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Conserved Fate and Function of Ferumoxides-Labeled Neural Precursor Cells In Vitro and In Vivo

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Abstract

Recent progress in cell therapy research for brain diseases has raised the need for non-invasive monitoring of transplanted cells. For therapeutic application in multiple sclerosis, transplanted cells need to be tracked both spatially and temporally, in order to assess their migration and survival in the host tissue. Magnetic resonance imaging (MRI) of superparamagnetic iron oxide-(SPIO)-labeled cells has been widely used for high resolution monitoring of the biodistribution of cells after transplantation into the central nervous system (CNS). Here we labeled mouse glial-committed neural precursor cells (NPCs) with the clinically approved SPIO contrast agent ferumoxides and examined their survival and differentiation in vitro, as well as their functional response to environmental signals present within the inflamed brain of experimental autoimmune encephalomyelitis (EAE) mice in vivo. We show that ferumoxides labeling does not affect NPC survival and pluripotency in vitro. Following intracerebroventricular (ICV) transplantation in EAE mice, ferumoxides-labeled NPCs responded to inflammatory cues in a similar fashion as unlabeled cells. Ferumoxides-labeled NPCs migrated over comparable distances in white matter tracts and differentiated equally into the glial lineages. Furthermore, ferumoxides-labeled NPCs inhibited lymph node cell proliferation in vitro, similarly to non-labeled cells, suggesting a preserved immunomodulatory function. These results demonstrate that ferumoxides-based MRI cell tracking is well suited for non-invasive monitoring of NPC transplantation.

Keywords

neural stem cells; cell therapy; MRI; cell tracking; SPIO; ferumoxides

Cell therapy has been suggested as a regenerative approach to replace CNS cells lost due to injury or disease. For clinical translation, there is an increasing need of technologies that can monitor the efficacy and safety of cell therapy by assessing the fate and bio-distribution of transplanted cells in a serial and non-invasive manner (Ben-Hur et al., 2005; Sykova and

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Jendelova, 2007). Magnetic resonance (MR) tracking of iron-labeled cells appears as a particularly suited technique for several reasons. First, resolution in MRI is constantly improving. In animals, high-field (>7 Tesla) magnets enable detection of small foci of superparamagnetic iron oxide (SPIO)-labeled cells in the rodent brain. In the clinical setting, a 3.0 Tesla magnet is sufficient to detect SPIO-labeled cells with sufficient sensitivity and resolution (de Vries et al., 2005). Second, MR imaging is non-invasive, making it possible to follow the migration of cells serially over time by imaging at regular intervals. Finally, MRI is already routinely applied clinically to follow the progression of neurological diseases and is thus widely available.

Magnetic labeling of cells is commonly achieved using biocompatible SPIO which provide the cells of interest with a large magnetic moment (Bulte and Kraitchman, 2004). One such contrast agent, ferumoxides (i.e. Feridex or Endorem), is FDA-approved and widely used in animal experiments and clinical trials (de Vries et al., 2005; Zhu et al., 2006; Toso et al., 2008). Following labeling with ferumoxides, the amount of cellular iron uptake is in the range of 10–20 pg Fe per cell (Bulte et al., 2004), enabling cellular visualization with T2*-weighted MRI.

