Magnetic Resonance Imaging Assessment of Macrophage Accumulation in Mouse Brain after Experimental Traumatic Brain Injury

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Abstract

Macrophages contribute to secondary damage and repair after central nervous system (CNS) injury. Micron-sized paramagnetic iron oxide (MPIO) particles can label macrophages *in situ*, facilitating three-dimensional (3D) mapping of macrophage accumulation following traumatic brain injury (TBI), via *ex vivo* magnetic resonance microscopy (MRM) and *in vivo* monitoring with magnetic resonance imaging (MRI). MPIO particles were injected intravenously (iv; 4.5 mg Fe/Kg) in male C57BL/6J mice ($n = 21$). A controlled cortical impact (CCI) was delivered to the left parietal cortex. Five protocols were used in naive and injured mice to assess feasibility, specificity, and optimal labeling time. *In vivo* imaging was carried out at 4.7 Tesla (T). Brains were then excised for 3D MRM at 11.7 T. Triple-label immunofluorescence (MPIO via Dragon Green, macrophages via F480, and nuclei via 4,6-diamidino-2-phenylindole [DAPI]) of brain sections confirmed MPIO particles within macrophages. MRM of naives showed an even distribution of a small number of MPIO-labeled macrophages in the brain. MRM at 48–72 h after CCI and MPIO injection revealed MPIO-labeled macrophages accumulated in the trauma region. When MPIO particles were injected 6 days before CCI, MRM 48 h after CCI also revealed labeled cells at the injury site. *In vivo* studies of macrophage accumulation by MRI suggest that this approach is feasible, but requires additional optimization. We conclude that MPIO labeling and *ex vivo* MRM mapping of macrophage accumulation for assessment of TBI is readily accomplished. This new technique could serve as an adjunct to conventional MR approaches by defining inflammatory mechanisms and therapeutic efficacy of anti-inflammatory agents in experimental TBI.

Key words: head trauma; inflammation; MRI

Introduction

SECONDARY DAMAGE AFTER TRAUMATIC BRAIN INJURY (TBI) results from a complex sequence of pathophysiological events involving vascular, cellular, and biochemical cascades (Baethmann et al., 1988; Dusart and Schwab, 1994; McIntosh et al., 1996). A number of mechanisms of secondary injury and repair are mediated in part by the inflammatory response, including contributions from neutrophils, activated microglia/macrophages, astrocytes, and even neurons (Hallenbeck, 1977; Giulian, 1987; Schoettle et al., 1990; Clark et al., 1994; Holmin et al., 1995; Soares et al., 1995; Sinz et al., 1998; Barone

and Feuerstein, 1999). Neuroinflammation is widely known to have both a beneficial and detrimental role after TBI: beneficial on the one hand, in repairing injured tissue, and detrimental on the other hand, in releasing neurotoxic substances that cause additional brain damage (Morganti-Kossmann et al., 2001; Scherbel et al., 1999; Sinz et al., 1999). Recent work by Gris et al. (2004) suggests that macrophages may play a pivotal role at the interface between early detrimental and delayed beneficial effects of inflammation. Therefore, the ability to detect the response of these cells *in vivo* after TBI may lead to a greater understanding of the cellular mechanisms behind secondary injury and repair.

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In experimental models of TBI, assessment of the inflammatory response has been dependent on histological evaluation of affected tissue after sacrifice. In humans, mediators of inflammation after TBI have been assessed in cerebrospinal fluid, urine, and dialysate, and in a few studies, immunohistochemically and/or on Western blot in resected cerebral contusions (Clark et al., 1999; Sinz et al., 1998; Berger and Kochanek, 2006; Hutchinson et al., 2007). With regard to macrophages, it has been particularly difficult to differentiate the contribution of endogenous microglia versus infiltrating monocytes. This might be important given the possibility that macrophages derived from these precursor cells could play different roles, as suggested in the recent important work of Longbrake et al. (2007). One elegant approach to address this question in experimental brain injury in mice is the use of bone marrow chimeras with bone marrow-derived macrophages expressing enhanced green fluorescent protein (Popovich and Hickey, 2001; Tanaka et al., 2003). That strategy requires total body irradiation and bone marrow transplantation, and is currently limited to mice.

It has been demonstrated recently that micron-sized paramagnetic iron oxide (MPIO) particles can label cells for *in vivo* detection by magnetic resonance imaging (MRI) (Hinds et al., 2003; Shapiro et al., 2004). These iron oxide particles create distinct areas of hypointensity on T_2^* -weighted images. Macrophages readily endocytose these particles; therefore, it is possible to label these cells directly *in vivo* (Wu et al., 2006). An advantage with the larger sized particles is that single particles can be visualized with MRI *in vivo* (Shapiro et al., 2006; Wu et al., 2006).

Cell-specific imaging has become an important field in MRI. The increased development of superparamagnetic iron oxide particles as contrast agents has allowed this field to grow. Phagocytic monocytes play an important role with phagocytic, antigen-presenting, and secretory functions; thus, mapping their accumulation with magnetic resonance microscopy (MRM) and tracking the response of these cells with MRI could lead to greater understanding of the pathophysiology of numerous diseases. To develop effective therapies for the treatment of TBI, a thorough appreciation of the role of macrophages could be essential. We report the use of MPIO particles to detect the response of macrophages using MRM

and *in vivo* MRI after experimentally induced TBI produced by controlled cortical impact (CCI) in a mouse model.

