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Long-Term MR Cell Tracking of Neural Stem Cells Grafted in Immunocompetent Versus Immunodeficient Mice Reveals Distinct Differences in Contrast Between Live and Dead Cells

Stacey Cromer Berman^{1,2}, Chulani Galpoththawela^{1,2}, Assaf A. Gilad^{1,2}, Jeff W. M. Bulte^{1,2,3,4}, and Piotr Walczak^{1,2,*}

¹ Division of MR Research, Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

² Cellular Imaging Section, Vascular Biology Program, Institute for Cell Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

³ Department of Chemical and Biomolecular Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

⁴ Department of Biomedical Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Abstract

Neural stem cell (NSC)-based therapy is actively being pursued in preclinical and clinical disease models. Magnetic resonance imaging (MRI) cell tracking promises to optimize current cell transplantation paradigms, however, it is limited by dilution of contrast agent during cellular proliferation, transfer of label from dying cells to surrounding endogenous host cells, and/or biodegradation of the label. Here, we evaluated the applicability of magnetic resonance imaging for long-term tracking of transplanted neural stem cells labeled with superparamagnetic iron oxide and transfected with the bioluminescence reporter gene luciferase. Mouse neural stem cells were transplanted into immunodeficient, graft-accepting *Rag2* mice or immunocompetent, graft-rejecting Balb/c mice. Hypointense voxel signals and bioluminescence were monitored over a period of 93 days. Unexpectedly, in mice that rejected the cells, the hypointense MR signal persisted throughout the entire time-course, whereas in the nonrejecting mice, the contrast cleared at a faster rate. In immunocompetent, graft-rejecting Balb/c mice, infiltrating leukocytes, and microglia were found surrounding dead cells and internalizing superparamagnetic iron oxide clusters. The present results indicate that live cell proliferation and associated label dilution may dominate contrast clearance as compared with cell death and subsequent transfer and retention of superparamagnetic iron oxide within phagocytes and brain interstitium. Thus, interpretation of signal changes during long-term MR cell tracking is complex and requires caution.

Keywords

superparamagnetic iron oxide; MR cell tracking; neural stem cells

*Correspondence to: Piotr Walczak, Division of MR Research, The Russell H. Morgan Department of Radiology and Radiological Science, Institute for Cell Engineering, Johns Hopkins University School of Medicine, Broadway Research Building Rm 649, 733 N Broadway, Baltimore, MD 21205. pwalczak@mri.jhu.edu.

The central nervous system (CNS) is the most complex of all systems in the human body, and it has extremely limited regeneration capabilities. Tissue repair of CNS damage is a difficult task, with two possible paradigms—manipulation of the endogenous progenitor cells or transplantation of exogenous cells. Although there have been some reports on mobilizing endogenous progenitors (1,2), the efficacy of this approach is limited. Alternatively, transplantation of neural precursors or stem cells introduces a large amount of exogenous cells with the potential to migrate, differentiate, and integrate into neural circuitries (3). In order for cell-based therapy to be applied clinically, techniques are required that can evaluate the migration and function of cells in vivo, help to guide treatment, and maximize the therapeutic effect.

There are currently several noninvasive imaging techniques available with which one can monitor cellular and biological processes, including noninvasive in vivo cellular imaging of neurografted stem cells in real time. For transplanted cells, a major goal is to monitor cells within the living organism and in their native environment, in particular to track the cell injection, migration, and viability, and to assess their ability to provide effective treatment. MR imaging of stem cells is a particularly useful noninvasive imaging modality, as it is clinically translatable, does not expose patients to radiation, allows whole body imaging, and has a high resolution. Indeed, MR imaging of magnetically labeled stem cells has been now been applied in several clinical cell therapy trials in patients (4).