Recent studies showed the migration properties and therapeutic value of multipotential neural precursor cells (NPCs) in experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis (Ben-Hur et al., 2003; Einstein et al., 2003; Pluchino et al., 2003, 2005). Ex vivo and in vivo imaging showed that MRI is a useful tool to follow ferumoxides-labeled, transplanted NPC migration in the EAE brain (Bulte et al., 2003; Ben-Hur et al., 2007). Crucial for the use of ferumoxides to label cells is its biocompatibility and lack of adverse effects on cell physiology. Specifically, for neural stem cells (NSC), it is important to determine whether any detrimental effects on cell lineage differentiation, survival, migration and immunomodulatory property are present following labeling, all of which are critical factors involved in the regenerative (therapeutic) potential of the cells. A thorough study defined the optimal incubation conditions for SPIO-labeling of human neural precursor cells, which maintains cell viability while enabling their visualization in the mouse brain by in vivo MRI (Neri et al., 2008). While ferumoxides labeling did not appear to adversely affect NPCs, a direct comparison between labeled and unlabeled NPCs has not heretofore been performed. In particular, no comparison has been performed on the functional capacity of transplanted cells to respond to tissue signals in disease conditions in vivo. We therefore compared the effect of ferumoxides labeling on the survival, differentiation, migration and immunomodulatory property of multipotential NPCs, with that of unlabeled cells.

MATERIALS AND METHODS

Isolation of Subventricular Cells and Growth of Neurospheres

Cerebral hemispheres were dissected from newborn C57Bl/6 mice. For transplantation experiments cells were grown from green fluorescent protein (GFP+) transgenic C57BL/6 mice (Japan SLC, Inc.) in order to enable their detection in the host brain, independently from iron labeling. Following removal of meninges, the tissue was minced, digested in 0.025% trypsin for 20 min and dissociated by trituration with a 5-ml pipette into a single cell suspension. The cells were suspended in serum-free F12/DMEM medium supplemented with 10 µg/ml human apo-transferin, 1 mM sodium-pyruvate, 0.05% BSA, 10 ng/ml d-biotin, 30 nM sodium-selenite, 20 nM progesterone, 60 µM putrescine, 25 µg/ml BSA, 2 mM l-glutamine and 25 µg/ml gentamycin (all from Sigma). The cells were plated at 10⁶ cells/ml in T-75 uncoated flasks and supplemented with 10 ng/ml of basic fibroblast growth factor (FGF2, R&D) and 20 ng/ml of epidermal growth factor (EGF, R&D), added daily.

Under these conditions, approximately 0.2% of cells proliferated into clusters of small round cells that grew into floating spheres containing multipotential NPCs.

Ferumoxides Labeling of Cells

Neurosphere cultures were incubated for 24 hr prior to use with 0.25 µl/ml ferumoxides (Feridex, Berlex, 25 µg Fe/ml), and 375 ng/ml poly-L-lysine (PLL, Mw = 388 kDa, Sigma) in N2 medium (Bulte et al., 2004). This resulted in labeling of >95% neurosphere cells. Labeled and unlabeled NPCs were prepared each time from the same batch of neurospheres.

Induction of MOG EAE in C57Bl/6 Mice

EAE was induced in 6–7 week old female C57BL/6 mice by immunization with an emulsion containing 300 µg of purified myelin oligodendrocyte glycoprotein (MOG) peptide (MEVGWYRSPFSRVVHLYRNGK, corresponding to residues 35–55) in saline and an equal volume of complete Freund's adjuvant containing 5 mg H37RA (Difco). 0.2 ml of the inoculum was injected subcutaneously. In addition, 300 ng of Bordetella pertussis toxin (Sigma) in 0.2 ml saline was injected intraperitoneally at the day of induction and two days later.

ICV Transplantation

All animal experiments were approved by our institutional committee. Neurospheres were expanded for 5 days and then harvested, centrifuged and counted. 3000 neurospheres in 5 µl DMEM/F12 medium were injected per mouse into the right lateral ventricle, using a stereotactic device, on day 7 post-EAE induction, under Ketamine (80 mg/kg) and Xylazine (20 mg/kg) anesthesia.

Histopathology

Two weeks following ICV transplantation (day 19–20 after induction of EAE), mice were deeply anesthetized (sodium pentobarbitol, 200 mg/kg i.p.) and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA). The brains were removed and further fixed by immersion in 4% PFA during 24 hr. Differentiation and migration of the cells were assessed in 8 µm coronal frozen sections.