Methods

Animal model

All experiments were approved by the Animal Care and Use Committee of Carnegie Mellon University and the University of Pittsburgh School of Medicine. Male C57BL/6J mice 11–15 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). The mice were fed with laboratory chow and water *ad libitum*, and maintained under temperature-controlled conditions with 12-h light/dark cycles. Mice were randomly assigned into one of five groups (Fig. 1). Group 1 included naives that only underwent MRI assessment ($n=3$). Group 2 included naives injected with MPIO and imaged 24 h later ($n=4$). Group 3 mice had MPIO injected at 24 h after CCI, which was then followed 24 h later by MRI evaluation ($n=5$). Group 4 mice underwent CCI, which was followed 48 h later by an MPIO injection and 24 h after injection by MRI examination ($n=4$). Group 5 mice were injected with MPIO particles, followed 6 days later by CCI and MRI assessment at 48 h after injury ($n=5$).

Controlled cortical impact

The mouse CCI model was used as previously described (Smith et al., 1995) with minor modifications (Whalen et al., 1999; Kochanek et al., 2006). Anesthesia was induced with 3% isoflurane in a plastic jar, and maintained with N_2O/O_2 (1:1) and 1–2% isoflurane via nose cone. Mice were placed in a stereotaxic holder, a temperature probe was inserted through a burr hole into the left frontal cortex, and the left parietal bone was removed for trauma. Once brain temperature reached $37 \pm 0.5^\circ\text{C}$ and was maintained at this temperature for 5 min, a vertically directed CCI was delivered (5.0 m/sec at a depth of 1.0 mm). After injury, the bone flap was replaced and sealed with dental cement, and the incision was closed. Anesthesia was discontinued, and the animals were monitored in supplemental O_2 for 30 min and returned to their cages until MRI assessment.

FIG. 1. Schematic of the five experimental groups studied depicting time of magnetic resonance imaging (MRI), micron-sized paramagnetic iron oxide (MPIO) particle injection, and controlled cortical impact (CCI) injury, where applicable.

Contrast agent

The contrast agent used was MPIO particles (Bangs Laboratories, Fishers, IN), which are 0.9- μm superparamagnetic styrene-divinyl benzene inert polymer microspheres that contain a magnetite core and a fluorescein-5-isothiocyanate dye (Dragon Green fluorescent probe) contained within the cross-linked polymer sphere. The contrast agent was administered in the mice as an intravenous bolus dose of 4.5 mg Fe/kg body weight, through a catheter placed in the femoral vein. The dose was chosen based on a previous study (Wu et al., 2006).

In vivo MRI

Anesthesia for imaging was induced as previously described (Foley et al., 2005). Mice were intubated and mechanically ventilated with 2% isoflurane in a 1:1 N₂O/O₂ gas mixture. A femoral artery catheter was surgically inserted for continuous blood pressure monitoring and arterial blood sampling. A rectal temperature probe was inserted for monitoring and maintenance of temperature at $37.0 \pm 0.5^\circ\text{C}$ using a warm air heating system (SA Instruments, New York, NY). Mean arterial blood pressure (MABP) and heart rate were also monitored throughout image acquisition. Arterial blood gases were collected at the beginning and end of the studies, and ventilator parameters were adjusted to maintain arterial CO₂ tension (PaCO₂) at 30–45 mm Hg.

MRI studies were performed on a 4.7-Tesla (T), 40-cm-bore Bruker AVANCE AV system (Billerica, MA), equipped with a 15-cm-diameter shielded gradient insert and a home-built RF coil. T₂*-weighted gradient-echo images were acquired with the following parameters: repetition time (TR) = 400 ms, echo time (TE) = 10 ms, field of view (FOV) = 2.5 cm, slice thickness = 1 mm, eight averages, and a 256 × 256 matrix, with an in-plane resolution of 100 μm .

Ex vivo MRM

After *in vivo* MRI evaluation, the brains were perfused and fixed in 4% paraformaldehyde. The fixed brains were imaged using an 11.7-T, 89-mm-bore Bruker AVANCE spectrometer, equipped with a Micro 2.5 gradient insert. High-resolution three-dimensional (3D) images were acquired with the following parameters: TR = 500 ms, TE = 6 ms, FOV = 1 × 1 × 1.7 cm, four averages, 256 × 256 × 128 matrix. The post-processing program AMIRA, version 2.3 (Visage Imaging, Carlsbad, CA), was used to create 3D surface renderings from this data set (see Fig. 4 below). Briefly, segmentation of the data set assigns to each pixel of the images a label describing what it belongs to, in this case either brain, contusion region, or particles. Surfaces were then extracted from the segmentation results and simplified. An isosurface is then created to provide an impression of the 3D shape of the object.