Current strategies for in vivo tracking of transplanted cells by magnetic resonance imaging (MRI) depend on labeling of cells with an MR contrast agent (5). Superparamagnetic iron oxide (SPIO) nanoparticles have previously been used clinically as cell tracking T_2^* contrast agents which, through T_2 relaxation effects, induce hypointensities on MR images. Unfortunately, Feridex[®] (Berlex Laboratories), the SPIO contrast agent approved for clinical use in liver imaging, is no longer manufactured and available commercially. Therefore, these experiments were performed using a similar SPIO—Molday ION Rhodamine B (BioPAL, Worcester, MA), which is tagged to a fluorescent marker and is detectable by fluorescence imaging. One advantage of SPIO particles over other agents is that they are biocompatible and biodegradable through the normal biochemical pathways for iron metabolism (6). However, there are several potential pitfalls with long-term MR tracking using SPIO contrast agents. Dilution of the iron oxide label is a major limitation, as the MR signal is lost over time due to cellular proliferation, especially in the case of rapidly dividing cells. In addition, the contrast agent could possibly be transferred to surrounding endogenous cells (e.g., macrophages of microglia) after death of transplanted cells (7). If endogenous cells take up the SPIO nanoparticles, it could be difficult to interpret the imaging data, which may then give inaccurate information on the transplanted cells. Another limitation of MRI cell tracking using SPIO labeling is that this technique does not offer information about the survival or function of cells. Such information can be provided by the use of reporter gene-based imaging techniques. Bioluminescence imaging (BLI) is a well-established reporter gene-based imaging modality that has been successfully used for monitoring cell survival in small animals (5,8,9). Although BLI is not clinically translatable, the technique is useful for following the survival of cells in animal models until a robust, clinically applicable MR-reporter gene (10–12) becomes available.

Several recent studies have been conducted on the reliability of long-term tracking of transplanted, magnetically labeled cells and the persistence of the contrast agent in various organs. Baligand et al. xenografted SPIO-labeled myoblasts into the mouse leg and showed that the SPIO label still visible 3 months after transplantation even though cells were only viable for less than 1 week (13). Therefore, evaluating the presence of viable cells during long-term studies can be difficult. Similar results were reported for cells transplanted into heart tissue, indicating that MRI of SPIO-labeled cells do not reliably track long-term

engrafted cells. MR signal from labeled cardiomyoblasts persisted significantly longer after cells died, as determined by BLI (14). Terrovitis also found that MRI overestimates ferumoxide-labeled stem cell survival following transplantation in the heart (15). Every tissue will likely interact with the contrast agent differently; therefore, it is important to carefully study these interactions and the potential long-term retention of released SPIO in local tissue. Studies designed to investigate the long-term fate of iron following intracerebral transplantation of magnetically labeled stem cells, to our knowledge, have not yet been reported.

We have chosen to allograft SPIO-labeled C17.2 neural stem cells (NSCs) in the brain of two groups of mice: immunocompetent, allograft-rejecting Balb/c mice in which all cells will be eliminated; and immunodeficient, allograft-accepting *Rag2* mice, in which the cells will survive, although some degree of cell death after grafting is expected. The primary goal of this study was to establish the long-term fate, at 95 days post-transplantation, of SPIO particles and the MRI signal changes following the death of grafted cells versus the proliferation of stem cells. Cell survival or rejection was monitored and validated by reporter-gene based BLI, and the persistence or clearance of SPIOs was analyzed by T_2 and T_2^* -weighted MRI.

MATERIALS AND METHODS

Culture and Labeling of C17.2 NSCs

The *LacZ*-transfected NSC line C17.2, derived from neonatal mouse cerebellum (courtesy of Dr. Evan Snyder) (16), was cultured in Dulbecco's modified Eagle's medium (Gibco, NY), supplemented with 10% fetal bovine serum (Gibco), 5% horse serum (Gibco), 2 mM L-glutamine (Gibco), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), and 1% amphotericin B (Sigma-Aldrich). For constitutive firefly luciferase expression, 2.5×10^6 cells were transduced for 6 h with the lentiviral vector, pLenti4-CMV-fLuc2, with a multiplicity of infection of 10 and 0.1% polybrene (Chemicon, MA). Cells were grown and cultured on 75-cm² polystyrene tissue culture dishes (Corning, NY) at 37°C and 5% CO₂.

