Neural Stem Cells Derived From Primitive Mesenchymal Stem Cells Reversed Disease Symptoms and Promoted Neurogenesis in an Experimental Autoimmune Encephalomyelitis Mouse Model of Multiple Sclerosis

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Research

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Abstract

Background

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system (CNS). MS affects millions of people and causes a great economic and societal burden. There is no cure for MS. We used a novel approach to investigate the therapeutic potential of neural stem cells (NSCs) derived from highly proliferative human primitive mesenchymal stem cells (MSCs) in an experimental autoimmune encephalomyelitis (EAE) mouse model of MS.

Methods

MSCs were differentiated into NSCs, labeled with PKH26 and injected into the tail vein of EAE mice. The effect of transplanted cells on the disease process was assessed by neurobehavioral changes in the mice. Two weeks following cell transplantation, the animals were sacrificed to collect blood, lymphatic, and CNS tissues for analysis. Transplanted cells were tracked in various tissues by flow cytometry. Immune infiltrates were determined and characterized by H&E and immunohistochemical staining, respectively. Levels of immune regulatory cells, Treg and Th17 were analyzed by flow cytometry. Myelination was determined by Luxol fast blue staining and immunostaining. *In vivo* fate of transplanted cells and expression of inflammation, astrogliosis, myelination, neural, neuroprotection, and neurogenesis markers were investigated by using immunohistochemical and qRT-PCR analysis.

Results

MSC derived NSCs expressed specific neural markers, NESTIN, TUJ1, VIMENTIN and PAX6. NSCs improved EAE symptoms more than MSCs when transplanted in EAE mice. Post-transplantation analyses also showed homing of MSCs and NSCs into the CNS with concomitant induction of anti-inflammatory response resulting in reduction of immune infiltrates. NSCs also modulated Treg and Th17 cell levels in EAE mice comparable to healthy controls. Luxol fast blue staining showed significant improvement in myelination in treated mice. Further analysis showed that NSCs upregulated genes involved in myelination and neuroprotection but downregulated inflammatory and astrogliosis genes more significantly than MSCs. Importantly, NSCs differentiated into neural derivatives in the CNS and promoted neurogenesis possibly by modulating BDNF and FGF signaling pathways.

Conclusions

NSC transplantation reversed the disease process by inducing an anti-inflammatory response and promoting myelination, neuroprotection, and neurogenesis in EAE disease animals. These promising results provide basis for clinical studies to treat MS using NSCs derived from primitive MSCs.

Background

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system (CNS) (1). MS affects approximately 400,000 individuals in the United States and 2.5 million people worldwide and as a result inflicts a substantial economic and societal burden due to the early age of disease onset and recurrent relapses (2). In MS, inflammatory responses are elicited by the intrinsic immune system as well as by parenchymal glial cells, which contribute to oligodendrocyte damage, demyelination, axonal damage and impaired or reduced neuronal signaling (1). Multiple factors including genetics and environmental agents seem to be involved with the onset of MS (1). Although, drug treatments help in reducing disease progression or minimizing disability in patients, they cause severe side effects and do not reverse the symptoms of MS (3). Therefore, it is imperative to design therapeutic strategies that not only stop disease progression, but also repair the damage to the CNS without severe side effects to MS patients. With the advent of recent progress in stem cell research, cell therapy is considered a more promising alternative to drug therapy (4). Mesenchymal stem cells (MSCs) have shown paracrine effects by secreting cytokines and trophic factors involved in immunomodulation (5). MSCs are known to home towards the site of injury and participate in the healing and repair processes throughout life (6). Therefore, there is tremendous interest in MSCs due to their medicinal properties. However, MSCs isolated from adult sources have poor growth as well as limited self-renewal and differentiation potential, and pose a significant risk of graft versus host disease (GvHD) (6). On the other hand, MSCs isolated from perinatal sources are more naïve, exhibiting a low risk of host immune response due to the lack of expression of the major histocompatibility complex (MHC) class II antigens and thus do not cause GvHD (6). Recently, we have isolated highly proliferative primitive MSCs, which can be rapidly amplified to large numbers, and differentiated into functional derivatives, for large-scale animal and clinical studies (6-8). MSCs are known to have homing capabilities to the site of injury as well as the ability to secrete cytokines and growth factors that may indirectly promote endogenous tissue regeneration (5). However, the ability of transplanted MSCs to cross the blood brain barrier (BBB) to repair the damaged CNS is not well established (9). Several reports describe the ability of neural stem cells (NSCs) to migrate to the area of pathology in experimental models of CNS diseases (10, 11). In addition, resident NSCs that produce new neurons throughout life are continuously decreased in MS patients (12). Therefore, stem cell-based therapies that promote endogenous neural regeneration to repair the damage in the CNS (13, 14), may prove ideal for the treatment of MS. In this study, we applied a novel cell therapy approach whereby highly proliferative and naïve primitive MSCs were first differentiated into NSCs that were transplanted into an experimental autoimmune encephalitis (EAE) mouse model, which is known to share similar characteristics and pathological features with MS (15). Our results demonstrated that EAE disease symptoms were reduced or reversed and significant improvements were observed in the pathology of the CNS in animals transplanted with MSCs and NSCs, with the latter being the more effective. The transplanted cells survived and migrated to the CNS where they promoted myelination and neural protection by modulating immune response and inhibiting astrogliosis as well as induced endogenous neurogenesis, thereby leading to functional recovery. Despite being highly proliferative MSCs or their derivatives, NSCs did not cause immune rejection or teratoma formation. These studies demonstrate that NSCs derived from primitive MSCs have therapeutic potential to treat MS and other neurodegenerative diseases.

Materials And Methods

Maintenance and culture of primitive MSCs

Previously isolated and characterized naïve primitive MSCs (8), were maintained using growth medium (GM) containing DMEM nutrient mix F12 medium (DMEM/F12; Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; VWR, Radnor, PA, USA), and 5.6% of antibiotic solution (0.1% gentamicin, 0.2% streptomycin, and 0.12% penicillin) (Sigma, St Louis, MO, USA), and incubated at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. Using this medium, primitive MSCs were maintained and expanded into large numbers without the loss of their self-renewal and differentiation potential.

Differentiation of primitive MSCs into NSCs and oligodendrocytes cells (ODCs)

Primitive MSCs were induced towards the neural lineage by culturing the cells in induction media containing 10 µg epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA) in DMEM/F12 basal medium supplemented with 5.6% antibiotic solution for 3 days. Then replaced with neural media containing 20 µg EGF, 20 ng basic fibroblast growth factor (bFGF), 2 mM Glutamine (Sigma), 1x B27 supplement (Thermo Fisher Scientific, Waltham, MA, USA), in neurobasal medium for 2 weeks. NSCs were differentiated into ODCs by culturing in neurobasal medium containing 2% B27, 10 ng bFGF, 10 ng PDGF-AA (PeproTech), and 100 ng SHH (PeproTech) for 2 weeks and then 0.5% FBS, 2% B27, 1x N2, and 30 ng T3 (Thermo Fisher Scientific) for an additional 10 days. Characterization of NSCs and ODCs for the expression of neural and ODC markers was performed by flow cytometry, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), immunocytochemical staining, and western blot analyses.