Immunohistochemistry

Macrophages were detected immunohistochemically in paraformaldehyde-fixed coronal brain sections, taken after MRM, through the dorsal hippocampus, which is in the peri-contusional region in this model directly beneath the contusion site. Endogenous peroxidase was quenched for 15 min with 0.3% hydrogen peroxide in methanol. Nonspecific binding was minimized by incubating the sections in 3%

normal goat serum in phosphate-buffered saline (PBS) for 1 h. Sections were then incubated overnight with a 1:500 dilution of primary anti-macrophage antibody (F480; Serotec, Oxford, UK), then specific labeling was detected with a biotin conjugated goat anti-rat IgG using a 30-min incubation with Texas Red Avidin D (Vector Laboratories, Burlingame, CA) for immunofluorescence studies. Then the slices were subjected to dual-channel fluorescence microscopic examination, i.e., in green (Dragon Green) for MPIO detection and red (Texas Red) for F480⁺ cell detection. Nuclei of hippocampal neurons were identified by incubation of the slides with 4,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) for 30 min, to confirm anatomical location. For light microscopic examination, immunolabeling was detected with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Appropriate primary delete control incubations were prepared.

Light microscopic evidence of macrophage accumulation (F480-immunopositive cells) was quantified in coronal brain sections (×20) from naive mice or mice at 48 or 72 h after CCI. Regions of interest in brain-injured mice included medial peri-contusional cortex, dorsal hippocampus (immediately beneath the contusion), and dorsal thalamus, all in the hemisphere ipsilateral to injury. Brain sections from naive mice were quantified in identical regions of interest in the left hemisphere.

MPIO quantification

Ex vivo 3D images were analyzed to quantify the number of MPIO particles present in the brains. The “analyze particles” feature of the Image J software (Abramoff et al., 2004) was used to automatically determine the number of hypointense spots present in the 3D data set. Since other features can generate hypointensity in T₂*-weighted MR images, such as hemorrhaging and defects in the sample, we used a highly circular area within a certain number of pixels (4–50) to define the search criteria (circularity of 0.75–1.0, where 1.0 is a perfect circle). This will most likely give an underestimate of the labeled cell infiltration, since areas of hypointensity that have non-circular shapes due to accumulation of large number of labeled cells, or labeled cells that are masked by artifacts will not fit the criteria and not be counted.

Statistical analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Data were analyzed using an analysis of variance (ANOVA) with Student-Newman-Keuls test. A probability of less than 0.05 ($p < 0.05$) was considered significant.

Results

Physiology

Temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ for all mice. Arterial blood gases were measured at both the beginning and conclusion of each MRI experiment and averaged. Mean PaCO₂, PO₂, pH, MABP, and hematocrit did not differ between groups (Table 1).

Ex vivo MRM

MPIO-labeled cells are best visualized with high-resolution MRM. Discrete black spots can be seen in the MRM images

TABLE 1. PHYSIOLOGICAL DATA^a

	Group 1: Naive (n = 3)	Group 2: Naive with MPIO (n = 4)	Group 3: Imaging at 48 h after CCI: MPIO injection at 24 h after CCI (n = 5)	Group 4: Imaging at 72 h after CCI: MPIO injection at 48 h after CCI (n = 4)	Group 5: Imaging at 48 h after CCI: MPIO injection 6 days before CCI (n = 5)
MABP (mm Hg)	88.0 ± 4.0	97.0 ± 4.0	78.0 ± 5.0	91.0 ± 6.0	79.0 ± 9.0
PaCO ₂ (mm Hg)	39.2 ± 5.1	41.7 ± 3.9	41.7 ± 3.7	35.4 ± 7.1	38.8 ± 3.9
PaO ₂ (mm Hg)	239.8 ± 38.6	210.5 ± 48.4	214.9 ± 34.4	227.0 ± 36.1	255.5 ± 10.0
pH	7.36 ± 0.05	7.34 ± 0.04	7.34 ± 0.01	7.36 ± 0.04	7.35 ± 0.02
Hematocrit (%)	44.0 ± 5.0	39.0 ± 8.1	42.0 ± 6.0	39.0 ± 7.0	41.0 ± 9.0
Heart rate (BPM)	434.0 ± 41.0	426.0 ± 94.0	432.0 ± 90.0	460.0 ± 96.0	447.0 ± 86.0

^aData are mean ± SD.

from the excised brains in mice that were injected with MPIO when compared with naive (Fig. 2). A low level of random particles was seen in naive brains in mice that were injected with MPIO and imaged 24 h later (group 2). Figure 2B shows representative data from group 2 mice. Mice imaged at 48 h

after CCI and 24 h after MPIO injection (group 3) revealed a few particles in the injured hemisphere in peri-contusional regions (Fig. 2C). Maximal particle accumulation in and around the contusion site was seen in mice in group 4, imaged at 72 h after CCI and 24 h after MPIO particle injection

FIG. 2. Visualization of macrophage accumulation by high-resolution magnetic resonance microscopy (MRM) of representative excised brains at 11.7 Tesla. (A) Naive mouse (group 1) with no contrast injection. (B) A low level of randomly localized areas of punctuate hypointensity (arrows and arrowheads) representing micron-sized paramagnetic iron oxide (MPIO) particles from a naive mouse (group 2) that was injected 24 h earlier with MPIO particles. (C) A small number of particles in brain from a mouse (group 3) that was injected with MPIO particles at 24 h after controlled cortical impact (CCI) and imaged at 48 h after CCI. (D) More robust contusional and peri-contusional accumulation of particles in a mouse (group 4) that was injected with MPIO particles at 48 h after CCI and imaged at 72 h after CCI. (E) Smaller number of particles in a mouse (group 5) that was injected with MPIO particles at 6 days before CCI and imaged at 48 h after CCI.

