Mesenchymal stem cells do not exert direct beneficial effects on CNS remyelination in the absence of the peripheral immune system

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\textbf{A R T I C L E  I N F O}

Article history:
Received 30 March 2015
Received in revised form 17 June 2015
Accepted 29 June 2015
Available online 30 June 2015

Keywords:
MSC
MS
Cuprizone
Intraventricular
Corpus callosum

\textbf{A B S T R A C T}

Remyelination is the natural repair mechanism in demyelinating disorders such as multiple sclerosis (MS) and it was proposed that it might protect from axonal loss. For unknown reasons, remyelination is often incomplete or fails in MS lesions and therapeutic treatments to enhance remyelination are not available. Recently, the transplantation of exogenous mesenchymal stem cells (MSC) has emerged as a promising tool to enhance repair processes. This included the animal model experimental autoimmune encephalomyelitis (EAE), a commonly used model for the autoimmune mechanisms of MS. However, in EAE it is not clear if the beneficial effect of MSC derives from a direct influence on brain resident cells or if this is an indirect phenomenon via modulation of the peripheral immune system. The aim of this study was to determine potential regenerative functions of MSC in the toxic cuprizone model of demyelination that allows studying direct effects on de- and remyelination without the influence of the peripheral immune system. MSC from three different species (human, murine, canine) were transplanted either intraventricularly into the cerebrospinal fluid or directly into the lesion of the corpus callosum at two time points: at the onset of oligodendrocyte progenitor cell (OPC) proliferation or the peak of OPC proliferation during cuprizone induced demyelination. Our results show that MSC did not exert any regenerative effects after cuprizone induced demyelination and oligodendrocyte loss. During remyelination, MSC did not influence the dynamics of OPC proliferation and myelin formation. In conclusion, MSC did not exert direct regenerative functions in a mouse model where peripheral immune cells and especially T lymphocytes do not play a role. We thus suggest that the peripheral immune system is required for MSC to exert their effects and this is independent from a direct influence of the central nervous system.

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\section{1. Introduction}

Multiple sclerosis is an inflammatory demyelinating disease of the central nervous system (CNS) that leads to progressive axonal damage and consequently to a loss of neurological functions (Lassmann, 2014). Remyelination is the natural mechanism of repair and it was supposed that it might protect from axonal loss and neurodegeneration (Tanaka and Yoshida, 2014). However, due to unknown reasons, remyelination is often incomplete or fails in MS patients (Bhatt et al., 2014). Currently licensed treatments for MS patients are limited to immunomodulatory drugs which are not able to directly enhance remyelination. Recently, the transplantation of exogenous mesenchymal stem cells (MSC) has been proposed to have such regenerative functions (Patel and Genovese, 2011).

MSC treatment has already been reported to be beneficial in the experimental autoimmune encephalomyelitis (EAE) model of MS (Morando et al., 2012). MSC are multipotent cells that can differentiate into adipocytes, chondrocytes, osteoblasts, and they have been observed to transdifferentiate into other cell types such as neural-like cells in vitro (Tondreau et al., 2008). Nevertheless, some controversy exists about the effect that MSC may have in CNS lesions and whether the homing of MSC to injured tissues and their engraftment is strictly required for their beneficial effect (Morando et al., 2012).
et al., 2012; Uccelli and Prockop, 2010). The main effects of MSC are suggested to be mediated by creating a neuroprotective and regenerative environment since MSC are able to secrete growth factors, matrix metalloproteinases, and chemokines such as transforming growth factor-beta (TGFβ), prostaglandin E2, hepatocyte growth factor, and interleukin 10, which could modulate both innate and adaptive immune responses (Uccelli et al., 2008; Rafei et al., 2009; Chen et al., 2011).