Flow Cytometry analysis

Cells were grown to 70% confluency, trypsinized, washed with PBS and pelleted. Cells were stained against CD44 and CD90 (FITC labeled antibodies) or CD29, CD73, CD105 (APC labeled antibodies) (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FACS Canto II (Becton Dickinson) and Diva Software (Becton Dickinson).

Immunocytochemical analysis

Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.5% Triton X-100 (Sigma) and blocked with 2% bovine serum albumin (Sigma) for 1 hour. Cells were then treated with primary antibodies at 1:100 dilution: NESTIN, TUJ1, VIMENTIN, PAX6, OLIG2, O4, MBP, and MOG at 4°C overnight, followed by staining with secondary antibodies at 1:200 dilution at room temperature for 2 hours. Cells were counterstained with DAPI at 1:100 dilution for 5 minutes at room temperature. Fluorescent images were captured using a confocal microscope (NIKON Instruments Inc.,

Melville, NY, USA). Fluorescent intensity and percentage of positive cells were calculated using ImageJ software (NIH, Bethesda, MD, USA).

Western blot analysis of cells

Cells were lysed using RIPA buffer (Sigma) and protein was quantified using the Pierce™ 660 nm protein assay kit and NanoDrop 1000 spectrophotometer (Fisher Scientific). The cell lysate (30 µg of total proteins) was resolved using SDS-PAGE with 12% resolving gel and 6% stacking gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at a continuous current of 100V for 90 minutes. For antibody staining, the membrane was blocked with 5% nonfat dry milk dissolved in TBS 1X containing 0.1% Tween-20 (TBST) for 30 minutes and incubated with primary antibodies at a 1:500 dilution in the blocking solution overnight at 4°C. The membrane was then washed with TBST and incubated with the secondary antibody conjugated with HRP at a 1:10000 dilution in blocking solution for 2 hours at room temperature. After washing with TBST, the blot was stained with Bio-Rad chemiluminescence for 5 minutes and bands were visualized using a chemidoc (Bio-Rad). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to GAPDH.

Induction of EAE in mice

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Oakland University, Rochester, Michigan (IACUC #18081) and the Institutional Biosafety Committee (IBC), Oakland University, Rochester, Michigan (IBC #2858). 6 week old C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and EAE was induced by subcutaneous immunization with 200 µg of myelin oligodendrocyte glycoprotein (MOG)_{33–35} peptide in complete Freud's adjuvant containing 2-5 mg killed mycobacterium tuberculosis H37Ra/mL emulsion (Hooke Laboratories, Lawrence, MA, USA). Mice were also injected intraperitoneally (i.p.) with pertussis toxin (100 ng, Hooke Laboratories) at the day of immunization and 24 hours later following the published protocol (16). Clinical score was blindly registered according to the following scale: 0: no clinical signs, 0.5: partial limp tail, 1: limp tail, 1.5: mild impaired righting, 2: severe impaired righting, 2.5: mild paresis of one hind limb, 3: severe paresis of one hind limb, 3.5: severe paresis of one hind limb and mild paresis of second hind limb, 4: paresis of two hind limbs, 4.5: paresis of both hind limb and partial fore limb, and 5: severe paralysis or death (16). Non-injected C57BL/6J mice were used as controls. All mice were monitored and assessed for clinical score twice a day and maintained at the Oakland University Animal Facility under a 12/12-hour light and dark cycle.

Cell transplantation in EAE mice

Cells were labeled with cell membrane labeling dye PKH26 (Sigma) following manufacturer's instructions (17). Confocal microscopy and flow cytometry analyses were used to confirm the efficiency of fluorescently labeling cells prior to transplantation. There were eight treatments (n=6 each), 1. Untreated healthy control, 2. healthy control+MSCs, 3. healthy control+NSCs, 4. Untreated EAE, 5. EAE score 1+MSCs, 6. EAE score 1+NSCs, 7. EAE score 2+MSCs, and 8. EAE score 2+NSCs. The experiments were

performed in triplicates. The mice were restrained in a rodent restrainer for cell transplantation. 10^6 primitive MSCs or NSCs were injected intravenously via the tail vein at either clinical score 1 or 2. The mice were monitored for 15 days following cell transplantation and then euthanized humanly to collect CNS, lymphoid tissues (spleen, brain, and spinal cord), and blood for analysis.

Tracking of transplanted cells

Blood and tissue samples were placed in red blood cell lysis buffer for 5 minutes and then homogenized, except blood samples, in a centrifuge tube. Cell culture medium was then added to the tube, inverted, and centrifuged at 1300 rpm for 5 minutes at 4°C. The supernatant was discarded, and cells were resuspended in PBS 1x. The cells were then filtered through a cell strainer. Labeled cells in each sample were determined using the FACS Canto II (Becton Dickinson) and Diva Software (Becton Dickinson).

Histochemical and immunofluorescence staining

For paraffin embedding, brain and spinal cord tissue samples were fixed with 4% paraformaldehyde for 24 hours. They were then dehydrated in 70% ethanol for 1 hour, 95% ethanol for 1 hour, 100% ethanol for 4 hours, xylene for 2 hours, and paraffin for 3 days. Tissue samples were then imbedded in paraffin and sectioned (5-10 µm thick) using a microtome. The paraffin embedded samples were stained with H&E (Hematoxylin and eosin; Thermo Fisher Scientific) to evaluate the cellular structure of the tissue and Luxol fast blue (LFB; Sigma) to examine myelination.

To detect the expression of human neural and inflammatory markers expressed by mouse brain and spinal cord tissue, the sections were stained with primary antibodies 1:100 dilution: CD45, CD68, CD3E, GFAP, MBP, MOG, TUJ1, NESTIN, OLIG2, O4 and HNA, respectively, at 4°C overnight followed by staining of secondary antibodies at 1:200 dilution at room temperature for 2 hours. They were counterstained with DAPI at 1:100 dilution for 5 minutes at room temperature. Representative fluorescent images/section of the randomly selected stained sections were captured using a confocal microscope (NIKON Instruments Inc.).