(Fig. 2D). When MPIO particles were injected 6 days before CCI and mice imaged 48 h after CCI, particles were still detected in the contusion and peri-contusional sites (group 5; Fig. 2E), suggesting that labeled macrophages were still present at 6–8 days after injection and were able to travel to the trauma site. This supports the argument that the hypointense signal imaged in our studies represents labeled macrophages, rather than a nonspecific accumulation of MPIO particles traversing an acutely injured blood-brain barrier (BBB). This is further supported by the extremely short half-life of these particles in blood, which has been found to be on the order of minutes in rats (Ye et al., 2008). It also supports the ability of labeled macrophages to be available to accumulate to an injury site, even when labeled 6 days prior to the insult.

Figure 3 shows quantification of MPIO particle accumulation in brain either after injury or control conditions (naive). A significant increase in MPIO signal was detected in mice injected with MPIO 48 h after CCI and imaged 24 h later (i.e., 72 h after CCI, group 4, $p < 0.05$ vs. all other groups).

Figure 4 depicts representative 3D surface renderings showing the distribution of the particles throughout the brain. The naive mice, 24 h after MPIO injection, exhibit a homogeneous low-level distribution of particles throughout the brain (group 2; Fig. 4A). At 48 h after injury and 24 h after MPIO injection (group 3), there is an obvious increase in the amount of particles present throughout the brain, with a small concentration in the contusion area (Fig. 4B). Consistent with the quantification in Figure 4, at 72 h post-CCI (group 4), there is a greater concentration of particles within the injury site, although there are still many present throughout the brain (Fig. 4C). When the particles were injected into the mice 6 days

before injury and imaging carried out at 48 h after CCI, particles are readily visualized, albeit highly concentrated within the trauma region (group 5; Fig. 4D).

In vivo MRI

The infiltration of macrophages labeled with MPIO was observed non-invasively with MRI. Discrete, punctuate, areas of hypointensity were seen in the trauma region of mice 48 h after CCI and 24 h after MPIO injection (group 3; Fig. 5B), and mice 72 h after CCI and 24 h after particle administration (group 4; Fig. 5C) when compared with naive mice (group 1; Fig. 5A). Based on the work of Wu et al. (2006), each discrete spot of hypointensity most likely indicates one or a few MPIO-labeled macrophages that have infiltrated the trauma site. However, *in vivo* assessment of macrophage accumulation was not as robust as seen with the MRM method and was, at best, qualitative with the labeling and imaging methods used in this study.

Immunohistochemistry

Immunofluorescence evaluation of the mouse brains after *in vivo* labeling with MPIO is shown in Figure 6. The region shown is the peri-contusional area of the dorsal hippocampus immediately beneath the contusion. Figure 6A depicts F480 staining of mouse macrophages (red) at 48 h after CCI and 24 h after MPIO injection (group 3). Figure 6B depicts the endogenous Dragon Green label of the MPIO particles (green). Figure 6C shows dual-labeled macrophages (yellow) along with staining for hippocampal nuclei (DAPI, blue) to confirm the anatomical location. The yellow color confirms the presence of MPIO particles within some of the macrophages that have

FIG. 3. Quantification of micron-sized paramagnetic iron oxide (MPIO) particles in injured brain from mice from the three controlled cortical impact (CCI) groups (groups 3, 4, and 5) along with naive mice injected with MPIO particles 24 h prior to imaging (group 2). MPIO particle accumulation in brain was significantly increased in mice injected with MPIO at 48 h after CCI and imaged at 72 h after CCI group 4 (* $p < 0.05$ versus all other groups; analysis of variance [ANOVA] and Student-Newman-Keuls test). Seventy-two hours after CCI has been previously shown to represent a time of robust macrophage accumulation after CCI in this model (Bayır et al., 2005) and after severe traumatic brain injury (TBI) in humans (Sinz et al., 1998). Data are mean $\pm$ standard error of the mean (SEM).

FIG. 4. Pseudocolor three-dimensional (3D) surface renderings from all slices from mice in four of the groups studied: (A) Group 2. (B) Group 3. (C) Group 4. (D) Group 5. In these images, micron-sized paramagnetic iron oxide (MPIO) particles appear red, and a surface map of the extent of the contusion (as defined on the magnetic resonance [MR] images) is outlined in green. (A) In naive mice (group 2) injected with MPIO particles 24 h before imaging, a low level of signal is seen homogeneously distributed throughout the brain. (B) In mice (group 3) injected 24 h after controlled cortical image (CCI) and imaged 48 h after injury, particle levels increase throughout the brain, compared with naive, with some concentration in the contusion site (green). (C) By 72 h after injury (group 4), accumulation progressed, consistent with the quantification shown in this figure, and more particles are within the contusion area compared with the rest of the brain. (D) In mice (group 5) injected with MPIO particles 6 days before CCI and imaged 48 h after injury, particles are readily detected, and an obvious signal highly focused in the contusion site is appreciated.

accumulated in the peri-contusional region. Similarly, Figure 6D–F shows macrophages, MPIO particles, and the dual-fluorescence correlation, respectively, for mice at 72 h after CCI and 24 h after particle injection (group 4), the time of peak macrophage accumulation in this model (Bayir et al., 2005). Many of the macrophages visualized contain MPIO particles, as previously reported in a model of acute cardiac transplant rejection in rats (Wu et al., 2006).