Here we investigated the direct effect of exogenously applied MSC on remyelination in the cuprizone model of toxic induced demyelination. The cuprizone model is widely accepted to study remyelination in the CNS (Gudi et al., 2014; Kipp et al., 2009; Skripuletz et al., 2011a). The aim of our experiments was to use an animal model of remyelination in which the peripheral immune system does not play an important role (Skripuletz et al., 2011a). This allows us to analyze the pathomechanisms of remyelination directly in the CNS bypassing interferences of peripheral immune cells, which might be found in inflammatory models such as EAE. EAE is a widely accepted animal model to study CNS inflammation and neurodegeneration, but assessment of remyelination may be complicated in a setting with concomitant de- and remyelination (Tanaka and Yoshida, 2014). To study remyelination, we have used different treatment protocols and MSC from three species (human, murine, canine) that were transplanted either intraventricularly into the cerebrospinal fluid or directly into the lesion of the corpus callosum.

2. Materials and methods

2.1. Experimental design

Demyelination was induced by feeding ten-week old mice a diet containing 0.2% cuprizone (bicyclic hexanone oxaldihydrazone, Sigma–Aldrich) for 5 weeks ad libitum. After this period, animals returned to normal diet for remyelination (Gudi et al., 2014).

To analyze the effects of MSC treatments, different injection protocols were applied including 16 groups of mice (for experimental design, see Fig. 1). First, MSC from three different species were injected in separate groups: human MSC, murine MSC, canine MSC, and PBS sham injections for controls. Second, two administration pathways were chosen: MSC or sham in controls were injected intraventricularly into the right ventricle or intracereosionally into the corpus callosum. Third, injections were performed at two different time points: week 3 which corresponds to a time point of onset of oligodendrocyte progenitor cell (OPC) proliferation and microglial recruitment in the cuprizone model or week 4 which corresponds to the peak of OPC and microglia proliferation. Brains were collected and studied at the peak of demyelination (week 5) or at early remyelination (week 5.5). Procedures involving animals were performed in compliance with the international guidelines on animal care in experimentation and approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany).

2.2. Animals

Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). Animals were housed two per cage and rested one week for adaptation to the housing conditions prior use. All procedures involving animals were performed in compliance with the international guidelines on animal care in experimentation and approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany). Human MSC were isolated from bone marrow of a healthy donor as previously described (Nessler et al., 2013; Schack et al., 2013). Human MSC were seeded in a 175 cm² culture flask and incubated at 37 °C, 5% CO₂, 85% humidity. After 24 h, non-adherent cells were removed by washing with PBS. Additional medium changes were performed every 3 days. Before reaching confluence, MSC were harvested with trypsin–EDTA (Biochrom) and trypsinization was stopped with medium containing 10% fetal calf serum. Afterwards, subcultures were prepared at a density of 4000 cells/cm². MSC from passages 6 to 8 were characterized by flow cytometric analysis (FACS) and used for transplantation.

Murine MSC derived from bone marrow of C57BL/6 mice expressing red fluorescent protein (RFP) were purchased from OriCell™ mouse MSC growth medium (Cyagen). Medium was changed every 2–3 days. Once 80–90% confluence was reached, cells were passaged by rinsing the cell surface 2 times with PBS without Ca²⁺ and Mg²⁺ (Gibco) and by incubating them with trypsin–EDTA for 5 min at 37 °C. Cells between passages 11–13 were used for FACS analysis and transplantation.
Canine MSC were isolated from abdominal adipose tissue of a male, healthy beagle dog. Cells were cultivated and expanded in Dulbecco’s Modified Eagle Medium (DMEM; Biochrom) containing 11.6% fetal bovine serum (HyClone, Thermo Fisher Scientific), hepes 1 M (Biochrom), 1.16% penicillin–streptomycin (PAA) and 0.002% fibroblast growth factor 2 (Preprotech). Medium was changed every 3 days until MSC reached a confluence of 80%. Dissociation was performed using Trypsin–EDTA (PAA) and cells of passage 3 were used for flow cytometry and transplantation.