Luciferase-expressing C17.2 cells were labeled with the fluorescently tagged SPIO formulation Molday ION Rhodamine B (BioPAL, Worcester, MA) having a colloidal size of 50 nm. Fe (25 µg/mL) of Molday ION Rhodamine B was mixed with culture medium and poly-L-lysine (375 ng/mL; Sigma-Aldrich, St. Louis, MO) (17), incubated at room temperature for 1 h, and added to cells for 24 h under normal culture conditions.

Cell Transplantation

All animal procedures were approved and conducted in accordance with the institutional guidelines for the care of laboratory animals. All cells were labeled in one batch and transplanted into all mice on the same day. For transplantation, the cells were harvested, washed, and suspended in phosphate-buffered saline (PBS) at a density of 5×10^4 cells/µL. Immunocompetent, Balb/c ($n = 15$, 6–8 weeks old, Charles River, Germantown, MD) and immunodeficient, *Rag2* mice ($n = 15$, 6–8 weeks old, 129S6/SvEvTac-*Rag2*^{tm1Fwa}, Taconic, Hudson, NY) were placed in a stereotaxic device (Stoelting, Wood Dale, IL) under 2% isoflurane anesthesia. Cells ($3 \times 10^5/3$ µL) were injected using a Hamilton 31G microinjection needle (Hamilton, Reno, NV) into the right corpus callosum. The coordinates were medial lateral (ML) = +2.0 mm and dorsal ventral (DV) = −1.5 mm relative to bregma 0.0.

Bioluminescence Imaging

BLI was performed weekly on all mice ($n = 30$) from 1 to 92 days after cell transplantation using a Xenogen IVIS 200 optical imaging device equipped with a high sensitivity, cryogenically cooled, charge-coupled device (CCD) detection system. Before imaging, each mouse was intraperitoneally injected with 150 mg/kg of the substrate luciferin (Caliper Life Sciences, Hopkinton, MA) to detect firefly luciferase activity. Mice were anesthetized with 1–2% isoflurane and imaged within 15 min after luciferin injection. BLI signal was processed using Xenogen Living Imaging 2.50 software. A region of interest was selected around each brain and the total flux (photons/sec) was calculated. The size of the region of interest was kept constant to compare the mice over time. The BLI signal was normalized for each mouse individually with the first imaging time point's total flux (photos/sec) being 100% and the signal from each subsequent time point relative to the initial total flux.

MRI

In vivo MRI was performed on a Bruker Biospec 9.4 T horizontal bore spectrometer equipped with an actively radiofrequency-decoupled coil system at 2, 11, 30, 50, 72, and 93 days after transplantation ($n = 3$ Balb/c mice, $n = 5$ *Rag2* mice). Two Balb/c mice from the MR group died early in the experiment. Mice were anesthetized by isoflurane inhalation (1–2%) and immobilized in a horizontal volume coil. Images were obtained using a T_2 -weighted spin echo sequence [echo time (TE) = 12 msec, pulse repetition time (TR) = 2000 msec, average (AV) = 4, field of view = 2.0×2.0 cm, matrix = 256×256 , RARE factor = 2] and a T_2^* -weighted multigradient echo sequence (TE = 5 msec, TR = 500 msec, AV = 4, field of view = 1.70×1.70 cm, matrix = 256×256). Both T_2 and T_2^* -weighted MR images were obtained for each mouse to compare the two sequences and fully characterize the hypointensity. Although T_2^* images display a better sensitivity to iron, shorter acquisition time and higher spatial resolution, the T_2 -weighted images do not have blooming effects and enable a more precise determination of the anatomical location of the contrast agent.

At day 95 of post-transplantation, mice were transcardially perfused with PBS followed by 4% paraformaldehyde fixation. The brains were removed and fixed in 4% paraformaldehyde overnight. For postmortem MR imaging, the brains were left intact in the skull and immersed in either Fomblin LC08 (Ausimont, USA) or PBS (Gibco, NY). Postmortem MR brain images were obtained on a Bruker 11.7 T vertical MR scanner using a transmitter/receiver coil. Then, 3D T_2^* -weighted gradient-echo images were acquired with TE = 4.99 msec, TR = 120 msec, AV = 4, matrix = $368 \times 256 \times 256$, and flip angle = 30° .

