixed cortex was homogenized with RIPA buffer, and the supernatant was collected for measuring protein levels. Equal amount of protein was run with SDS-PAGE, blotted on PVDF membrane and probed with different heat shock proteins antibodies and its signal was detected by chemiluminescent reagents. **A**: Representative Western blot data showed the HSPs levels in mixed cortex tissue from different animal groups. **B-F**: Densitometric analysis revealed that TBI in the MSCs-IL-10 and MSCs-GFP groups of rats showed an improvement of different HSPs and CHIP levels when compared to TBI rats. **G-H**: Western blot analysis indicated that the
chaperone-mediated autophagy markers, such as HSC70 (G) and LAMP2A (H) were
downregulated in TBI rats and transplantation of MSCs-IL-10 and MSCs-GFP restored their
levels. *p<0.05 and **p<0.01 in comparison to Sham + HBSS, TBI + MSCs-IL-10 and TBI +
MSCs-GFP, #p<0.05 in comparison to TBI + MSCs-GFP.
Table-1: Sources of different antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Source</th>
<th>Type</th>
<th>Company</th>
<th>Catalog no.</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>12163</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Wako</td>
<td>019-19741</td>
<td>Richmond, VA</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>12389</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>HSP90</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>4877</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>HSP70</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>4872</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>HSP60</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>12165</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>HSC70</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>8444</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>HSP40</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>4871</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>CHIP</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>2080</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Atg5</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>12994</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Atg7</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>8558</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>3738</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>LC3A/B</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>12741</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>p62</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>5114S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>2983</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>p-mTOR</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>2971</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>LAMP2A</td>
<td>rat</td>
<td>Monoclonal</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-8100</td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>NIX</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>12396</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>BNIP3</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>Santa Cruz Biotechnology</td>
<td>Sc-56167</td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>PINK1</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>6946S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>FUNDC1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Abcam</td>
<td>ab74834</td>
<td>Cambridge, MA</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>14179</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>pAkt (Ser473)</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>9271</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Akt</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>2972</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Antibody</td>
<td>Species</td>
<td>Type</td>
<td>Company</td>
<td>Catalog Number</td>
<td>Location</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------</td>
<td>--------</td>
<td>--------------------------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>4292S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>PSD95</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-71933</td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>Rabbit</td>
<td>Mouse</td>
<td>Cell signaling Technology</td>
<td>12270S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>p53</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>9282</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>Santa Cruz Biotechnology</td>
<td></td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>Bax</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>2772S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Cytochrome-C</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>11940S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Cell signaling Technology</td>
<td>9662S</td>
<td>Danvers, MA</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>Cell signaling Technology</td>
<td>15115</td>
<td>Danvers, MA</td>
</tr>
</tbody>
</table>