Prussian Blue Staining with DAB Enhancement

200–300 neurospheres were adhered for 4 hr to poly-D-lysine 10 µg/ml (Sigma) and fibronectin (0.1% from bovine plasma, Sigma) coated plates and stained immediately or after 4 days of culture. Cell cultures and brain sections were incubated with freshly prepared Perls' reagent for 30 min and with diaminobenzidine (DAB) for 15 min followed by another 15 min incubation with H₂O₂-activated DAB (Bulte et al., 2004).

Immunostaining

200–300 neurospheres were plated on poly-D-lysine 10 µg/ml (Sigma) and fibronectin (0.1% from bovine plasma, Sigma) coated sterile plates for adhesion. 3% normal goat serum (NGS) in phosphate buffered saline (PBS) was used for blocking. Primary antibodies included mouse IgM anti-PSA-NCAM (Chemicon), mouse IgG anti-Nestin (Chemicon), rabbit anti-NG2 (Chemicon), mouse IgM anti-O4 (Chemicon), rabbit IgG anti-GalC (Chemicon), and rabbit IgG anti-GFAP (Dako). Secondary antibodies used included goat anti-mouse IgM Texas red (Jackson ImmunoResearch Laboratories), goat anti-mouse IgG Alexa Fluor 488 (Molecular probes), and goat anti-rabbit IgG Texas red (Jackson ImmunoResearch Laboratories). Cells were fixed with a solution of 5% acetic acid and 5% ethanol and covered with mounting medium containing DAPI (Santa Cruz Biotech.)

Brain sections were blocked for 1 hr using PBS with 5% NGS and 5% BSA and then incubated overnight with the primary antibody at 4°C, followed by 1 hr incubation with the secondary antibody at room temperature. Brain slices were covered with mounting medium containing DAPI (Santa Cruz Biotech). Images of specific immunofluorescent reactivity were obtained using a Nikon DXm1200F camera attached to an Olympus BX51 microscope.

Assessment of Differentiation and Migration

Astrocytes were identified by GFAP staining, oligodendrocyte lineage cells by NG2, GalC and O4 stainings, and neurons by NeuN staining. For in vitro differentiation, the fraction of each lineage was determined by counting the labeled cells from a total of 20 microscopic fields (and at least 3,000 cells per condition) in plates containing ferumoxides-labeled neurospheres and plates containing non-labeled cells. For in vivo differentiation, the fraction of GFP+ cells stained with a differentiation marker was determined from the total GFP+ cells per section. Differentiation was assessed in 5 sections per brain from 5 transplanted brains with ferumoxides-labeled cells and 5 brains with non-labeled cells. The migration of transplanted cells was evaluated microscopically by GFP fluorescence for both ferumoxides-labeled and unlabeled NPCs in order to avoid any possible discrepancies between MR images and histological analysis. An ocular grid (of determined scale) was used to count the number of squares spanning the entire distance of migration, viewed as a straight line. The maximal distance of migration was determined as distance from the edge of the ventricle to the leading edge of migrating NPC in the corpus callosum. This was measured in 5 brains transplanted with labeled cells and 5 brains transplanted with unlabeled cells.

Caspase Activity

Caspase activity was determined using the Caspase-Glo 3/7 (Promega, Madison, WI) and the Apo-One Homogeneous Caspase 3/7 (Promega) assay. The Caspase-Glo 3/7 assay utilizes a luminogenic caspase 3/7 substrate (DEVD-luciferin) that reacts with recombinant luciferin following cleavage of the DEVD tetrapeptide by caspase 3/7 whereas the Apo-One Caspase 3/7 assay utilizes a profluorescent caspase substrate (Z-DEVD-R110) that fluoresces at 521 nm following cleavage of dual amide-linked DEVD peptides. Labeled neurospheres were washed twice in PBS (5 min, 80 × g) and suspended in PBS (1×10^4 spheres/ml) for distribution (100 µl; 1×10^3 spheres/well) into either white-walled luminometer plates (Nunc) or black-walled, clear bottom 96-well assay plates (Optilux, BD Falcon, Franklin Lakes, NJ) for measurements of luminescence and fluorescence, respectively. Caspase 3/7 reagent diluted in lysis buffer (100 µl) was added to each well and 2 cycles of freeze thaw (from -80°C to 25°C) were applied to facilitate neurosphere lysis. Luminescence and fluorescence were measured after a 90 min substrate incubation using a Wallac Victor 3 plate reader with plate shaking (60 sec) prior to photon counting (0.1 sec exposure).