Figure 7A–E depicts the distribution of F480-immunopositive cells in coronal brain sections in naive mice, and in mice at 72 h after CCI. Pericontusional immunoreactivity is obvious, and was seen in cortex, hippocampus, and thalamus ipsilateral to impact. Some immunoreactivity was also seen in the contralateral corpus callosum at 72 h after injury.

Semiquantitative assessment in these three regions of interest in naive mice and from mice at both 48 and 72 h after injury revealed readily identifiable immunopositive cells at both 48 and 72 h after injury, with the largest number (~10–15-fold greater than naive) seen at 72 h. Accumulation in the contusion proper could not be assessed related to the loss of friable necrotic contusional tissue during processing.

Discussion

Macrophages were labeled *in vivo* via injection of MPIO particles into the femoral vein of mice. Macrophages take up the label and are thus rendered visible to MRI or MRM (Wu et al., 2006). These cells then migrate to the trauma region after

FIG. 5. Non-invasive visualization of macrophage accumulation by magnetic resonance imaging (MRI) at 4.7 Tesla after experimental traumatic brain injury (TBI). (A) A representative *in vivo* image from a naive mouse (group 1). (B) Representative mouse image from group 3, which represents controlled cortical impact (CCI), followed 24 h later by injection of micron-sized paramagnetic iron oxide (MPIO) particles. MRI was then carried out at 48 h after CCI. The image slice is taken through the contusion site. A small number of punctate areas of hypointensity (arrows) are seen in and around the contusion site. Macrophages labeled with MPIO particles appear 35–50 times their normal size (Wu et al., 2006), suggesting that each discrete spot corresponds to one or a few labeled macrophages. (C) Representative mouse image from group 4, which represents CCI followed by MPIO injection and MRI at 48 and 72 h, respectively.

injury. Immunofluorescence examination of brain sections showed the presence of MPIO particles within macrophages in an area of decreased MR signal intensity, confirming a correlation between MRI findings and histology, and supporting the specificity of the MRM and MRI signals as being derived from accumulated macrophages rather than free iron particles.

We also studied a variety of paradigms of post-injury labeling and imaging to determine the optimal approach to the application of this method, to explore the kinetics of macrophage accumulation, and to confirm specificity of labeling in experimental TBI. Our data are consistent with clinical and experimental reports that suggest macrophage accumulation after

FIG. 6. Immunofluorescence evaluation of representative coronal mouse brain sections through the injury after *in vivo* labeling with micron-sized paramagnetic iron oxide (MPIO) particles. The pericontusional area of the dorsal hippocampus immediately beneath the contusion is shown in each example. (A) F480 staining of macrophages (red) in mice (group 3) at 48 h after controlled cortical impact (CCI) and 24 h after MPIO particle injection. Robust accumulation is readily seen. (B) The endogenous Dragon Green label of the MPIO particles (green). (C) Dual-labeled macrophages (yellow, F480, and Dragon Green) along with staining for nuclei (DAPI, blue) to confirm both the cellular localization of the label in macrophages and the anatomical location of hippocampal neurons in the section chosen. The yellow color confirms the presence of MPIO particles within some of the macrophages that have accumulated in the pericontusional region. (D–F) Macrophages (F480), MPIO particles (Dragon Green endogenous label), and the dual-labeled correlation (yellow), along with DAPI (blue) staining, respectively, for mice (group 4) injected with MPIO at 48 h after CCI and imaged at 72 h after CCI. Many of the macrophages visualized contain MPIO particles. Scale bar = 40 μ m (for A–C, provided in C); 20 μ m (D–F, provided in F).

FIG. 7. Light microscopic assessment of the pattern and time course of macrophage accumulation in mouse brain using F480 immunostaining. (A) A representative coronal section ($\times 4$ magnification) from a mouse brain ipsilateral to the impact at 72 h after controlled cortical impact (CCI). Immunoreactivity is obvious in peri-contusional cortex and hippocampus (arrow), in contrast to naive (B), where immunopositive cells are rarely seen. (C) A representative coronal brain section through the medial peri-contusional cortex from a mouse at 72 h after CCI revealing robust immunoreactivity. (D) A representative coronal brain section taken through the corpus callosum and underlying hippocampus contralateral to the impact. A modest amount of F480 immunoreactivity is seen (arrow) in cells within the corpus callosum. Scale bar = $20 \mu\text{m}$ (C,D). (E) Semi-quantitative assessment (mean $\pm$ standard error of the mean [SEM]) of the distribution and time course of macrophage accumulation (F480 immunoreactivity; immunopositive cells per high-power field [HPF]) in medial pericontusional cortex (open bars), dorsal thalamus (solid bars), and dorsal hippocampus (hatched bars) in the hemisphere ipsilateral to the contusion in injured mice, and the left hemisphere in naive mice. F480 immunoreactivity was greatest at 72 h after injury. $n = 2$, per group per time point.