Quantification of Treg and Th17 cells in the blood, spleen, and CNS tissues

For flow cytometry analysis, the tissue samples were processed as stated above for cell tracking. Isolated cells (10^6) from each tissue were stained with CD25, IL-17A (FITC labeled antibodies), or CD4 (APC labeled antibodies) (Becton Dickinson). Labeled cells were analyzed on a FACS Canto II (Becton Dickinson) using Diva Software (Becton Dickinson).

qRT-PCR analysis of cells and mouse tissues

Isolation of the total cellular mRNA was performed using the GeneJET RNA purification kit (Thermo Fisher Scientific) following manufacturer's instructions. Total RNA was purified with DNase and incubated at 37°C for 30 minutes using a thermocycler (Bio-Rad, Hercules, CA, USA). cDNA was

synthesized using iScript kit (Bio-Rad) and qRT-PCR was performed by using Sso-Advanced Universal SYBR Green Supermix Kit (Bio-Rad) on CFX96 Real-Time System (Bio-Rad). A 10 μ L reaction was used which included 5 μ L Sybr green, 3 μ L of distilled water, 0.5 μ L of forward primer, 0.5 μ L of reverse primer, and 1 μ L of 1:10 diluted cDNA. Each reaction was exposed to the following conditions: 98°C for 10 minutes, followed by 30 seconds of 98°C, 20 seconds of 60°C, and 30 seconds of 72°C for 44 cycles in 96-well optical reaction plates (Bio-Rad). Human and mouse *GAPDH/Gapdh* and *ACTIN/Actin* genes were used to normalize the targeted genes. Primer sequences are listed in Additional file 1.

Experimental design and statistical analysis

Statistical analysis was carried out by using one-way analysis of variance (ANOVA) test for multiple comparisons followed by post hoc tests and ANOVA for repeated measures followed by Tukey's post-hoc analysis. Data are presented as the mean $\pm$ standard error of the mean (SEM) of triplicates per analysis. Results were analyzed using SPSS version 26 (SPSS Inc. USA) and p values ≤ 0.01 were considered statistically significant. All statistical graphs were created using Microsoft Excel (Microsoft, Redmond, WA, USA).

Results

Characterization and differentiation of primitive MSC-derived NSCs.

We first differentiated the primitive MSCs into NSCs (18), which displayed typical neural extension morphology (Fig. 1a), loss of MSC markers (Fig. 1b, c) and expression of the neural genes, *NESTIN*, *TUJ1*, *VIMENTIN*, and *PAX6*, as well as neurotrophic factors, *CNTF*, *BMP2*, *PDGF*, *BDNF*, *GDNF*, *IGF*, *EGF*, and *FGF* (Fig. 1d, e). Similarly, translational expression of several neural proteins, NESTIN, TUJ1, VIMENTIN, and PAX6 was displayed by NSCs (Fig. 1f, g). These results were further validated by western blot analysis showing the expression of the neural proteins in NSCs (Fig. 1h, i). This protocol reproducibly yielded 73% differentiation of MSCs into NSCs (Fig. 1j). The ability of NSCs to differentiate to the glial lineage, particular ODCs (Fig. 1a), was demonstrated by the expression of *OLIG2*, *SOX10*, *O4*, and *MBP* at the transcriptional level (Fig. 1k) and OLIG2, O4, MBP, and MOG at the translational level (Fig. 1l, m) by the NSC-derived ODCs. NSCs also differentiated into neuronal lineage (data not shown).

Improvement in the neurobehavior and reduction of disease severity in EAE mice transplanted with cells.

To investigate the effects of MSCs and NSCs on EAE disease process, we used the EAE mouse model. EAE was induced in mice by MOG peptide immunization using an established protocol (16) and disease progression was followed by neurobehavioral analysis according to the well-establish disease symptom scale (Additional file 2, Hooke labs). The first signs of EAE induction in animals were noticed 11-12 days after MOG injections. Then animals with EAE score 1 and 2 were intravenously injected with PKH26 labeled 10^6 cells. They were evaluated for neurobehavioral changes twice a day until they were sacrificed for post-transplantation analysis. Neurobehavioral analysis results showed that average clinical score of the EAE control (4.5 ± 0.5) was significantly higher than the average score of mice

transplanted with MSCs (1.7 ± 0.3) and NSCs (0.8 ± 0.1) at score 1 and MSCs (2.4 ± 0.2) and NSCs (2.2 ± 0.1) at score 2 (Fig. 2a and Additional file 3). In addition, the average weight of the untreated EAE control ($13\text{g} \pm 0.6\text{g}$) was significantly lower than the average weights of the mice transplanted with healthy controls (Fig. 2b and Additional file 3), MSCs ($15.7\text{g} \pm 0.7\text{g}$) and NSCs ($16\text{g} \pm 0.4\text{g}$) at score 1 and MSCs ($14\text{g} \pm 0.7\text{g}$) and NSCs ($14.6\text{g} \pm 0.7\text{g}$) at score 2. When MSCs were transplanted at EAE score 1, they only slowed and halted the disease progression. Whereas NSCs not only slowed but also reversed the disease process, and animal condition was improved to near normal levels within two weeks after cell transplantation. Overall, NSCs showed greater efficacy than MSCs when transplanted at both EAE score 1 and 2 but the results were more significant in case of score 1. Therefore, we selected EAE score 1 transplanted animals for detailed post-transplantation investigation. Animals were sacrificed two weeks post-transplantation, and blood, lungs, lymphatic and CNS tissues were collected for histological, biochemical, immunological and molecular analyses.

Tracking of transplanted cells.

Following transplantation, the labeled transplanted cells homed to the blood, spleen, brain, and spinal cord (Fig. 3). Only an insignificant number of transplanted cells were found in the lungs of all animals, suggesting minimal lung trapping. Interestingly, significantly more transplanted MSCs (10.0%) than NSCs (6.7%) were observed in the blood of healthy animals, suggesting greater survivability of MSCs in the vascular system. In contrast, the number of transplanted MSCs and NSCs was 9.3% and 10.3% in the blood of EAE animals, respectively. Nearly half of transplanted cells were found in the spleen in all animals. Importantly, a significant number of transplanted cells were present in the CNS of EAE animals but not in healthy controls. A similar number of transplanted MSCs and NSCs were found in the brain (7.0% and 7.5%, respectively). Notably, NSCs (12.3%) were significantly more in the spinal cord than MSCs (10.5%). Overall, NSCs exhibited greater survivability in EAE animals than MSCs. Tissue samples were then subjected to histological analysis to investigate the effects of the transplanted cells on immune infiltrates.

Cell transplantation reduced immune infiltrates in CNS.

EAE induction mediates T cell activation resulting in increased infiltrates in the CNS by permeation of the BBB, and progression of disease pathogenesis (19). As expected, the brain and spinal cord (Fig. 4a, b) showed presence of cell infiltrates in EAE animals but not in healthy controls. The number of infiltrates in both brain and spinal cord were significantly decreased in animals treated with MSCs and NSCs; however, NSCs exhibited a greater reduction in infiltrates in the cortex region. Taken together, these results suggest that transplanted NSCs reduced cell infiltrates more significantly.