2.4. Microglia isolation and generation of supernatants

Primary cultures of mixed glial cells were prepared from brains of postnatal 1–3 day old C57BL/6 mice, as previously described (Prajeth et al., 2014). Shortly, meninges were removed and brains were treated enzymatically with 0.1% trypsin (Sigma–Aldrich) and 0.25% DNAse (Roche). Digested brains were chopped in small pieces and single cell suspensions were cultured into poly-l-lysine-coated T75mm2 culture flasks with DMEM + l-Glutamine + 4.5 g/L l-Glucose (Gibco) medium supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin (all Biochrom AG). Medium was changed the next day and every fourth day. Finally, microglia cells were isolated at day 9–11 by shaking the flask at 37 °C and 180 rpm for 30 min on an orbital shaker. Microglia were seeded in a 6-well plate at 5 × 10^5 cells per well. On the following day the medium was replaced with fresh medium or with medium containing 100 ng/ml LPS from Escherichia coli 055:BS (Sigma–Aldrich) and 50 ng/ml recombinant murine IFNγ (PeproTech). After 8 h of stimulation at 37 °C, 5% CO2 in a humidified incubator, cells were washed four times with pre-warmed medium to remove LPS and IFNγ and cells were further incubated by adding fresh medium. After 16 h culture supernatants were collected, centrifuged at 4000 rpm for 5 min to remove cells and aliquots were stored at −80 °C until further use.

2.5. Stimulation of MSC with microglia supernatants, RNA isolation and quantitative real time PCR

Five hundred thousand murine MSC were plated into each well of a 6 well plate. After reaching confluency, cells were incubated with three different conditions: medium, supernatant from unactivated microglia (1:2), and supernatant from LPS + IFNγ activated microglia (1:2). After 18 h incubation, cells were harvest by adding fresh medium. Total RNA isolation was performed using RNeasy® Mini Kit (Qiagen) as previously described (Gudi et al., 2011). Gene expressions of interleukin 6 (IL-6), glial cell-derived neurotrophic factor (Gdnf), insulin growth factor 1 (Igf1) and tumor growth factor 1 (Tgf1b) were analyzed by quantitative real time PCR. The ΔΔCt method was used to determine differences in the expression levels of each condition. Changes in mRNA expression levels were calculated after normalization to hypoxanthin phosphoribosyltransferase (Hprt1) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

2.6. MSC characterization by flow cytometric analysis

Characterization of MSC was performed prior to transplantation using a FACS scalpilur (BD Biosciences) as previously described (Nessler et al., 2013). Human MSC were analyzed using the following markers: PE anti-human CD73, APC anti-human CD90, PE/Cy7 anti-human CD105, PE/Cy7 anti-human CD49d (BioLegend), FITC anti-human CD14 (eBioscience). Mouse MSC were labeled with PE-anti-mouse CD29, PerCP/Cy5.5 anti-mouse CD44, FITC anti-mouse CD45, APC anti-mouse Sca-1, FITC anti-mouse CD49d (BioLegend). Canine MSC were phenotyped using APC anti-canine CD90 (1:20, eBioscence), Dylight 647 anti-CD29 (1:5, Biobybt), Biotin anti-CD45 (1:5, AbD Serotec) and CD44 (1:100, AbD Serotec).

2.7. Transplantation

2.7.1. Preoperative procedure

General anesthesia was induced by a combination of the intra-peritoneal administration of medetomidine (Domitor®, 0.5 mg/kg) and ketamine (100 mg/kg). Following this procedure, the toe- and eyelid-reflex were checked for ensuring deep anesthesia levels. Anesthesia continued up to four hours and a single dose of tramadol was administered subcutaneously (15 mg/kg).

2.7.2. Stereotaxic surgery

The fur on the skull was shaved and skin was disinfected. Eye dehyration was prevented with eye ointment (Bepanthen®, Bayer). Animals were fixed in the stereotaxic apparatus. A midline incision in the skin was made and small surgical hooks were used to maintain the area open. A solution of 30% hydrogen peroxide was used to clean the skull and to improve the visualization of the bregma point. The coordinates from this point were measured. The coordinates of the injection site, which were determined from The Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2001), were calculated. For targeting the right ventricle, the following coordinates were used: −1 mm lateral, 0.5 mm anterior and −3 mm ventral. For intraleisional injection the coordinates were: −1 mm lateral, 0.2 mm anterior and −1.9 mm ventral. The cranial bone at the injection site was opened with a drill head of 1.4 mm diameter. Following this preparation, a 10 μL Hamilton syringe was filled with 5 μL of PBS (control group) or 1 × 10^6 MSC resuspended in 5 μL PBS (treated groups). Once the syringe was inserted into the brain, the content was gradually released. Finally, after 5 min the syringe was slowly withdrawn. Wound closure was achieved with a tissue adhesive (Epiglu®, Meyer-Haake) and animals received a single dose of carprofen (4 mg/kg subcutaneously) for analgesia.