TBI 48–72 h after injury. Our studies also indicate that labeling nearly a week before injury still allows visualization of a macrophage signal in brain at 48 h after injury. This latter approach suggests that the macrophage labeling is specific, since the free MPIO particles are known to have an extremely short circulating half-life in rats (Ye et al., 2008).

In a previous study by our group, immunohistochemical detection of inflammatory cells in mice after CCI showed that polymorphonuclear leukocytes (PMN) were abundant 24 h after CCI, while macrophages progressively accumulate and are readily detected at 72 h (Bayir et al., 2005). We did not carry out a comprehensive quantitative assessment macrophage accumulation in this initial feasibility study of *in vivo* labeling. To achieve that goal, based on these initial data, it likely would be best to consider labeling one or more days before the injury (to minimize any potential transit of particles through an injured BBB after injury) and then carry out MRM mapping at various time points after injury. Given that normalization of the data to the level of systemic labeling could be desirable, approaches considering either an injured versus non-injured hemispheric ratio of accumulation, or a ratio of the signal in injured brain versus an extracerebral site might be considered. Such an approach would mirror the classic studies from the work of Hallenbeck's laboratory early in the study of leukocyte recruitment in stroke using radioactively labeled cell with Indium-111 (Hallenbeck et al., 1986). Further studies are needed in this regard.

MPIO particles were seen more clearly in our *ex vivo* studies than in our *in vivo* studies. This is most likely due to the different imaging resolutions. In particular, the *in vivo* images were collected with a 1-mm slice thickness versus a 10–20-fold increase in resolution with *ex vivo* MRM. Lower resolution is also a consequence of the reduced scan times that are necessary when imaging *in vivo* (20 min as compared to 18 h *ex vivo*). Another factor that may have made differentiation of the MPIO particles difficult *in vivo* was the presence of hemorrhage after TBI, which produces areas of low signal intensity, making it difficult to discern discrete areas of hypointensity on the images. However, local hemorrhage would not be anticipated to be a major concern for application of this method to most experimental models of focal or global cerebral ischemia, or neurodegenerative diseases. Better strategies are needed to be able to distinguish the MPIO particles within the trauma site *in vivo*. Work by Shapiro et al. (2005) found that the role that field strength plays in the size of the hypointense spots is not significant, and therefore recommend a strategy based on maximizing the TE and perhaps using a T_2^- rather than a T_2^* -based imaging technique.

The MPIO particles were sparse in the brains of naive mice. This is consistent with the possibility of an intravascular and/or peri-vascular site for these particles in brain. We cannot, however, completely rule out that a small number of particles may cross the brain by transcytosis or exclude the possibility that a few particles are phagocytosed by circulating monocytes which then migrate to the brain as part of the normal turnover of peri-vascular microglia (Ford et al., 1995). After injury, our immunochemical studies suggest that most of the particles represent accumulated macrophages containing MPIO particles derived from systemic labeling. However, again, we cannot rule out the possibility that some free particles directly diffuse across an injured BBB and are then taken up by accumulated macrophages. Our immunohistochemical

work and paradigm where labeling preceded injury by 6 days further argue that selective *in vivo* labeling of macrophages can be accomplished. To maximize specificity of the labeling macrophages, we suggest that MPIO particles should be injected outside of the immediate peri-trauma period (i.e., either one or more days before or after injury). We have not, however, proven that such a strategy is optimal.

The physiochemical pathways, pharmacokinetics, and toxicity of another iron particle used for macrophage labeling, namely, Ultrasmall Particles of Iron Oxide (USPIO), has been well characterized (Frank et al., 2003). After administration, USPIO accumulate in Kupffer cells (and other reticuloendothelial cells), and are thus loaded with label for imaging once they accumulate in an injury or infection focus. Eventually, the particles enter the normal iron pool of the body (Weisleder et al., 1990). In contrast, the bio-distribution of MPIO particles is less well characterized. This work is currently being carried out in our laboratory. Due to its non-degradable coating, it is likely that MPIO particles will not follow the same pathway as USPIO, as we have monitored the presence of MPIO particles for over 120 days following a single injection (Ye et al., 2008).

Siglienti et al. (2006) showed that macrophage uptake of small and ultra-small superparamagnetic iron oxide contrast agents led to a modest deviation towards an anti-inflammatory and less-responsive phenotype in mice. The immunomodulatory effects of MPIO particles are unknown, and work is needed to examine if their effects are negligible. That finding, however, would suggest the possibility of an underestimation of macrophage accumulation—and supports the diagnostic opportunity of this approach in CNS injury.

Once recruited into the brain, macrophages have been proposed to secrete a large number of pro-inflammatory cytokines, nitric oxide, oxygen radicals, and an up-regulation of major histocompatibility complex antigens (Csuka et al., 2000; Kreutzberg, 1996; Morganti-Kossmann et al., 2002). However, they may play a role in regeneration and repair, including secretion of neurotrophins and other growth factors and their receptors (Mitrasinovic et al., 2005; Ricci et al., 2000; Schwartz and Yoles, 2006), particularly at more delayed time points after injury (Gris et al., 2004). The technique of detecting MPIO-labeled macrophages using MRI may help in defining the role of accumulated macrophages and the effect of various therapies targeting macrophage accumulation, particularly if additional refinements can be made to enhance resolution of the *in vivo* MRI component of this work.