To investigate the effect of transplanted cells on the inflammatory response in EAE mice and characterize the infiltrates observed in the H&E results, we analyzed the expression of CD45, CD68, and CD3E markers, representing leukocytes, macrophages, and T cells, respectively. The results depicted in Fig. 4c, d, e show high expression of these markers in the brain and spinal cord of EAE animals but not in healthy controls. Importantly, NSCs were more effective in reducing the expression of all three markers in the spinal cord.

It is well known that EAE and MS are caused by an inflammatory response (20). Therefore, to determine the effects of transplanted cells on the expression of inflammatory genes, transcriptional analysis of the brain, spinal cord, and spleen tissues was performed. As expected, expression of the inflammatory markers, *Cd3e*, *Il-17a*, *Il-2*, *Il-1 β* , *Il-6*, *Tnfa*, and *Ifny* was increased in the CNS and spleen in EAE animals compared to the healthy controls (Fig. 4f, g, Additional file 4). When EAE animals were transplanted with cells, expression of inflammatory genes was significantly reduced in the CNS of MSC and NSC treated EAE animals. The expression of inflammatory genes was also reduced in the spleen (Additional file 4). In general, NSCs displayed greater modulatory effect on the inflammatory gene expression than MSCs. Overall, NSCs exerted a greater anti-inflammatory response, potentially by reducing the number of pro-inflammatory cells, macrophages, leukocytes, and T cells.

Immunomodulation of Treg and Th17 by transplanted cells

Treg (CD4⁺ and CD25⁺) and Th17 (CD4⁺ and IL17A⁺) are the main effector cells of cell mediated immune response in EAE and MS (19). We investigated the levels of these cells in the blood, spleen, and CNS by flow cytometry. Treg cells were significantly reduced to 8.6% and 7.3% in EAE animals from 35.6% and 43.0% in healthy controls in both the blood and spleen (Fig. 4h). In contrast, Treg cells were substantially increased in EAE animals transplanted with cells in the blood and spleen. In EAE animals treated with MSCs, Treg cells were 27.4% and 41.1% in the blood and spleen, respectively. Whereas in NSC treated animals, Treg cell levels were 39.3% and 42.0% in the blood and spleen, respectively. In conclusion, Treg cell numbers increased in animals treated with both MSCs and NSCs; however, more significant improvements in the restoration of Treg levels in the blood were observed in the case of NSCs than MSCs. In the CNS of EAE animals, Treg levels were 14.6% and 22.6%, respectively. Although cell transplantation reduced Treg cells in the brain, they were more significantly reduced to 14.8% and 10.0% in the spinal cord of EAE animals treated with MSCs and NSCs, respectively. Taken together, NSCs showed greater efficacy than MSCs in reducing Treg cells in the spinal cord in EAE animals. It is known that Treg cells increase during the chronic late scores of EAE (21). Therefore, it is plausible that reduced Treg levels in animals treated with cells could contribute to improved disease symptoms.

As expected, Th17 cells were significantly increased from 10.3% and 6.9% in the blood and spleen of healthy controls to 15.3% and 13.7% in EAE animals, respectively. When treated with MSCs, Th17 cell levels were not significantly affected in the blood (14.9%) but were reduced to normal levels (10.1%) in the spleen (Fig. 4h). Importantly, NSC transplantation reduced Th17 to levels similar to the healthy control animals. Both the brain and spinal cord of EAE animals had significantly high levels of Th17 cells (22.5% and 30.5%, respectively) as compared to healthy control animals (1.6% and 0.9%, respectively). Upon MSC transplantation, Th17 cells were significantly reduced in the brain and spinal cord (12.6% and 20.3%, respectively). On the other hand, NSC transplantation had a more prominent effect on reducing Th17 cells in both the brain and spinal cord (11.5% and 13.7%, respectively) in EAE animals. Clearly, NSCs showed greater immunomodulatory effects in EAE mice.

Cell transplantation reduced reactive astrocyte and gliosis in CNS.

Upon induction of EAE, the expression of GFAP is increased (Fig. 5a-d), due to the activation of astrocytes in response to gliosis (22). We first examined the expression of *Cntfra*, *Bmpr*, *Gfap*, and *Nfl-b* associated with astrogliosis (23, 24) via qRT-PCR. As expected, these genes were increased in the CNS of EAE animals (Fig. 5a, b). However, the expression of the gliosis associated genes significantly decreased in the CNS tissues of EAE animals transplanted with cells. Specifically, EAE animals transplanted with NSCs displayed a greater effect in reducing these genes in the spinal cord. We then examined GFAP expression at a protein level through immunostaining of CNS tissue sections. Overall, GFAP expression was reduced in the CNS of EAE animals transplanted with cells but was only significant in the brain and not in the spinal cord (Fig. 5c, d). Transplantation of NSCs promoted greater reduction of gliosis in the EAE animals.

Cell treatment promoted remyelination

LFB staining of CNS tissue sections was performed to investigate the effect of transplanted cells on the degree of myelination. While LFB stain intensity was reduced in EAE mice, it was significantly improved in transplanted animals (Fig. 6a, b). The stain intensity was greater in NSC treated animals compared to those treated with MSCs. Increase in the stain intensity in the cortex and spinal cord suggest that transplanted cells induced endogenous remyelination.

To investigate the cause of increased myelin, we then examined myelin associated gene expression in the CNS tissue by qRT-PCR. The expression of all tested myelination associated genes, *Erk2*, *Krox-20*, *Oct-6*, *Mpz*, *Mbp*, and *Mog* was significantly upregulated in the CNS of the transplanted EAE mice (Fig. 6c, d), except *Krox-20* and *Mbp* was not upregulated in the spinal cord in the case of MSCs. We validated these results by performing immunostaining of myelin-associated proteins, MBP and MOG. The expression of MBP and MOG in the brain of EAE animals (Fig. 6e, f), was significantly increased upon transplantation of MSCs and NSCs. The trend in the expression of MBP and MOG in the spinal cord of EAE animals treated with cells was similar to the brain, while MBP expression was greater in EAE animals treated with NSCs in the spinal cord. Taken together, these results suggest that transplanted NSCs showed increased remyelination in the CNS in EAE animals than MSCs.

Cell transplantation upregulated genes involved in neuroprotection.

Neurotrophic factors support the growth, survival, and differentiation of both developing and mature neurons in CNS (25). It has been suggested that neurotrophic factor, BDNF plays an important role in axon protection during autoimmune demyelination of the CNS (26). Our analysis showed extensive decrease in expression of all the tested genes, *Bdnf*, *Trkb*, *Ras*, *Pi3k*, *Akt*, *Creb*, *Fgfr*, *Raf*, *Mek1*, *Mek2*, and *Erk1* associated with neuroprotection in EAE mice (Fig. 7a, b). When EAE mice were transplanted with cells, the expression of all genes, (except *Trkb* in the brain) was significantly increased and NSCs had a greater effect than MSCs. In most cases, the expression was near or higher than the levels of the healthy control in both the brain and spinal cord. These results clearly show that NSCs provided greater neuroprotection than MSCs.