2.8. Immunohistochemistry and immunofluorescence staining

Immunohistochemistry was performed as previously described (Skripuletz et al., 2011b, 2015; Gudi et al., 2009). Animals were killed with an overdose of anaesthesia of Rompun® (4 mg/kg) and Ketamine (100 mg/kg) and perfused with 4% PFA via the left cardiac ventricle at week 5 for demyelination and week 5.5 for remyelination of cuprizone treatment. Brains were removed and kept in 4% PFA overnight. Afterwards the tissue was dehydrated for paraffin embedding and 7 μm serial sections were cut and collected. Paraffin embedded brain sections between bregma −1.46 and −2.06 mm were de-waxed, rehydrated, and microwaved for 10 min in 10 mM citrate buffer (pH 6.0). Then sections were quenched with H2O2, blocked for 1 h in PBS containing 3% normal goat serum, 0.1% Triton X-100, and then incubated overnight with the primary antibody. The following primary antibodies were used: for myelin proteolipid protein (PLP) (1:500, mouse monoclonal immunoglobulin G2a, clone plpc1, Serotec), myelin associated glycoprotein (MAG) (1:1000 mouse monoclonal immunoglobulin G1, clone AB89780, Abcam), and myelin oligodendrocyte glycoprotein (MOG) (1:2 hybridoma supernatant, mouse monoclonal immunoglobulin G, generous gift from C. Linnington), for oligodendroglial cells neurite outgrowth inhibitor (NogoA) (1:750, rabbit polyclonal immunoglobulin G, Millipore), adenosomatous polyposis coli (APC) (1:200, mouse monoclonal immunoglobulin G2b, clone CC-1, Calbiochem), and Olig2 (1:500, rabbit polyclonal immunoglobulin G, Millipore), for proliferation Ki-67 (1:100, mouse monoclonal immunoglobulin G1, clone B56, BD Pharmingen), for astrocytes glial fibrillary acidic protein (GFAP) (1:200, rabbit polyclonal immunoglobulin G, DakoCytonation), for
activated microglia Mac3 (1:500, rat immunoglobulin G1, clone M3/84, BD Pharmingen), for human MSC anti-human CD44 Phagocytic Glycoprotein-1 (1:100 human monoclonal immunoglobulin G1, clone DF1485, DAKO), for canine MSC CD44 (1:100 dog monoclonal immunoglobulin G2a, clone YKX337.87, Serotec).

The next day, sections were washed and incubated with biotinylated anti-mouse IgG (H + L), anti-rat IgG (H + L), and anti-rabbit IgG (H + L) secondary antibodies (1:500, Vector Laboratories) for 1 h followed by peroxidase-coupled avidin–biotin complex (ABC Kit, Vector Laboratories). Reactivity was visualized with 3,3′-diaminobenzidine (DAB, Vector Laboratories). Stained slides were analyzed by light microscopy (Olympus BX61, Hamburg, Germany).

For immunofluorescence stainings treatment with H2O2 was omitted, and blocking was performed with PBS containing 1% normal goat serum and 0.1% Triton X-100. Sections were incubated overnight. On the next day, slides were washed with PBS and incubated for 1 h with the corresponding secondary antibodies: anti-mouse immunoglobulin G2b Alexa-555 conjugated (1:500, Invitrogen), anti-mouse immunoglobulin G2a Alexa-555 conjugated (1:500, Invitrogen), anti-rabbit immunoglobulin G (H + L) Alexa-488 conjugated (1:500, Invitrogen), anti-rat immunoglobulin G (H + L) Alexa-488 conjugated (1:500, Invitrogen), and anti-rat immunoglobulin G (H + L) Alexa-555 conjugated (1:500, Invitrogen). Finally, slides were mounted with Mowiol (Calbiochem) containing 4,6-diamidino-2-phenylindole (DAPI; Invitrogen).