Apoptosis Assay

The presence or absence of cell apoptosis in response to ferumoxides labeling was determined by high affinity binding of recombinant annexin-V FITC (ApoTarget; Invitrogen, Carlsbad, CA) to phosphatidylserine found on the outer cell membrane during early phases of programmed cell death and propidium iodide (PI) to detect cells undergoing apoptosis. After incubation in the presence of a 4-fold concentration of ferumoxides/PLL (100 µg/ml ferumoxides), neurospheres were concentrated by centrifugation (5 min, 50 × g), washed twice in PBS and resuspended (1×10^4 spheres/ml) in annexin-V binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) containing annexin-V FITC (1:20 dilution) and PI (500 ng/ml) for 20 min and protected from light exposure. To reduce nonspecific apoptosis neurospheres were collected by centrifugation at 50 × g and gently

resuspended in assay buffer. Neurospheres were viewed using an Olympus IX 71 inverted fluorescence microscope equipped with an Olympus DP70 CCD camera.

Ex vivo MRI

Individual perfused-fixed brains were immersed in Fomblin (Ausimont USA, Thorofare, NJ), an anhydrous perfluoropolyether, and 3D multigradient echo MR images were obtained at 70 μ m resolution using a 11.7 T Bruker NMR spectrometer and a 15 mm Litz coil. The scan parameters were: FOV = $13 \times 10.5 \times 7.9$ mm; matrix = $192 \times 148 \times 112$; NEX = 8; TR = 150 msec; TE = 4.5 msec; n echoes = 4, flip angle = 90° ; TA = 5 hr, 31 min.

In vivo MRI

The biodistribution of ferumoxides-labeled neurospheres within the CNS of naive and EAE mice was monitored at 9.4T (Bruker Advance) using T2-weighted 3D RARE on days 1, 4, and 7 after ICV transplantation. The mice were anesthetized with isoflurane and their breathing monitored during the entire imaging procedure. The head was centered within a 30-mm receiving coil and an 8 mm region of the CNS was imaged using 3D RARE with parameters: FOV $1.9 \times 1.9 \times 0.8$ cm, $148 \times 148 \times 250$ μ m, TE 11.4 msec, TR = 1500 msec, RARE Factor = 2, AVG = 1.

Lymph Node Cell Proliferation Assay

Lymph nodes were excised from naïve mice, dissociated into single cell suspension and 2×10^5 cells/well were seeded in 96 well plates, as previously described (Einstein et al., 2003). Lymph node cells (LNCs) were cultured in 10% fetal calf serum in RPMI medium (10% RPMI), and stimulated with 2.5 μ g/ml Concanavalin A (ConA). NPC spheres were irradiated with 3000 Rad and then added directly to non-stimulated LNCs or ConA-stimulated LNCs.

Statistical Analysis

Values of differentiation, survival, proliferation and migration in the in vivo and in vitro experiments are the mean $\pm$ S.E.M. of 3 experiments. All comparisons between ferumoxides-labeled cells versus unlabeled cells were performed using the standard 2-tail student's t-test.