We next examined the effect of transplanted cells on the expression of neural proteins. We observed a significant decrease in the expression of TUJ1 and NESTIN in the brain of EAE animals (Fig. 7c, d). When EAE animals were transplanted with cells, TUJ1 but not NESTIN expression was significantly improved and was near normal levels in the brain. Whereas the expression of both TUJ1 and NESTIN was significantly improved and was near normal levels in the spinal cord. These results suggest cell transplantation also promoted neural development.

Differentiation of transplanted cells in CNS.

To investigate the fate of transplanted cells, CNS tissue sections were co-stained with cell specific antibodies, TUJ1, OLIG2, and O4, against neural cells, oligodendrocyte progenitors, and ODCs, respectively, as well as human nuclear antigen, and visualized by fluorescent microscopy. The results depicted in Fig. 8a, b, showed HNA positive cells were found in the CNS of the transplanted EAE animals. Whereas the results in Fig. 8a-d showed that while the cell specific markers decreased in EAE animals, their expression was co-localized with HNA and significantly improved in transplanted cells. Of the HNA positive cells, 70.9% and 72.0% cells were positive for TUJ1 in the brain and spinal cord of MSC injected animals, respectively. Whereas, only 51.3% and 42.9% of the HNA positive cells expressed TUJ1 in the brain and spinal cord of the NSC treated animals, respectively. Further analysis revealed that 46.7% and 37.9% of HNA positive cells were also OLIG2 positive while 36.5% and 34.4% of HNA positive cells expressed O4 in the brain and spinal cord in MSC treated animals, respectively. Whereas in the case of NSC treated animals, 65.6% and 86% of HNA positive cells were OLIG2 positive in brain and spinal cord. A higher number of HNA positive cells (70.2% and 87.8%) were positive for O4 in the brain and spinal cord in NSC treated animals, respectively. Evidently, a significantly higher number of transplanted cells differentiated towards the ODC lineage in the case of NSCs than MSCs. These results suggest that transplanted cells promoted neural development and regeneration.

Cell transplantation result in upregulation of neurogenesis genes

Recent studies have demonstrated that neurogenesis genes are downregulated in the CNS tissues of EAE animals (27). Since cell fate analysis indicated *in vivo* differentiation of transplanted cells (Fig. 8), we performed transcriptional analysis of genes, which are known to promote neuron development and growth. As anticipated, neurogenesis genes, early (*S100b*, *Nestin*, *Blbp*, *Pax6*, and *Hes5*), intermediate (*Tuj1*, *Ncam*, *Rest*, *Tlx*, *Ascl1*, *Tbr2*, *Ccnd1*, *Mcm2*, and *Sstr2*) and late (*NeuroD1*, *Dcx*, *Prox1*, *Calretinin*, *NeuN*, *Calbindin*, *Pou4f2*, and *Tuc-4*) were downregulated in both the brain and spinal cord of EAE animals. However, transplanted cells rescued the expression of these genes in the CNS (Fig. 9a, b). NSCs had a significantly greater expression of the neurogenesis genes in both the brain and spinal cord when compared to animals transplanted with MSCs. Taken together, NSCs resulted in a greater improvement in the promotion of neurogenesis via the upregulation of *Ascl1*, *Sstr2*, *Prox1*, *Pou4f2* and *Dcx* in the CNS tissues.

Our results showed that expression of *Ras*, *Pi3k*, *AKT* and *Creb* were downregulated in EAE animals but increased in NSCs transplanted animals (Fig. 7). This may suggest that the BDNF-TrkB signaling

pathway, which plays a crucial role in the promotion of cell survival and neuroprotection *in vivo* (28, 29), is at least partially responsible for functional neural recovery in transplanted animals. Furthermore, several genes including *Fgfr*, *Raf*, *Mek1/2*, *Erk1/2*, and *Olig2* as well as *Ascl1* and *Prox1*, which are associated with the FGF signaling pathway and involved in neuronal and glial differentiation (28, 30), were down regulated in EAE mice, but their expression was significantly increased in transplanted NSCs. Taken together, our results suggest that NSC transplantation modulated the BDNF-TrkB and FGF signaling pathways as illustrated in Fig. 9c.

Discussion

In the current study, we used highly proliferative and naïve primitive MSCs isolated from a specific region of the umbilical cord tissue (7, 8) to differentiate them into a large amount of NSCs required for animal studies. Primitive MSC-derived NSCs expressed known neural markers including NESTIN, TUJ1, VIMENTIN, and PAX6 (31). Although published reports indicate expression of NESTIN (32) and VIMENTIN (33) by MSCs, our results showed that MSCs exhibited very low expression of these markers. Further differentiation of NSCs yielded cells with ODC characteristics as they expressed typical markers, OLIG2, SOX10, O4, MBP and MOG (34).

EAE disease progression and symptoms in the established mouse model followed a typical pattern and scored according to published reports (16). EAE was induced 10-11 days following MOG immunization and clinical symptoms got progressively worse, as predicted. Animals at score 1 and 2 of EAE were randomly transplanted with labeled MSCs and NSCs. PKH26 lipophilic tracer was used to label the cells, which has been successfully used to track cells *in vivo* for more than 8 weeks as reported in our previous studies (17, 35). Following cell transplantation, animals showed significant improvement in the clinical symptoms. The symptoms progressively and reproducibly improved in EAE disease score 1 animals than score 2 animals. Although, MSCs slowed and halted the disease progression, results of NSCs transplantation were even more encouraging at disease score 1. NSCs not only slowed but also reversed the disease process, as the neurobehavior of transplanted EAE animals improved to near normal levels. In brief, NSCs treatment proved to be more efficacious based on the clinical symptoms, particularly in EAE disease score 1. Therefore, post-transplantation analysis was focused on EAE disease score 1 animals.

Our cell tracking data showed that the majority of the transplanted labeled cells were localized in the blood, spleen, and CNS in EAE animals. This is consistent with previous studies where MSCs were transplanted in EAE mice and were found to home towards the spinal cord tissue (36). However, in our study, a significant number of injected MSCs and NSCs were found in not only the spleen but also the brain and spinal cord, demonstrating their homing potential to migrate to multiple tissues typically affected by EAE. Interestingly, significantly more transplanted NSCs than MSCs were observed in the spinal cord. Although labeled cells transplanted in healthy animals were not found in CNS, they were able to permeate the BBB in diseased animals as BBB is compromised in EAE (19).