2.9. Determination of de- and remyelination, quantification of glial cells and localization of MSC

De- and remyelination were analyzed in the corpus callosum by immunohistochemistry for the myelin markers PLP, MAG, and MOG as previously described (Lindner et al., 2008; Skripuletz et al., 2008; Bénardais et al., 2014). Stained sections were scored in a blinded manner by three observers and graded on a scale from 0 (complete demyelination) to 3 (normal myelin). Quantification of glial cells was performed for oligodendrocytes (APC, NogoA, and Olig2), astrocytes (GFAP), and activated microglia (Mac3) (Skripuletz et al., 2013). Gial cells were recognized as double positive cells for immunohistochemistry and hematoxylin staining. OPC were determined as Olig2+ and APC−, and proliferating OPC were considered double positive cells for Olig2 and Ki67. Positive cells were counted in the median part of the corpus callosum at a magnification of ×200. The counted area was larger than 0.08 mm² and the results are represented as number of cells per mm².

Murine MSC expressed red fluorescent protein (RFP), which made them easy to trace in the brain. Human and canine MSC localization was observed by immunofluorescence staining, using a specie specific marker (CD44), as described above.

2.10. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by the Fisher-protected least–significant difference test for post hoc comparison if appropriate. All data are given as arithmetic means ± standard error of the mean (SEM). P values of the different ANOVAs are given in the results.

3. Results

3.1. MSC characterization

Prior to the injections, human, murine and canine MSCs were characterized by FACS analysis for the typical cellular surface markers. Human MSC expressed the markers CD90, CD73, CD105, CD49d and were negative for the marker CD14. Murine MSC expressed RFP+, CD44, and Sca-1, but did not show the marker CD49d. Canine MSC expressed CD29, CD44, CD90 and were negative for CD45. Thus, our results show that cells from all three species correspond to the surface marker criteria for MSC (De Bakker et al., 2013; Dominici et al., 2006; Screven et al., 2014; Sung et al., 2008).

To evaluate the possibility of MSC to produce trophic factors that are of relevance to remyelination, we analyzed mRNA expression of selected factors that have been associated with remyelination. The response of MSC was analyzed in vitro using three different medium conditions (normal medium, supernatant from unactivated microglia, and supernatant from LPS + IFNγ activated microglia). In the presence of an inflammatory environment, MSC overexpressed the mRNA for the growth factor Gdnf and the interleukin Il6 (Suppl. Fig. 1). These results show that upon stimulation by other glial cells the MSC used in our experiments were capable to produce growth factors involved in remyelination.

3.2. No influence of stereotactic injection on de- and remyelination

To investigate (or exclude) an effect of the stereotactic injection procedure on de- and remyelination, two treatment groups were analyzed. Both groups were fed with cuprizone for 5 weeks. While the control group received no additional treatment, the other group received an intraventricular PBS injection at week 4. Brain sections were analyzed for the myelin marker PLP, MAG, and MOG, the oligodendrocyte marker APC and NogoA, the microglia marker Mac3, and the astrocyte marker GFAP. Results show that the injection did not change demyelination at week 5 or remyelination at week 5.5 (Suppl. Fig. 2). In addition, no injection effects were found on oligodendrocytes, activated microglia, and astrocytes at both de- and remyelination (Suppl. Fig. 3). Furthermore, we investigated the injection area. At the level of injection the BBB was partially disrupted and we observed a slight infiltration of very few CD3+ cells and albumin extravasation on both sides of the cannula’s track. We did not find T cell infiltration and albumin extravasation in other regions of the brain (data not shown).

3.3. MSC localization after intraventricular and intraslesional transplantation

To analyze the effects of human, murine, and canine MSC on cuprizone induced demyelination and the subsequent remyelination two different administration routes and two different injection time points were investigated. MSC were administered intraventricularly into the CSF or intrasesionally into the corpus callosum 3 or 4 weeks after the start of the cuprizone feeding. Seven days after intraventricular injections, human, murine, and canine MSC were located in the ventricle attached to the parenchyma forming spherical masses (Fig. 2). In animals that received MSC into the corpus callosum, cells were integrated into the surrounding tissue at seven days after application (Fig. 2).