Quantitative MRI Signal Analysis

MRI black pixel analysis for T_2 and T_2^* -weighted images was performed for *Rag2* and Balb/c mice for all in vivo MRI images as previously described (18). For each MR image, a Z-projection of the minimum intensity was made in ImageJ software (National Institutes of Health, Bethesda, MD). A region of interest was manually drawn around the control hemisphere, contralateral to the injection site, and a baseline pixel intensity histogram was created, using ImageJ, to establish the minimum signal intensity in the absence of iron (Fig. 1). A second region of interest was drawn around the ipsilateral hemisphere with the hypointensity, and a second pixel intensity histogram was created. The number of pixels in the ipsilateral hemisphere below the minimum signal intensity threshold was summed in Microsoft Excel and reported as black pixel count for the SPIO hypointensity. Then, the number of black pixels was normalized for each mouse with the day 2 number of black pixels being 100%. We chose to normalize the data as normalized data allows for a simpler and straightforward comparison between the two groups of mice. Normalizing the data removes the variable of differences in initial cell distribution and allows for a direct

comparison as to how that contrast hypointensity changes over time. The data is analyzed in terms of percentages of change from the day of transplantation as taking into account the biological variability/differences between mice. Postmortem 3D MRI images were processed using Amira software (Mercury Computer Systems, San Diego, CA), and 3D reconstructions were made using the Amira label voxel module.

Histology and Immunohistochemistry

Mice undergoing MR imaging were sacrificed for histology at day 95 after transplantation ($n = 3$ for Balb/c, $n = 5$ for *Rag2*). Additional groups of mice dedicated for histological analysis at earlier time points were sacrificed on days 16 and 57 ($n = 5$ for Balb/c, $n = 5$ for *Rag2* per time point). Before immunohistochemical analysis, the brains were fixed in 4% paraformaldehyde overnight, then cryopreserved in 30% sucrose, frozen in dry ice, and cryosectioned at 30 μ m slices.

For immunohistochemistry, the following primary antibodies were utilized: anti- β -galactosidase rabbit polyclonal (55976, 1:200, Cappel MP Biomedicals, Solon, OH), anti-Iba1 rabbit polyclonal (019-19741, 1:250, Wako, Richmond, VA), anti-CD45 rat monoclonal (MCA1388, 1:100, AbD Serotec, Raleigh, NC), and anti-GFAP rabbit polyclonal (Z0334, 1:400, Dako, Carpinteria, CA). Goat anti-rat 488 (A11042, 1:200) and goat anti-rabbit 488 (A11008, 1:200) from Molecular Probes (Eugene, OR) were used as secondary antibodies for immunohistochemistry. Tissue samples were blocked for 1 h at room temperature with PBS containing 10% normal goat serum and 3% Triton, incubated overnight at 4°C with primary antibody diluted with PBS, 2% normal goat serum and 3% Triton, and then incubated with the secondary antibody for 2 h at room temperature with PBS and 3% Triton. Nuclei were stained using 1 μ g/mL Hoechst dye (Invitrogen, Carlsbad, CA). Intracellular iron oxide nanoparticles were detected by rhodamine fluorescence or Prussian blue staining (17). The slides were embedded with Vectashield mounting medium (Vector, Burlingame, CA) to preserve the fluorescence signal.

Microscope analysis was performed using an Olympus BX51 fluorescence microscope equipped with an Olympus DP-70 digital acquisition system. Confocal microscopy images were obtained on a Zeiss Axiovert 200 microscope with 510-Meta confocal module and LSM Image Browser software.