In EAE, activated T cells travel through the BBB leading to an increase of cell infiltrates in the CNS (19). We also observed a significant increase of cell infiltrates in the brain and spinal cord of EAE animals. However, in EAE animals transplanted with cells the number of infiltrates in the CNS decreased significantly. Previously, adipose MSCs have been shown to inhibit cell infiltrates in the EAE animal model (37). However, in our study NSCs displayed a greater effect than MSCs in reducing the number of infiltrates in the CNS. Although NSCs derived from various sources have shown to modulate immune response in EAE animal models (38-41), this is the first report where NSCs derived from primitive MSCs were used and showed greater effectiveness compared to MSCs. As anticipated our immunostaining results showed that infiltrates were comprised of leukocytes, macrophages, and T cells (42). Since EAE induce a pro-inflammatory response, results of this study showed that transplanted cells modulated the immune response resulting in significantly reducing the expression of cytokines, *Cd3e*, *Il-17a*, *Il-2*, *Il-1β*, *Il-6*, *Tnfa*, and *Ifnγ*, in the CNS and spleen. In EAE, IL-6 aggravates clinical symptoms and spinal cord pathology mainly by promoting pathogenic Th17 cells, which initiate and perpetuate inflammation and demyelination (43, 44). Our results showed that cell transplantation was effective at countering the induction of *Il-6* gene expression in the spleen and the CNS tissues. This suggests that transplanted cell exerted anti-inflammatory response.

Studies have shown that Treg and Th17 play an important role in the development of EAE and the immune response correlated with the disease (45-47). Treg maintains cell immune tolerance, whereas Th17 mediates the inflammatory response under abnormal conditions such as during an injury (46). As expected, we observed an imbalance between Treg and Th17 in EAE animals. At the onset of EAE, the levels of Treg decreased and Th17 increased in the blood and spleen, but both increased in the CNS. However, after NSC transplantation, the imbalance was corrected and the levels of Treg and Th17 resembled those found in the blood and spleen of the healthy controls. Whereas levels of both Treg and Th17 were reduced in the CNS of animals transplanted with cells. The underlying mechanism for the restoration of the Treg/Th17 levels could be due to the transplanted cells modulating the function of T cells by inhibiting the production of TNFα and IFNγ as shown in our study and reported previously (48-50).

In order to elucidate the mechanism that might explain the therapeutic effects of MSCs and NSCs in EAE mice, we investigated gliosis in the CNS. It is known that in chronic EAE, lesions become inactive resulting in accumulation of reactive astrocyte (GFAP+) filaments in the CNS (51). We found that astrogliosis gene, *Gfap*, was upregulated in EAE mice as well as GFAP+ mouse cells, which were integrated into the brain and grey matter of the spinal cord. Upon cell transplantation, a significant decrease in the expression of astrogliosis genes, *Cntfra*, *Bmpr*, *Gfap*, and *Nfl-b*, as well as a recession of GFAP+ cells in the grey matter was in agreement with previous reports (48, 52, 53).

Since cell transplantation reduced infiltrates, induced anti-inflammatory response, and inhibited gliosis, they were likely to promote remyelination as reported previously (54, 55). Our approach significantly restored the lost myelin in EAE animals, particularly in the case of transplanted NSCs. Loss of ODCs and activated T cells in MS/EAE contributes to demyelination of neurons (56). Our results showed significant

upregulation of myelin associated genes, *Erk2*, *Krox-20*, *Oct-6*, *Mpz*, *Mbp*, and *Mog*, and proteins, MBP and MOG, suggesting restoration of ODCs resulting in significant improvement in myelination observed in animals transplanted with cells, particularly with NSCs.

Therefore, we investigated whether transplanted cells themselves differentiate into the glial lineage *in vivo*. Analysis of CNS tissues showed co-localization of expression of HNA and genes, TUJ1, as well as OLIG2 and O4, which are key factors in oligodendrogenesis (57, 58). Interestingly, MSCs displayed an upregulation of TUJ1 providing evidence that the MSCs had entered into the early stages of neural differentiation. Whereas the upregulation of OLIG2 and O4 in the case of NSC transplantation suggested that, they differentiated towards the ODC lineage. Therefore, it is plausible to suggest that NSCs have potential to differentiate *in vivo* to replace, regenerate, and repair the lost cells due to EAE.

Typically, NSCs reside in the hippocampus hilus and subventricular zone, which promote proliferation and migration in different diseases (27). Moreover, these cells have the potential to differentiate to produce mature astrocytes and neurons through the enhancement of neurogenesis (59). However, the therapeutic potential of self-neurogenesis in the mature CNS is very limited due to the sharp decline of this process after birth (27, 60). In the current study, results showed that in NSC transplanted animals, there was an overall increase in the expression of neurogenesis specific genes in the CNS tissues. Additionally, NSC transplanted animals displayed the greatest upregulation of *Ascl1*, *Sstr2*, and *Prox1* in the spinal cord, which is known to promote neuronal and ODC differentiation (28, 30). Presumably, these factors contributed to the greater efficacy of NSCs for neuroprotection and the reversal of EAE symptoms by preserving the neurons. The fact that the expression of these factors is improved more significantly in animals transplanted with NSCs, and that the animals recovered to near normal provides a 'proof of concept' that the use of NSC therapy in MS patients would more effective than using MSCs.

One of the possible reasons for NSCs being more effective is their ability to express neurotrophins, such as BDNF, a regulator of adult neurogenesis (61). Studies have revealed that TrkB has the greatest relative affinity for BDNF, and is expressed on a large proportion of stem cells during differentiation and maturation towards the neural lineages (61). When BDNF binds to TrkB, a downstream cascade effect is triggered by activating RAS, PI3K, and AKT. This activation leads to upregulation of the nuclear gene transcription of *CREB* (29). Studies have shown that when *CREB* is upregulated in differentiating neurons, it will enhance neuronal proliferation and cell survival (62). In this study, we observed the upregulation of markers associated with BDNF-TrkB signaling which resulted in the increase expression of *Ras*, *Pi3k*, and *Akt* as well as *Creb* and *Bdnf* in animals treated with NSCs. Although EAE animals transplanted with MSCs displayed a significant increase of *Bdnf* and *Trkb*, downstream genes expression was insignificant when compared to untreated EAE animals. Therefore, NSCs displayed a greater effect in promoting proliferation and neuron maturation via activation of the BDNF-TrkB pathway through BDNF stimulation.