3.4. MSC did not modulate cuprizone induced demyelination and oligodendrocyte loss

To evaluate whether MSC affect the toxin-induced demyelination, brain sections were analyzed for the myelin marker PLP, MAG, and MOG and the oligodendrocyte marker APC and NogoA. Naive control mice fed with normal food showed normal myelin patterns in the corpus callosum and cerebral cortex. After 5 weeks of cuprizone feeding, nearly complete demyelination was found in the corpus callosum of PBS-control mice (Fig. 3A and Suppl. Fig. 4).
In the cerebral cortex severe demyelination was observed (Suppl. Fig. 5). No difference was found between mice treated with MSC independently of the application route, the time point of treatment and MSC origin and the corresponding cuprizone-controls.

In the corpus callosum, a severe loss of oligodendrocytes was found after 5 weeks of cuprizone feeding (Fig. 4A and Suppl. Fig. 6). Oligodendrocytes were nearly completely depleted in all cuprizone groups, which is in accordance with the myelin results.

3.5. MSC did not influence remyelination and regeneration of oligodendrocytes

After 5 weeks of cuprizone feeding, mice were returned to normal chow to allow remyelination. At week 5.5 (0.5 week of remyelination) new myelin proteins and new oligodendrocytes were visible in the corpus callosum of cuprizone control mice. For each of the markers studied in immunohistochemical stainings (PLP, MAG, MOG) human, murine, or canine MSC treated mice did not show any effect on remyelination compared to cuprizone controls (Fig. 3B and Suppl. Fig. 4C). Analysis of the myelin protein PLP in the cerebral cortex showed similar results in all groups (Suppl. Fig. 5). Analogous to myelin protein re-expression, MSC did not influence the re-appearance of OPC and mature oligodendrocytes in the corpus callosum compared to cuprizone controls (Fig. 4B, Suppl. Figs. 4 and 6).

3.6. MSC did not influence glial reactions during demyelination and remyelination

Accumulation of activated microglia was investigated by Mac3 staining. In naïve normal fed control mice activated microglia were not found. In the corpus callosum high numbers of activated Mac3 positive microglia were found in cuprizone control mice during demyelination (Fig. 5A and Suppl. Fig. 6). During remyelination decreased microglia numbers were found in the corpus callosum of cuprizone control mice (Fig. 5B and Suppl. Fig. 6). Neither human nor murine or canine MSC had an influence on the degree of microgliosis during de- and remyelination compared to cuprizone controls.

The degree of astrogliosis during de- and remyelination was visualized by GFAP immunostaining. There was a marked increase in GFAP positive astrocytes in the corpus callosum of cuprizone fed control mice, but no significant changes after MSC treatment were observed (Fig. 6A, B and Suppl. Fig. 6).

4. Discussion

The aim of our study was to clarify the potential direct beneficial effect of MSC on remyelination in a mouse model of multiple sclerosis. The effects of MSC on CNS de- and remyelination were investigated in a well-described toxic mouse model that enables the analysis of direct effects on remyelination bypassing the role of the peripheral immune system. We showed that MSC did not change the course of demyelination and did not influence regenerative functions during remyelination.

On the contrary, several potentially neuroprotective and regenerative approaches using MSC have been described in EAE, an autoimmune mediated inflammatory driven animal model for MS. Intravenous treatment with a million murine MSC ameliorated the disease course of MOG-induced EAE when injected at onset or at peak of the disease (Zappia et al., 2005). Further studies reported that intraventricular administration of murine MSC offered neuronal protection and improved clinical features (Kassis et al., 2008). However, there is still a controversy on the local and direct effect of MSC in the CNS and whether the homing of MSC to injured tissues and their engraftment is strictly required for their beneficial effect (Morando et al., 2012; Uccelli and Prockop, 2010). An alternative explanation in EAE could be the modulation of the autoimmune response and a downregulation of immune cell infiltration into the CNS by mechanisms that exclusively take place in peripheral immune organs.