RESULTS

Bioluminescence Imaging of Cell Survival and Immune Rejection

Implanted SPIO-labeled, luciferase-expressing C17.2 cells generated detectable BLI signal one day after transplantation. Over the course of the experiment (92 days), all immunodeficient *Rag2* mice had a detectable BLI signal at every time point, indicating acceptance of the cells (Fig. 2a). In immunodeficient *Rag2* mice, BLI signal gradually increased during the first 35 days and was followed by a plateau at the level of 600-fold that of the initial signal, indicating acceptance of the graft and proliferation of the cells (Fig. 2b). In immunocompetent Balb/c mice, initially there was a BLI signal increase, but after day 15, the BLI signal dropped below the background level for all but one of the Balb/c mice, indicating rejection of the graft (Fig. 2b). The one Balb/c mouse that accepted the cells had a detectable BLI signal throughout the experiment; however, it never reached the level observed in immunodeficient mice.

Histological Analysis of Cell Survival and Immune Rejection

β -Galactosidase staining for the presence of *LacZ*-expressing C17.2 cells was in good agreement with the BLI findings. β -Gal positive cells were only detected in *Rag2* mice at all

time points—days 16, 57, and 95 (Fig. 2c). In immunocompetent Balb/c mice, the immune system rejected the transplanted C17.2 cells and β -gal positive cells were not detected, except for the one Balb/c mouse that accepted the cells, further confirming that BLI is a reliable method for determining survivability of the cells.

The extent of the host immune response was evaluated using anti-CD45 staining, which is a pan-leukocyte marker that detects infiltrating macrophages, neutrophils, and B and T cells. Anti-Iba1 staining was used to detect microglia. In Balb/c mice, extensive infiltration with CD45-positive cells was observed within the graft site, correlating with immune rejection of the transplanted cells (Fig. 3a). In contrast, in immunodeficient *Rag2* mice, very few CD45-positive cells were present (Fig. 3b). A similar result was seen with anti-Iba1 staining, where the Iba1-positive microglia were often colocalized with the iron deposits in Balb/c mice (Fig. 3c), but there were less Iba1-positive cells in the graft site of *Rag2* mice (Fig. 3d).

MRI Results

In both T_2 - and T_2^* -weighted MRI of immunodeficient *Rag2* and immunocompetent Balb/c mice, hypointensities from the SPIO labeled C17.2 cells were detected within the corpus callosum the day after transplantation (Fig. 4a–d). MR hypointense signals persisted in all mice throughout the experiment and were still detectable 93 days after transplantation. Black pixel analysis demonstrated that MRI signal hypointensities decreased gradually over time in both immunocompetent Balb/c mice and immunodeficient *Rag2* mice (Fig. 5a,b). Unexpectedly, the MR signal clearance was more pronounced in the immunodeficient *Rag2* mice that accepted the cells than in the rejecting, immunocompetent Balb/c mice, which is exactly the opposite to what was previously suggested (19,20). At 93 days after transplantation, MR signal dropped in *Rag2* mice to 22.3 ± 12.3 black pixels compared with day 1 baseline, whereas Balb/c mice had 51.6 ± 6.4 black pixels from the T_2 -weighted images (Fig. 5a). For the T_2 -weighted MRI images, the differences in the number of black pixels between Balb/c and *Rag2* mice was statistically significant ($P < 0.05$, Student's *t*-test) at every time point except day 30. For the T_2^* -weighted images, the difference was statistically significant at every time point (Fig. 5b). High-resolution postmortem MRI confirmed the distribution of the hypointensities at 95 days after transplantation and was used to reconstruct the 3D view of hypointense pixels (Fig. 6).