FGF has been found to be a potent modulator of proliferation and differentiation of neural progenitor cells within the CNS (63). Additionally, FGF signaling is important for the regulation of neurogenesis in these tissues (64). A study revealed that upregulation of FGF signaling led to an increase in the neural

differentiation process (65). Upon FGF binding to the FGF receptor, this triggers the intracellular pathway that leads to the cascade activation of RAS, RAF, MEK1, MEK2, ERK1, and ERK2 (66). *Ascl1* is then transcribed as a result of this cascade effect leading to neuronal and ODC differentiation (67). Our results showed that there was a significant upregulation of *Fgfr*, *Ras*, *Raf*, *Mek1*, *Mek2*, *Erk1*, and *Erk2* as well as *Ascl1* and *Prox1*, particularly in NSCs transplanted animals with the greatest effect in the spinal cord. Significantly, higher expression of *Ascl1* in animals transplanted with NSCs suggest that the cells promoted neuronal differentiation as well. Taken together, our results suggest that BDNF-TrkB and FGF signaling plays an important role in the promotion of proliferation, cell survival and neuronal as well as glial differentiation observed when transplanted NSCs in an EAE animal.

Conclusion

In brief, this study demonstrated the potential of NSCs to induce anti-inflammatory responses and provide neuroprotection as well promoted endogenous neurogenesis resulting in the reversal of EAE clinical symptoms and repair the damaged CNS. Although, there have been a number of clinical trials utilizing autologous bone marrow MSCs and umbilical cord derived MSCs, the results are not always conclusive (68-70). Furthermore, most of these studies were limited to phase I and II trials due to limited availability of cells. Two clinical trials used human fetal-derived NSCs (71); however, results of these phase I/II studies have not been reported. Furthermore, NSCs were isolated from their fetal tissue and their use could pose ethical and moral concerns (72). The results of our study are highly promising in reversing the disease process and improving the EAE pathology of CNS, particularly in case of NSCs. Importantly, since primitive MSCs used here are highly proliferative and can be maintained for many passages without losing their differentiation potential, they can be rapidly amplified and differentiated into NSCs in the amounts required for large animals and clinical studies. This has been one of the major impediments for conducting phase III and IV using MSCs or their derivatives. Further studies should be pursued to determine if higher or multiple doses of cells injected at advanced/chronic stages of EAE could also be effective in reversing the EAE disease process. It would also be interesting to know if the cell treatment at the time of MOG injection could block induction of EAE in mice. In addition, studies aimed at elucidating the cellular and molecular mechanism of functional recovery in detail would be helpful. Nonetheless, our study provided proof of concept and basis for investigating the efficacy of primitive MSC-derived NSCs to treat MS in clinical trials.

Abbreviations

BBB: Blood brain barrier

bFGF: Basic fibroblast growth factor

CNS: Central nervous system

EAE: Experimental autoimmune encephalomyelitis

EGF: Epidermal growth factor

FBS: Fetal bovine serum

GM: Growth medium

GvHD: Graft versus host disease

H&E: Hematoxylin and eosin

i.p: Intraperitoneally

IACUC: Institutional Animal Care and Use Committee

IBC: Institutional Biosafety Committee

LFB: Luxol fast blue

ODCs: Oligodendrocytes cells

MHC: Major histocompatibility complex

MOG: Myelin oligodendrocyte glycoprotein

MS: Multiple sclerosis

MSCs: Mesenchymal stem cells

NSCs: Neural stem cells

qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction

SEM: Standard error of the mean

TBST: TBS 1X containing 0.1% Tween-20

Declarations

Ethics approval and consent to participate

All procedures and handling of animals during the investigations were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC #18081) and the Institutional Biosafety Committee (IBC #2858) at Oakland University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization: G.R.C., C.B., and C.M.; Methodology: G.R.C., C.B., C.M., S.H., S.K., and D.L.F.; Software: C.B. and C.M.; Validation: C.B., C.M., D.L.F., and G.R.C.; Formal Analysis: C.B., C.M., D.L.F., and G.R.C.; Investigation: C.B., C.M., S.H., S.K., D.L.F. and G.R.C.; Resources: G.R.C.; Data curation: C.B., C.M., and G.R.C.; Supervision: G.R.C.; Visualization: C.B., C.M., S.H., S.K., D.L.F., and G.R.C.; Funding acquisition: G.R.C.; Project administration: G.R.C.; Writing – original draft: C.B. and G.R.C.; Writing – review & editing: all authors.

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Figures

Figure 1

Characterization of NSCs and ODCs derived from primitive MSCs. (a) Phase contrast images of the primitive MSCs, NSCs and ODCs. Scale bars represent 100 μ m (magnification: 4 $\times$). (b and c) Histograms and graphical representation of expression of MSC markers as determined by flow cytometry,

respectively. Expression of MSC markers, CD29, CD44, CD73, CD90, and CD105, was significantly reduced in NSCs (** $p \leq 0.01$). (d and e) Expression of neural and neurotrophic genes, respectively, as determined by qRT-PCR. NSCs expressed neural markers, NESTIN, TUJ1, VIMENTIN, and PAX6. NSCs also expressed neurotrophic factors, CNTF, BMP2, PDGF, BDNF, GDNF, IGF, EGF, and FGF at higher levels. (f) Expression of neural proteins as determined by immunocytochemical staining. Shown are merged images of DAPI (blue) and human antibodies (green and red). NSCs had significantly higher expression of neural proteins, NESTIN, TUJ1, VIMENTIN, and PAX6 than MSCs. (g) Quantification of fluorescent intensity of immunocytochemical stained proteins (** $p \leq 0.01$). (h and i) Western blot and quantitative analysis of normalized neural protein expression using ImageJ software, respectively (** $p \leq 0.01$). All proteins were normalized to GAPDH expression. MSCs expressed low levels of NESTIN and VIMENTIN, but not TUJ1. Whereas, NSCs had significantly high level of expression of these markers. (j) Percentage of immunocytochemical stained cells positive for NESTIN, TUJ1, VIMENTIN and PAX6 (** $p \leq 0.01$). (k) Expression of genes, OLIG2, SOX10, O4, and MBP, in ODCs as determined by qRT-PCR. (l) Expression of proteins, OLIG2, O4, MBP, and MOG in ODCs, as determined by immunocytochemical staining. Shown are merged images of DAPI (blue) and human antibodies (green and red). (m) Quantification of fluorescent intensity of immunocytochemical stained proteins (** $p \leq 0.01$). Gene expression (in d, e, k) was normalized to GAPDH and ACTIN and error bars represent the SEM of triplicate experiments (** $p \leq 0.01$). All scale bars (f and l) represent 50 μm (magnification: 40 $\times$).

Figure 2

Effect of cell transplantation on the EAE clinical symptoms. (a and b) Changes in clinical score of disease symptoms and weight in EAE mice (averages of $n=6$ each) following transplantation of cells, respectively. Primitive MSCs and NSCs were transplanted on day 14 (indicated by black arrow) at EAE score 1 and score 2 (** $p \leq 0.01$). The animals were monitored and clinical symptoms as well as weight were recorded on daily basis.