RESULTS

In vitro Differentiation of Ferumoxides-Labeled Neural Precursor Cells (NPCs)

Newborn mice sub-ventricular zone (SVZ) derived neural precursors were expanded in vitro for 5 days into nestin+, PSA-NCAM+ neurospheres (Fig. 1A,B). During the last 24 hr, half of the neurosphere culture was labeled with the MRI contrast agent ferumoxides (Fig. 1C–E) and the differentiation of NPC into neural cell lineages was compared between ferumoxides-labeled versus unlabeled cultures. After 5 days of differentiation, the cultures were stained for GFAP to detect astrocytes, GalC and O4 to detect oligodendrocytes and NeuN to detect neurons. Immunofluorescent staining was performed 5 days after the induction of differentiation (Fig. 1F–J). These newborn SVZ-derived NPC spheres generated mainly glial progeny and very few neurons. There was no significant difference in the differentiation pattern of the cells between the ferumoxides-labeled and unlabeled groups (Table I). Thus, ferumoxides labeling does not affect lineage commitment during NPC differentiation.

Survival of Ferumoxides-Labeled Neurospheres in vitro

To determine whether ferumoxides labeling was pro-apoptotic, caspase 3/7 activity was examined in NPCs labeled with a 4-fold concentration of ferumoxides (100 μ g/ml). Caspase

3/7 activity in neurospheres labeled with ferumoxides did not differ significantly ($p = 0.88$) from control unlabeled neurospheres (Fig. 2A). Neurospheres were further stained with annexin V -FITC to detect phosphatidylserine translocation to the extracellular membrane, an early marker of apoptosis, and with propidium iodide (PI) to detect the loss of cell membrane integrity in late apoptotic and necrotic cells. Early experiments indicated that high centrifugal forces ($>80 \times g$) during routine neurosphere collection and rapid (0.5 ml/sec) or repeated ($>2\times$) pipet ejection increased neurosphere apoptosis as indicated by a characteristic ring pattern of annexin V -FITC cell membrane labeling (data not shown). Under optimized cell handling conditions, binding of annexin V -FITC was essentially absent in neurospheres cultured with or without ferumoxides (Fig. 2B, C). Equivalent PI binding was evident in solitary cells (control: 40 ± 5 cells/40 $\times$ field; ferumoxides-labeled: 39 ± 6 cells per 40 $\times$ field) and in cells associated with the surface of the neurospheres (control: 15 ± 3 cells/neurosphere; ferumoxides-labeled: 13 ± 4 cells/neurosphere).

Migration of ICV Transplanted Ferumoxides-Labeled NPCs

Ferumoxides-labeled and unlabeled neurospheres from green fluorescent protein (GFP) transgenic mice were transplanted into the cerebral ventricles (ICV) of mice 7 days after the induction of EAE. The mice were sacrificed at the peak of the disease (day 20 post induction) and the brains were imaged ex vivo using MRI (11.7 T) and then processed for histopathology.

Ferumoxides labeling was sufficient to enable visualization of the migration of the NPCs on ex vivo MRI (Fig. 3A,B). Migration along white matter tracts was examined using iron staining with Perls' reagent and GFP fluorescence. There was an excellent agreement between the pattern of iron staining and GFP fluorescence (Fig. 3C,D). However, in view of studies showing uptake of iron particles by local macrophages (Pawelczyk et al., 2008), we used the GFP fluorescence to measure ferumoxides-labeled NPCs migration and to compare it to that of unlabeled cells. As we observed previously, there was a significant variability between the extent of transplanted cell migration in individual EAE mice (Ben-Hur et al., 2007). On average, ferumoxides-labeled NPCs migrated up to 1.9 ± 0.56 mm, as compared to 1.7 ± 0.43 mm for control cells ($p = 0.74$). Thus, ferumoxides labeling did not inhibit NPC migration in the brain. To further examine the applicability of ferumoxides labeling in the clinical setup, the spatial and temporal dynamics of NPC migration was visualized by in-vivo MRI. At one day after transplantation into the lateral ventricle of EAE mice, the neurospheres were located exclusively in the ventricle (Fig. 4A). Migration of cells into the corpus callosum was observed in MRIs at 4 and 7 days after transplantation (Fig. 4B, C). This was corroborated by ex vivo MRI, performed at 22 days post-transplantation (Fig. 4D).