Histological Characterization of the Contrast Agent Deposits in the Brain Tissue

Red fluorescent iron oxide nanoparticles (Molday ION Rhodamine B) were detected in both rejecting and nonrejecting mice in brain slices at all time points (days 16, 57, and 95) (Fig. 7a–f). There was a gradual loss of red fluorescence over time that correlated well with the loss of MR signal. At day 16, there was quite good colocalization of the red fluorescent iron oxide nanoparticles with β -galactosidase-positive C17.2 cells in *Rag2* mice, but, at days 57 and 95, the transplanted cells had migrated extensively from the injection site and most of them did not show positivity for red fluorescence (SPIO). At day 16 after transplantation, the time of completed rejection in Balb/c mice, the core of the residual iron oxide deposits remained in the proximity of the injection site, forming clusters up to 30 μ m in size (Fig. 8a). Similar clusters were found in *Rag2* mice (Fig. 8d) indicating that even in these immunodeficient animals shortly after transplantation a significant amount of transplanted cells undergo cell death followed by release of the SPIO particles. Brain areas containing SPIO iron deposits were hyponucleated and in Balb/c mice were surrounded by CD45-positive immune cells (Fig. 8a–c). This strongly suggests that most of iron deposits were extracellular. Some of the iron oxide nanoparticles were also internalized by endogenous cells in the brain. Anti-CD45 staining (Fig. 8a–c) or anti-Iba1 staining (Fig. 8g) showed red fluorescent iron oxide nanoparticles taken up by immune cells or microglia. Prussian blue

staining confirmed the presence of iron oxide nanoparticles and the hypointense area where they were found to persist in the brain (Fig. 8h).

DISCUSSION

In this study, we demonstrated that magnetic cell labeling with SPIO results in a strong MR signal detected as hypointensities on T_2 - and T_2^* -weighted MR images. The signal persists for at least 95 days after transplantation, regardless of the survival or death of transplanted cells. Longitudinal in vivo MR imaging showed that the extent of MR hypointensity gradually reduced over time in both graft-rejecting and nonrejecting animals; however, the decrease was more pronounced and at a faster rate in surviving and proliferating stem cell grafts (in *Rag2* mice) than in grafts that were immunorejected (in Balb/c mice). After 95 days, about 40% of the original number of black pixels remained in the Balb/c mice, compared with about 20% in the *Rag2* mice from the T_2 data. This is contradictory to previous results from Zhang et al., which reported that, when killed, SPIO-labeled neural progenitor cells were transplanted into the cisterna magna, there were no Prussian blue-positive cells histologically, implying that the iron is cleared rather than being held in endogenous cells or in the interstitium (19). In this study by Zhang et al., labeled neural progenitor cells were injected into the cerebrospinal fluid, whereas in our study, they were injected into the brain parenchyma, which could account for the difference in iron clearance from dead cells. However, viable neural progenitor cells transplanted in the parenchyma were shown to extensively migrate to the tumor, which resulted in a complete loss of MR signal from the injection site (19). They also reported a near-complete clearance of hypointense MR signals for SPIO-labeled NSCs cells that were killed with five cycles of freeze-thawing before injection, as compared with the contralateral site where the same cells were injected but not killed before transplantation (20). This differential contrast was observed at 4 weeks post-transplantation and is in stark contrast to our current results. One possible explanation is the rate of cell proliferation; C17.2 cells are immortalized and divide more rapidly than SVZ-derived progenitor cells.

We have shown that C17.2 cells, transplanted into the brain parenchyma of immunodeficient *Rag2* mice, were accepted, and migrated extensively. Based on the level of BLI signal, the surviving C17.2 cells underwent many cell divisions to reach the plateau of BLI signal at day 30 implying that each cell would only have a negligible amount of iron remaining. This explains why transplanted cells that were detected further away from the injection site were usually negative for the contrast agent. Another previously proposed possibility (21) is that the C17.2 cells divide asymmetrically, and if one of the daughter cells receives the entire contrast agent load, the other daughter cell that is free of contrast becomes undetectable immediately.

In Balb/c mice, the MR signal from the iron oxide nanoparticles remains high-despite cell rejection. The death of labeled cells due to immunorejection leads to a sudden release of contrast agent and the cell debris is phagocytosed by brain phagocytes and infiltrating immune cells. The brain is considered as an immune-privileged site, and it is separated from the other organ systems, including the immune system, by the blood-brain barrier. In physiological conditions, microglia are the main immune defense of the central nervous system, surveying the local environment for potential inflammation or foreign agents and coming out of quiescence when activated (22–24). Activated CNS-specific immune cells travel across the blood-brain barrier in the case of inflammation, and in concert with activated microglia, mount an immune response. Leukocytes and microglia that immunogenically respond to the transplanted cells are programmed to undergo apoptosis in the CNS after dealing with the foreign agent (25–27). The apoptosis response became evident in the experimental autoimmune encephalomyelitis (EAE) disease model where the