In a previous study, we could show that MSC applied intravenously or intranasally did not exert any protective effects on demyelination and oligodendrocytes (Nessler et al., 2013). This effect was attributed to the fact that the cells did not reach the lesions in the brain, presumably, because in the cuprizone model the BBB remains intact without T cell infiltration and consequently MSC either could not cross the BBB or were not attracted by other...
No effects of MSC on de- and remyelination. Cells or PBS in controls were applied at week 4 of cuprizone induced demyelination. In A, all groups, except for the untreated group (naïve control), show nearly complete loss of the myelin protein PLP (red fluorescence) in the corpus callosum. In B, early remyelination is observed in a similar pattern in all cuprizone treated groups. Naïve controls show more compact and dense myelin. Representative brain sections are shown for each group. Each graphic bar represents the mean ± SEM. Comparable results were obtained when cells were transplanted at week three of cuprizone feeding (Suppl. Fig. 1).
Fig. 4. No effects of MSC on oligodendrocytes during de- and remyelination. Cells or PBS in controls were applied at week 4 of cuprizone induced demyelination. In A, severe loss of APC (red fluorescence) and NogoA (green fluorescence) positive oligodendrocytes in the corpus callosum occurred in all cuprizone groups after 5 weeks of cuprizone feeding. In B, high numbers of new oligodendrocytes were found in the corpus callosum in all cuprizone groups at week 5.5. Representative brain sections of the corpus callosum are presented. Each graphic bar represents the mean ± SEM. Comparable results were obtained when cells were transplanted at week three of cuprizone feeding (Suppl. Fig. 2).
Fig. 5. No effects of MSC on microglia activation during de- and remyelination. Cells or PBS were applied at week 4 of cuprizone induced demyelination. In A, high numbers of Mac3 positive microglia (green fluorescence) were found in all cuprizone treated groups at peak of demyelination (week 5). At early remyelination (week 5.5) microglial numbers decreased and no effects were found between the cuprizone groups (B). Representative brain sections of corpus callosum are shown. Each graphic bar represents the mean ± SEM. Comparable results were obtained when cells were transplanted at week three of cuprizone feeding (Suppl. Fig. 1).
No effect of MSC on the degree of astrogliosis. Cells or PBS in controls were applied at week 4 of cuprizone induced demyelination. In all cuprizone groups higher numbers of GFAP positive astrocytes (green fluorescence) were found in the corpus callosum during demyelination (week 5) and the subsequent remyelination (week 5.5). Representative brain sections of the corpus callosum are presented. Each graphic bar represents the mean ± SEM. Comparable results were obtained when cells were transplanted at week three of cuprizone feeding (Suppl. Fig. 1).
immune cells. Thus, in the present study, MSC were injected into the CNS either into the lateral ventricle or directly into the lesion of the corpus callosum. Since an injection into the lesion leads to disturbance of the blood–brain barrier, control experiments were performed to exclude that the injury might cause an inflammatory reaction. We found that stereotactic injections neither changed the course of demyelination and the subsequent remyelination, nor modified glial reactions compared to cuprizone only controls. Thus, we are convinced that our model is suitable to investigate the role of MSC on CNS remyelination.

When MSC were injected intraventricularly or directly into the lesion of the corpus callosum, we could not find any modulation of ongoing demyelination, astrocyte and microglia activation and myelin debris removal. More importantly, MSC did not exert any regenerative functions on oligodendrocytes and remyelination. Thus, our results show that MSC do not provide direct beneficial effects on remyelination in our toxic model. The reason for the different findings might be explained by the modulation of the peripheral immune system in inflammatory models of MS. Peripheral immune cells and especially T lymphocytes play a key role in EAE induction and it was shown that (Lanz et al., 2010; Zappia et al., 2005; Zhang et al., 2005) T cells reside within the bronchus-associated lymphoid tissues of the lungs and lung-draining mediastinal lymph nodes where priming takes place before entering the CNS (Odoardi et al., 2012). For MSC it was demonstrated that MSC applied intravenously become trapped in the lungs (Gao et al., 2001). Thus, it might be speculated that MSC there exert their immune modulatory functions by both direct cellular contact and/or by secreting factors that could induce T cell anergy and inhibit their migration towards the CNS. In contrast to inflammation driven demyelination, in the toxic cuprizone model T cells are not affected and the peripheral immune system does not play a role in de- and remyelination, which seems to be the important target for MSC to exert beneficial effects in EAE.