Figure 3

Transplanted cells homed to the blood, spleen, lungs and CNS. To determine labeled cells in various organs, collected tissue samples prepared as described in the Methods and Materials were subjected to flow cytometry. Symbols, **, #, %, &, §, and ¥ indicate significant difference at $p \leq 0.01$ between all experimental conditions: healthy control, healthy control+MSCs, healthy control+NSCs, EAE, EAE+MSCs, and EAE+NSCs. Significant number of labeled cells were found in the blood and spleen, but not lungs of both healthy and EAE mice indicating that there was no cell trapping in the lungs. Labeled cells were found in the CNS (both brain and spinal cord) of transplanted EAE animals.

Figure 4

Reduction of inflammation and modulation of Treg and Th17 cells by the transplanted cells. (a and b) Histological analysis of paraffin sections stained with H&E and inflammation scores of the brain and spinal cord, respectively. Cellular infiltrates reduced significantly in the CNS in EAE animals transplanted with MSCs and NSCs. Symbols, **, &, §, and ¥ indicate significant difference at $p \leq 0.01$ between all experimental conditions: healthy control, EAE, EAE+MSCs, and EAE+NSCs. (c) Immunohistochemical

staining of paraffin sections of the brain and spinal cord with cell specific antibodies, CD45, CD68, and CD3E, representing leukocytes, macrophages, and T cells, respectively. (d and e) Quantification of fluorescent intensity of brain and spinal cord sections shown in c, respectively ($**p \leq 0.01$). (f and g) Transcription of inflammation markers, Cd3e, Il-17a, Il-2, Il-1 β , Il-6, Tnfa and Ifny, in the brain and spinal cord, using qRT-PCR. Gene expression was normalized to Gapdh and Actin and error bars represent the SEM of triplicate measures ($**p \leq 0.01$). (h) Graphical representation of Treg (CD4+CD25+) and Th17 (CD4+IL-17A+) cells in the blood, spleen, brain, and spinal cord as determined by flow cytometry ($**p \leq 0.01$). Treg cell numbers went down but Th17 went up in the blood and spleen and both of these cells were significantly increased in the CNS of EAE animals. Transplanted cells significantly increased Treg but significantly reduced Th17 cells in the blood and spleen and both of these cells were significantly reduced in the CNS of EAE animals.

Figure 5

Suppression of gliosis by transplanted cells. (a and b) Transcription of genes, Cntfr, Bmpr, Gfap, and Nfl-b, involved in gliosis in the brain and spinal cord, using qRT-PCR. Gene expression was normalized to Gapdh and Actin and error bars represent the SEM of triplicate measures ($**p \leq 0.01$). Expression of gliosis genes was significantly upregulated in EAE mice but reduced significantly in treated animals. (c) Immunohistochemical staining of paraffin sections of the brain and spinal cord with antibody astroglisis marker, GFAP. Scale bars represent 50 μ m scale bars (magnification: 40 $\times$). (d) Quantification of fluorescent intensity of GFAP in the brain and spinal cord sections depicted in c, respectively ($**p \leq$

0.01). GFAP levels significantly increased in EAE mice. However, they were significantly reduced in transplanted animals.

Figure 6

Cell transplantation resulted improvement in myelination of CNS. (a) Analysis of paraffin sections of the brain and spinal cord stained with LFB to detect myelination. Scale bars represent 50 μ m (magnification: 40 $\times$). (b) Quantification of LFB stain intensity of paraffin sections shown in a. Symbols, **, &, §, and ¥ indicate significant difference at $p \leq 0.01$ between all experimental conditions: healthy control, EAE, EAE + MSCs, and EAE + NSCs. LFB stain intensity was significantly improved in the cortex, cerebellum and spinal cord in transplanted EAE animals. (c and d) Transcription of myelination markers, Erk2, Krox-20,

Oct-6, Mpz, Mbp, and Mog in the brain and spinal cord. Gene expression was normalized to Gapdh and Actin and error bars represent the SEM of triplicate measures (**p ≤ 0.01). (e) Immunohistochemical staining of myelin proteins, MBP and MOG, in paraffin sections of the brain and spinal cord. Scale bars represent 50 μm scale bars (magnification: 40×). (f) Quantification of fluorescent intensity of myelin proteins in the brain and spinal cord sections depicted in e (**p ≤ 0.01).

Figure 7

Cell transplantation upregulated neuroprotection markers. (a and b) Transcription of neuroprotection markers, Bdnf, Trkb, Ras, Pi3k, Akt, Creb, Fgfr, Raf, Mek1, Mek2, Erk1, in the brain and spinal cord, respectively, analyzed using qRT-PCR. Gene expression was normalized to Gapdh and Actin and error bars represent the SEM of triplicate measures (**p ≤ 0.01). All of the tested markers were increased to normal or higher levels in NSCs, but not MSCs transplanted animals as compared to the healthy controls. (c) Immunohistochemical staining of paraffin sections of brain and spinal cord using antibodies against neural proteins, TUJ1 and NESTIN. Scale bars represent 50 μm scale bars (magnification: 40×). (d) Quantification of fluorescent intensity of immunostaining in the brain and spinal cord sections depicted in c. (**p ≤ 0.01). Expression of the neural proteins was more significantly increased in transplanted NSCs than MSCs in EAE animals.

Figure 8

Transplanted cells expressed neural and oligodendrocyte proteins that co-localized with human nuclear antigen in vivo. (a and b) Immunohistochemical staining of paraffin sections of the brain and spinal cord with antibodies against cell specific proteins, TUJ1 (NSCs), OLIG1 (oligodendrocyte progenitor cells), and O4 (ODCs), and counterstained with antibody against HNA. Scale bars represent 50 μ m scale bars (magnification: 40 $\times$). (c and d) Percentage of TUJ1+/HNA+, OLIG2+/HNA+, and O4+/HNA+ cells in the brain and spinal cord sections shown in a and b, respectively (** $p \leq 0.01$). TUJ1 staining intensity was greater in both brain and spinal cord in case of MSCs and OLIG2 and O4 staining intensities were greater in case of NSCs.

Figure 9

Cell transplantation resulted in upregulation of neurogenesis markers in the CNS. (a and b) Determination of expression of genes associated with various stages of neurogenesis, early (S100b, Nestin, Blbp, Pax6, Hes5), intermediate (Tuj1, Ncam, Rest, Tlx, Ascl1, Tbr2, Cnd1, Mcm2, Sstr2) and late (NeuroD1, Dcx, Prox1, Calretinin, NeuN, Calbindin, Pou4f2, Tuc-4) using qRT-PCR. Gene expression was normalized to mouse Gapdh and Actin and error bars represent the SEM of triplicate measures (** $p \leq 0.01$). Expression

of genes representing all stages of neurogenesis was greater in case of NSCs than MSCs, but it was more pronounced in middle and late stages of neurogenesis. (c) Proposed molecular mechanism of cell survival/neuroprotection and differentiation promoted by NSCs.

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