disease is monophasic and recovery occurs rather than the autoimmune response perpetuating itself (26). Because of the delicate nature of the CNS, the immune response is modulated to avoid rampant damage (25,26,28). Some immune cells are able to cross the blood-brain barrier back into the circulation, but it is believed that the main fate of activated microglia and leukocytes in the CNS following the immune response is apoptosis (25–27). Because the immune cells die and remain in the area of immune response, this may explain why the iron oxide clusters that are initially released from rejected cells remain in the CNS and are not cleared from the brain parenchyma.

The two strains of mice were chosen for this study to compare the differences of cell rejection versus cell acceptance. There is initial cell death after transplantation in both strains of mice, which may confound the interpretation of our results, but as this cell death will occur in both groups of mice, the differences in contrast over time must be derived from the biological differences between surviving versus rejected cells. In *Rag2* mice, a significant amount of free iron clusters remain near the injection site, even at 95 days after transplantation. This suggests that some of the labeled, transplanted cells died upon transplantation or shortly thereafter, and therefore, the iron load remained high in the area surrounding the dead cells causing a persistence of MR signal. It has been documented that a large percentage (possibly 90%) of transplanted cells into the CNS may die shortly after transplantation (29–32). A large portion of the cell death occurs as apoptosis during the first week after transplantation (32). If the cells die during or immediately after transplantation, their entire iron load is released and remains in the CNS. Based on our results, this extracellular iron primarily accounts for the persistent, localized MR hypointensity for up to 95 days in both immunodeficient and immunocompetent mice. Administration of neuro-protective and pro-survival factors can be considered to reduce the amount of cell death following transplantation during this critical period and potentially change the proportion of MR signal originating from dead versus surviving, labeled cells (30). Pro-survival factors have been shown to enhance the function of cardiomyocytes transplanted into the rat heart by Laflamme et al. (33).

The interpretation of long-term MR cellular imaging is difficult, as we have shown in this study. The MR hypointensity can originate from particles within microglia or from deposits persisting in the interstitium after the immune rejection of transplanted C17.2 cells. Deposits of iron oxide in the brain continue to produce high MR signal long after transplanted cells are dead. Therefore, MRI of magnetically labeled cells alone is currently unable to distinguish transplanted labeled cells from free iron oxide released upon cell death or iron taken up by endogenous cells. The recent studies by Baligand et al. in leg muscle labeled cell transplants (13) as well as Chen et al. and Terrovitis et al. in intracardiac transplants (14,34) are in agreement with our findings in the brain. Previous studies involving SPIOs in the brain and spinal cord have shown that SPIO-labeled cells could be used to track transplanted cells in the brain (3) and spinal cord (7); however, the limitations of long-term tracking were also recognized.

Unquestionably, the strength of MR tracking of labeled cells is short-term imaging and real-time MR-guided delivery of cellular therapeutics (4,35,36). For the brain, this will be of particular importance for intra-arterial injections in stroke treatment (37). This will be especially important for human translational studies, where needle positioning and verification of accurate delivery of cells into target organs is critical (4,38). Long-term stem cell tracking in the clinic would benefit from a permanent method of tagging cells that cannot be taken up by endogenous immune cells, diluted, or held in the interstitium. A reliable, cell-specific reporter would lead to more accurate long-term MR tracking of cells. One solution to this problem may be the development of an MR reporter gene based on the expression of an existing or artificial protein that will have MR signal only when the

transplanted cells are viable and functioning. Although there is significant effort to develop such a reporter system (10,11), a robust MR reporter gene system with sensitivity sufficient for stem cell tracking is currently not available.

In conclusion, MRI of magnetically labeled cells should be undertaken cautiously, and preferably with an additional imaging method that would provide data on the viability of grafted cells. The significant persistence of SPIO-hypointensities in MRI, long after transplanted stem cells are dead, and the fact that current cell transplantation procedures are typically associated with substantial cell death following transplantation indicates that this technique cannot reliably report long-term on stem cell engraftment in the brain. Thus, MRI cell tracking with SPIO particles should only be considered for long-term use if there is a certainty that the majority of transplanted cells remain viable throughout the study. However, MRI cell tracking still remains the method of choice for visualizing the injection and location of cells shortly after transplantation.