Since cuprizone intoxication is in strong contrast to human MS, it does not reflect the complex pathomechanisms of the human scenario where the peripheral immune system plays an important part in lesion development. However, rodent models such as the cuprizone induced toxic demyelination have become helpful in exploring the pathomechanisms of remyelination, which is not possible in humans and also difficult in other animal models like EAE. Nevertheless, all animal models only partly mimic the processes of MS with every model having its advantages and disadvantages. To our opinion, the different animal models are needed since they complement each other and help to understand parts of the human disease MS. The aim of this study was to concentrate on pure remyelination excluding the effects of the immune system that may also be modulated by MSC.

In contrast to our observations, a recent study has shown that murine bone marrow-derived MSC injected intravenously reached the brain and induced beneficial effects on cuprizone induced demyelination and the subsequent remyelination (El-Akabawy and Rashed, 2015). However, in this work the animals were not perfused prior to brain removal, and thus, it is uncertain whether the detected cells were inside the blood vessels or in the CNS tissue. Furthermore, evaluation of demyelination was not performed in the middle area of the corpus callosum, which corresponds to the well-characterized and defined area to evaluate de- and remyelination in the cuprizone model (Matsushima and Morell, 2001; Torkildsen et al., 2008; Kipp et al., 2009; Skripuletz et al., 2011a). It was shown that the lateral parts of the corpus callosum, which were investigated by the authors, are less vulnerable to the toxin (Schmidt et al., 2013). In addition, El-Akabawy and Rashed, 2015 described less demyelination and a better remyelination as consequence of MSC treatment but only one time point was investigated. The authors investigated week 4 of cuprizone exposure. During this time point nearly all oligodendrocytes are lost and there is severe demyelination (Matsushima and Morell, 2001; Torkildsen et al., 2008; Kipp et al., 2009; Skripuletz et al., 2011a; Bénardais et al., 2013). In the cuprizone model, new oligodendrocytes and new myelin occur first at week 5 (Matsushima and Morell, 2001; Torkildsen et al., 2008; Kipp et al., 2009; Skripuletz et al., 2011a). In another study Hedayatpour et al., 2013 showed beneficial effects of intravenously applied MSC on remyelination. However, the authors performed a six week cuprizone diet to induce demyelination. In the cuprizone model, regeneration of oligodendrocytes and new myelin occur despite of continuous cuprizone exposure at week 6 (Gudi et al., 2009; Matsushima and Morell, 2001; Kipp et al., 2009; Skripuletz et al., 2011a). In the work of Hedayatpour et al., 2013 the corpus callosum was dissected and a single cell suspension was prepared for FACS analyses of glial reactions including OPC. Although, this method is suitable to isolate microglia, analysis of OPC and astrocytes seems to be rather artificial. Furthermore, mice were not perfused prior to brain removal, and thus, blood cells were included in the cell suspension. Another study has reported that MSC transplantation into the fimbria enhanced remyelination after 12 weeks of cuprizone exposure (Jaramillo-Merchán et al., 2013). We could not confirm these results in the acute model of cuprizone performing a 5 week cuprizone treatment. However, we did not evaluated white matter regions outside the midline of the corpus callosum in order to avoid regions which are less vulnerable to cuprizone (Schmidt et al., 2013).

In conclusion, we demonstrate that MSC do not exert any direct effects on remyelination in CNS. We thus propose that the peripheral immune system is required for MSC to mediate regenerative effects in CNS inflammatory diseases and the effect is independent from the CNS.

Acknowledgments

We thank I. Cierpka-Leja, S. Lang, and A. Niesel for excellent technical assistance. This work is part of L. Salinas Tejedor’s PhD thesis. T. Skripuletz, M. Stangel, and W. Baumgärtner are supported by the German Research Foundation (DFG, FOR 1103, TP1b, STA 518/4–1, BA 815/10–2).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.06.024.

References