Differentiation of NPCs in the EAE Mice Brains

The in vivo differentiation of ferumoxides-labeled and unlabeled GFP+ NPCs was examined following ICV transplantation into EAE brain ($n = 5$ mice per group). For that purpose, we quantified the fraction of GFAP⁺ astrocytes, NG2⁺ oligodendrocytes progenitors, and NeuN⁺ neurons among GFP transplanted cells (Fig. 5). In accordance to our previous transplantation studies in EAE, the NPCs acquired only glial lineage markers and no graft-derived neurons could be found. There was no difference in the fraction of GFAP⁺ astrocytes and NG2⁺ oligodendrocyte progenitors between the ferumoxides-labeled and unlabeled grafts. ($p = 0.17$ for GFAP⁺ and $p = 0.8$ for NG2⁺ cells; Table II).

Inhibition of Lymph Node Cell Proliferation by Ferumoxides-Labeled Neurospheres

We have previously shown that NPCs inhibit lymph node cell (LNC) proliferation without inducing apoptosis (Fainstein et al., 2008). In order to determine whether the NPCs maintain this immunomodulatory property after ferumoxides labeling, we co-cultured labeled and

unlabeled NPCs with ConA activated LNCs. ConA stimulation caused a 51-fold increase in ^3H -thymidine incorporation as compared to naïve LNCs. Co-culturing with NPCs inhibited ConA induced proliferation in a dose dependent manner, reaching $85 \pm 5\%$ inhibition at a NPC to LNC ratio of 1:2, as compared to control-stimulated LNCs. Ferumoxides-labeled NPCs caused a $83 \pm 6\%$ inhibition in similar conditions, indicating that their immunomodulatory properties were intact. (Fig. 6)

DISCUSSION

The clinical application of cell therapy requires efficient methods to track the graft in the host tissue non-invasively. MRI of SPIO-labeled cells appears to be a highly sensitive method to visualize even small numbers of cells in the brain (Bulte and Kraitchman, 2004; de Vries et al., 2005; Stroh et al., 2005), and suitable for following the migration and homing of transplanted cells. Previous studies have shown that labeled cells maintain their contrast for at least 6 weeks (Bulte et al., 2001) suggesting that SPIO labeling may be a suitable method for cell tracking over time. However, it has recently been observed that SPIOs could cause morphological changes and alterations in the differentiation pattern of human adult mesenchymal stem cells (Kostura et al., 2004; Farrell et al., 2008), although others did not find any alterations in differentiation of these cells (Arbab et al., 2005). Therefore, we deemed it necessary to perform an in-depth evaluation as to whether or not incorporation of ferumoxides exerts any significant negative effects on the functions of grafted cells.

Here, we studied both in vitro and in vivo whether ferumoxides, an FDA-approved SPIO formulation, exhibited any significant detrimental effects on the survival, differentiation, migration and immunomodulatory properties of NPCs. The survival of NPCs was measured in vitro using different apoptosis markers with a concentration of ferumoxides that was 4-fold higher than the concentration used to detect cells by MRI. Basal caspase 3/7 activity was present in neurosphere lysates. Interestingly, basal caspase-3 activity is necessary for the differentiation of neural progenitor cells (Fernando et al., 2005), indicating a normal, non-apoptotic role for caspase proteases (Fernando and Megeney, 2007). This baseline activity was not affected by ferumoxides labeling (Fig. 2), and did not increase to activity levels associated with apoptosis. As indicated by additional methods to measure apoptosis (annexin V-FITC and PI staining), routine cell culture handling of floating neurospheres induced significant apoptotic cell death. Gentle handling of neurospheres reduced significantly Annexin V-FITC reactivity but a low degree of PI staining remained in solitary cells and cells located on the surface of the neurosphere. Importantly, ferumoxides labeling did not affect Annexin V - FITC and PI staining (Fig. 2), indicating that its effect on survival was negligible as compared to the effect of culture handling. Thus, our findings confirm recent observations on SPIO-labeling of human NPCs that had no adverse effects on cell survival when using similar labeling conditions (Neri et al., 2008).