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FIG. 1.

MRI black pixel analysis. A Z-projection of minimum intensity was created and regions of interest were manually drawn for the ipsilateral hemisphere (red) and contralateral hemisphere (yellow). Pixel intensity histograms were created for each region of interest (top—contralateral, bottom—ipsilateral) and the minimum pixel value for the contralateral hemisphere served as the baseline threshold (blue line). The threshold was applied to the hemisphere with cell injection and the total number of pixels below this baseline threshold (red box) was counted as the number of black pixels due to the iron oxide.

FIG. 2.

Monitoring survival and rejection of transplanted C17.2 cells. **a:** Bioluminescence imaging of C17.2 cells transplanted into graft accepting *Rag2* and graft rejecting Balb/c mice. Mice were imaged weekly from day 2 to day 92. **b:** Quantification of BLI signal normalized to the signal on day 2 for each mouse. **c:** Anti- β -galactosidase staining (green) for transplanted C17.2 cells 16 days after transplantation. Grafted cells could be detected in *Rag2* mice but not in Balb/c mice, except for one mouse that did not reject.

FIG. 3.

Histopathological assessment of immunological/inflammatory response within the graft site 16 days after transplantation. **a** and **b**: Anti-CD45 staining for infiltrating leukocytes (green), iron oxide (red), and Hoechst dye (blue) in Balb/c (**a**) and *Rag2* (**b**) mice. **c** and **d**: Anti-Iba1 staining for microglia (green) and iron oxide (red) in Balb/c (**c**) and *Rag2* (**d**) mice.

FIG. 4. Serial in vivo MRI. **a** and **b**: T_2 -weighted MR images of Balb/c (**a**) and Rag2 (**b**) mice at days 2, 11, 30, 50, 72, and 93 after transplantation. In all mice, hypointensities were detectable within the corpus callosum the day after transplantation and persisted for up to 93 days. **c** and **d**: T_2^* -weighted MR images of Balb/c (**c**) and Rag2 (**d**) mice at days 2, 11, 30, 50, 72, and 93.

FIG. 5. MRI black pixel quantification for T_2 -weighted (**a**) and T_2^* -weighted (**b**) MR images for Balb/c ($n = 3$) and *Rag2* ($n = 5$) mice on day 2, 11, 30, 50, 72, and 93 (*Significant, $P < 0.05$; **Significant, $P < 0.01$).

FIG. 6.
3D Reconstruction of postmortem high-resolution MRI of a Balb/c mouse brain at 95 days after transplantation (about 80 days after complete rejection of the graft).

FIG. 7. Histological detection of iron oxide label dilution. Anti- β -galactosidase staining (green) for C17.2 cells is positive in *Rag2* mice at days 16, 57, and 95 (**a–c**) and negative in Balb/c mice (**d–f**). The amount of iron oxide nanoparticles (red) gradually decreased over time in both mouse strains but was detected at all time points. Scale bar 200 μ m for (a–f).

FIG. 8. Histological detection of iron oxide label and host tissue response at day 16 after grafting. Staining for anti-CD45 pan-leukocyte antigen (green), iron oxide (red), and nuclei (blue) in Balb/c mice (**a–c**) and *Rag2* mice (**d–f**) demonstrates large deposits of SPIO contrast agent (asterisks) that are surrounded by CD45⁺ immune cells in Balb/c mice (arrows) very little CD45 positivity can be detected in *Rag2* animals. The tissue space occupied by these deposits is anucleated, indicating that these are extracellular deposits. Anti-Iba1 staining (**g**, green) shows that some iron oxide nanoparticles colocalize within microglia (arrows). Prussian blue staining (**h**) shows iron oxide nanoparticles (blue) clustered together in a hyponucleated brain region. Scale bar 20 μm for (a–g), 100 μm for (h).