There are several limitations to this initial study of macrophage accumulation in TBI as assessed by MRM. First, although we assessed multiple temporal labeling strategies, we did not carry out a comprehensive assessment of the time course of macrophage accumulation with either immunohistochemistry or MPIO. Chen et al. (2003) studied CCI in rats and reported that macrophage accumulation (quantified by OX-42 staining) peaked at 4 days after injury. Zhang et al. (2006) demonstrated peak ED-1-positive macrophage accumulation at 96 h after CCI in rats. Thomale et al. (2007) built on these findings and, relevant to our work, assessed the topography of macrophage accumulation in rats after CCI. Macrophage accumulation in the contusion (assessed via ED-1) peaked at 72 h after injury, while accumulation in pericontusional cortex was more delayed, peaking at 7 days. Our robust MPIO signal in the contusion at 72 h after injury thus

parallels this finding. We also recognize that we were unable to differentiate immunohistochemically whether a given F480-positive cell is derived from microglia or a peripheral blood monocyte. Strategies such as the use of green fluorescent protein-expressing macrophages are required (Tanaka et al., 2003). That approach has been used in stroke models, but to our knowledge, it has not been applied to TBI. Germane to that point, we noted some MPIO accumulation and also some F480 labeling in the contralateral hemisphere after CCI, suggesting some contralateral damage, as reported by Hall et al. (2008). It is not clear, however, based on the methodologies used in our study, whether the MPIO signal is from intravascular or parenchymal macrophages, or whether the F480-positive cells in the contralateral hemisphere represent activated microglia or accumulated macrophages. Finally, we recognize that our MR quantification approach likely gives a conservative estimate of macrophage accumulation. Further study is needed.

We conclude that MPIO particles can be used to label macrophages for MR assessment of the response to experimental TBI in mice. MRM provides valuable 3D maps of macrophage accumulation, minimizing the need for preparation and quantification of stained brain sections. In addition, ultimately, serial and non-invasive *in vivo* assessment of macrophages after TBI could have great potential in defining the cellular mechanisms of secondary injury and repair and response to novel treatments. Additional work is needed to optimize the *in vivo* approach, and to more completely characterize this exciting opportunity for experimental and potentially clinical CNS injury.

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Author Disclosure Statement

No competing financial interests exist.

References

- Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image processing with Image. *J. Biophotonics Int.* 11, 36–42.
- Baethmann, A., Maier-Hauff, K., Kempfski, O., Unterberg, A., Wahl, M., and Schürer, L. (1988). Mediators of brain edema and secondary brain damage. *Crit. Care Med.* 16, 972–978.
- Barone, F.C., and Feuerstein, G.Z. (1999). Inflammatory mediators and stroke: new opportunities for novel therapeutics. *J. Cereb. Blood Flow Metab.* 19, 819–834.
- Bayir, H., Kagan, V.E., Borisenko, G.G., Tyurina, Y.Y., Janesko, K.L., Vagni, V.A., Billiar, T.R., Williams, D.L., and Kochanek, P.M. (2005). Enhanced oxidative stress in iNOS-deficient mice after traumatic brain injury: support for a neuroprotective role of iNOS. *J. Cereb. Blood Flow Metab.* 25, 673–684.
- Berger, R.P., and Kochanek, P.M. (2006). Urinary S100B concentrations are increased after brain injury in children: a preliminary study. *Pediatr. Crit. Care Med.* 7, 557–561.
- Chen, S., Pickard, J.D., and Harris, N.G. (2003). Time course of cellular pathology after controlled cortical impact injury. *Exp. Neurol.* 182, 87–102.
- Clark, R.S., Kochanek, P.M., Adelson, P.D., Bell, M.J., Carcillo, J.A., Chen, M., Wisniewski, S.R., Janesko, K., Whalen, M.J., and Graham, S.H. (1999). Increases in Bcl-2 and cleavage of caspase-1 and caspase-3 in human brain after head injury. *FASEB J.* 13, 813–821.
- Clark, R.S., Schiding, J.K., Kaczorowski, S.L., Marion, D.W., and Kochanek, P.M. (1994). Neutrophil accumulation after traumatic brain injury in rats: comparison of weight drop and controlled cortical impact models. *J. Neurotrauma* 11, 499–506.
- Csuka, E., Hans, V.H., Ammann, E., Trentz, O., Kossmann, T., and Morganti-Kossmann, M.C. (2000). Cell activation and inflammatory response following traumatic axonal injury in the rat. *NeuroReport* 11, 2587–2590.
- Dusart, I., and Schwab, M.E. (1994). Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. *Eur. J. Neurosci.* 6, 712–724.
- Foley, L.M., Hitchens, T.K., Kochanek, P.M., Melick, J.A., Jackson, E.K., and Ho, C. (2005). Murine orthostatic response during prolonged vertical studies: effect on cerebral blood flow measured by arterial spin labeled MRI. *Magn. Reson. Med.* 54, 798–806.
- Ford, A.L., Goodsall, A.L., Hickey, W.F., and Sedgwick, J.D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct *ex vivo* antigen presentation to myelin basic protein-reactive CD4⁺ T cells compared. *J. Immunol.* 154, 4309–4321.
- Frank, J.A., Miller, B.R., Arbab, A.S., Zywicke, H.A., Jordan, E.K., Lewis, B.K., Bryant, L.H., Jr., and Bulte, J.W. (2003). Clinically applicable labeling of mammalian and stem cells by combining superparamagnetic iron oxides and transfection agents. *Radiology* 228, 480–487.
- Giulian, D. (1987). Ameboid microglia as effectors of inflammation in the central nervous system. *J. Neurosci. Res.* 18, 155–171.
- Gris, D., Marsh, D.R., Oatway, M.A., Chen, Y., Hamilton, E.F., Dekaban, G.A., and Weaver, L.C. (2004). Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. *J. Neurosci.* 24, 4043–4051.
- Hall, E.D., Bryant, Y.D., Cho, W., and Sullivan, P.G. (2008). Evolution of post-traumatic neurodegeneration after controlled cortical impact traumatic brain injury in mice and rats as assessed by the de Olmos silver and fluorojade staining methods. *J. Neurotrauma* 25, 235–247.
- Hallenbeck, J.M., Dutka, A.J., Tanishima, T., Kochanek, P.M., Kumaroo, K.K., Thompson, C.B., Obrenovitch, T.P., and Contreras, T.J. (1986). Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. *Stroke* 17, 246–253.
- Hallenbeck, J.M. (1977). Prevention of postischemic impairment of microvascular perfusion. *Neurology* 27, 3–10.
- Hinds, K.A., Hill, J.M., Shapiro, E.M., Laukkanen, M.O., Silva, A.C., Combs, C.A., Varney, T.R., Balaban, R.S., Koretsky, A.P., and Dunbar, C.E. (2003). Highly efficient endosomal labeling of progenitor and stem cells with large magnetic particles allows magnetic resonance imaging of single cells. *Blood* 102, 867–872.
- Holmin, S., and Mathiesen, T. (1995). Intracerebral inflammatory response to experimental brain contusion. *Acta Neurochir.* 132, 110–119.