SPIO-labeling of human NPCs did not adversely affect their differentiation pattern in vitro (Neri et al., 2008). Here we used newborn mouse SVZ -derived NPCs that are partially committed to glial lineages (Ben-Hur et al., 1998). This characteristic makes them particularly suitable for replacement therapy in demyelinating diseases. Differentiation was studied both in vitro and in vivo at a ferumoxides concentration commonly used to detect cells by MRI. Ferumoxides-labeled cells did not exhibit any abnormal modification of their differentiation pattern in vitro or in the EAE brain as detected by lineage-specific antibodies (Figs. 1, 4 and Tables I, II). In addition, we did not observe any morphological alteration of the labeled differentiated cells in vitro.

Crucial for successful integration of graft cells into the host tissue is their ability to react to the host tissue signals. Particularly in EAE, transplanted neural precursors are attracted by the inflammatory process to migrate in white matter tracts towards the involved CNS regions (Ben-Hur et al., 2003). This is especially important for multiple sclerosis given the multifocal and inflammatory nature of the disease. Since it is technically impossible to transplant cells into each one of the lesions in MS, a rather practical approach is to deliver them ICV or intrathecally. Injection of NPCs into either of these fluid spaces allows wide dispersion within the CNS adjacent to the white matter tracts that are most commonly involved in the disease, and the cells can migrate toward the lesions to express their local regenerative, trophic and immunomodulatory properties (Einstein et al., 2006). Thus, it is of critical importance that ferumoxides-labeling does not limit cell motility even to a minor extent. We found that the migration of NPCs into the white matter tracts of EAE mice brains was not inhibited by ferumoxides labeling. Notably, the excellent correspondence between Prussian blue staining and GFP fluorescence proves that the ferumoxides-induced hypointense MRI signal for assessing migration is specific. Moreover, this also indicates that GFP fluorescence represents accurately the migration of ferumoxides-labeled cells, thus allowing direct comparison of experimental groups by simple GFP fluorescence microscopy.

Finally, while recent studies showed that transplanted NPCs ameliorated EAE predominantly by an immunomodulatory mechanism (Ben-Hur et al., 2003; Einstein et al., 2003; Pluchino et al., 2003, 2005). It was therefore important to study the effect of ferumoxides labeling on NPC immunomodulatory function. Indeed, ferumoxides-labeled NPCs retained their function to inhibit the proliferation of lymph node cells.

In conclusion, we have shown that ferumoxides labeling does not affect NPC functions, as indicated by their survival, migration, and differentiation in vitro and in vivo and immunomodulatory property in vitro. These findings support the further use of SPIOs in clinical trials of NPC transplantation in demyelinating disease.

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Fig. 1.

In vitro differentiation of ferumoxides-labeled NPCs. **A:** PSA-NCAM immunoreactivity on the day of neurosphere plating. **B:** Nestin immunoreactivity on the day of neurosphere plating. **C–E:** Prussian blue staining of control neurospheres (**C**), ferumoxides-labeled neurospheres 4 hr after adhesion to plate (**D**), and ferumoxides-labeled cells 4 days after adhesion to plate (**E**). **F:** NG2 immunostaining of oligodendrocytes progenitors after 1 day of differentiation. **G–J:** After 5 days of differentiation, neurospheres generate GFAP+ astrocytes (**G**), GalC+ oligodendrocytes (**H**), O4+ oligodendrocytes (**I**), and NeuN+ neurons (**J**). **A, C, D:** scale bar = 200 μ m. **B, F–J:** scale bar = 100 μ m. **E:** scale bar = 10 μ m.

Fig. 2.

Ferumoxides labeling does not increase apoptotic death in NPCs. Caspase 3/7 activity in ferumoxides/PLL-labeled neurospheres did not differ significantly from baseline activity in unlabeled neurospheres (A, $p > 0.05$). Annexin V-FITC (green) and PI (red) binding in unlabeled neurospheres (B) did not differ from that detected in ferumoxides-labeled neurospheres (C). Scale bar = 100 μm .

Fig. 3. Migration of ferumoxides-labeled NPCs in EAE mice brains. **A:** Ex vivo MRI (axial section) showing migration of ferumoxides labeled neural precursor cells, appearing as hypointensities (black) in the corpus callosum of an EAE mouse. **B:** Prussian blue histochemistry of the corresponding axial brain section of the MRI, showing many iron-labeled cells. **C:** Coronal section showing migration of GFP+, ferumoxides-labeled, transplanted NPCs in the corpus callosum of EAE mice. **D:** Prussian blue histochemistry of an adjacent section shows excellent correspondence between GFP fluorescence and iron staining. Scale bar: 200 μ m.

Fig. 4.

Serial in vivo MRI tracking of ICV transplanted NPCs in EAE. Ferumoxides-labeled NPCs were transplanted to the right ventricle of EAE mice (black arrow). At day 1 after ICV transplantation (A), cells indicated by hypointense (black) MRI signal are found exclusively within the cerebral ventricles and are absent within the corpus callosum (white arrow). At four (B) and seven (C) days after ICV transplantation some cells had migrated into the corpus callosum (white arrow). Ex-vivo MRI at day 22 post transplantation confirmed this pattern of migration (D).

Fig. 5.

In vivo differentiation of GFP⁺, ferumoxides-labeled transplanted cells. **A:** NG2⁺ oligodendrocyte progenitor. Red: anti-NG2 immunostaining. Green: GFP. **B:** GFAP⁺ astrocyte. Red: anti-GFAP immunostaining. Green: GFP. Scale bar = 10 μ m.

Fig. 6. Inhibition of ConA-induced lymph node cell (LNC) proliferation by NPCs. Ferumoxides-labeled NPCs inhibited ConA-induced LNC proliferation in a dose dependent manner similar to non-labeled NPCs.

TABLE I**In vitro Differentiation of Ferumoxides-Labeled vs. Unlabeled Cells**

Marker	Feridex-labeled Cells	Unlabeled cells	P value
NG2	71.9% $\pm$ 4.8%	70.8% $\pm$ 5.7%	0.69
GFAP	36.5% $\pm$ 8.8%	33.1% $\pm$ 9.4%	0.45
GalC	7.6% $\pm$ 2.3%	11.2% $\pm$ 3.9%	0.10
O4	9.4% $\pm$ 5.1%	10.1% $\pm$ 4.3%	0.75
NeuN	1.7% $\pm$ 1.6%	1.8% $\pm$ 1.5%	0.86

The values represent the fraction of NG2+ oligodendrocytes progenitors in labeled and unlabeled neurospheres after 1 day of differentiation, and the fraction of GFAP+ astrocytes, GalC+ and O4+ oligodendrocytes, and NeuN+ neurons after 5 days of differentiation.

TABLE II

In vivo Differentiation of Ferumoxides-Labeled vs. Unlabeled Cells

Marker	Feridex-labeled cells	Unlabeled cells	P value
NG2	41.5% $\pm$ 12.9%	39.3% $\pm$ 14.5%	0.80
GFAP	14.6% $\pm$ 0.04%	11.8% $\pm$ 2.5%	0.17
NeuN	<1%	<1%	Not relevant

The values represent the fraction of GFAP+ astrocytes and NG2+ oligodendrocyte at 2 weeks after transplantation in acute EAE brains.