- Hutchinson, P.J., O'Connell, M.T., Rothwell, N.J., Hopkins, S.J., Nortje, J., Carpenter, K.L., Timofeev, I., Al-Rawi, P.G., Menon, D.K., and Pickard, J.D. (2007). Inflammation in human brain injury: Intracerebral concentrations of IL-1 α , IL-1 β , and their endogenous inhibitor IL-1ra. *J. Neurotrauma* 24, 1545–1557.
- Kochanek, P.M., Vagni, V., Janesko, K.L., Washington, C.B., Crumrine, P.K., Garman, R.H., Jenkins, L.W., Clark, R.S., Homanics, G.E., Dixon, C.E., Schnermann, J., and Jackson, E.K. (2006). Adenosine A1 receptor knockout mice develop lethal status epilepticus after experimental traumatic brain injury. *J. Cereb. Blood Flow Metab.* 4, 565–575.
- Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19, 312–318.
- Longbrake, E.E., Lai, W., Ankeny, D.P., and Popovich, P.G. (2007). Characterization and modeling of monocyte-derived macrophages after spinal cord injury. *J. Neurochem.* 102, 1083–1094.
- McIntosh, T.K., Smith, D.H., Meaney, D.F., Kotapka, M.J., Genarelli, T.A., and Graham, D.I. (1996). Neuropathological sequelae of traumatic brain injury: relationship to neurochemical and biomechanical mechanisms. *Lab. Invest.* 74, 315–342.
- Mitrasinovic, O.M., Grattan, A., Robinson, C.C., Lapustea, N.B., Poon, C., Ryan, H., Phong, C., and Murphy, G.M., Jr. (2005). Microglia overexpressing the macrophage colony-stimulating factor receptor are neuroprotective in a microglial-hippocampal organotypic coculture system. *J. Neurosci.* 25, 4442–4451.
- Morganti-Kossmann, M.C., Rancan, M., Otto, V.I., Stahel, P.F., and Kossmann, T. (2001). Role of cerebral inflammation after traumatic brain injury: a revisited concept. *Shock* 16, 165–177.
- Morganti-Kossmann, M.C., Rancan, M., Stahel, P.F., and Kossmann, T. (2002). Inflammatory response in acute traumatic brain injury: a double-edged sword. *Curr. Opin. Crit. Care* 8, 101–105.
- Popovich, P.G., and Hickey, W.F. (2001). Bone marrow chimeric rats reveal the unique distribution of resident and recruited macrophages in the contused rat spinal cord. *J. Neuropathol. Exp. Neurol.* 60, 676–685.
- Ricci, A., Greco, S., Mariotta, S., Felici, L., Amenta, F., and Bronzetti, E. (2000). Neurotrophin and neurotrophin receptor expression in alveolar macrophages: an immunocytochemical study. *Growth Factors* 18, 193–202.
- Scherbel, U., Raghupathi, R., Nakamura, M., Saatman, K.E., Trojanowski, J.Q., Neugebauer, E., Marino, M.W., and McIntosh, T.K. (1999). Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. *Proc. Natl. Acad. Sci. USA* 96, 8721–8726.
- Schwartz, M., and Yoles, E. (2006). Immune-based therapy for spinal cord repair: autologous macrophages and beyond. *J. Neurotrauma* 23, 360–370.
- Schoettle, R.J., Kochanek, P.M., Magargee, M.J., Uhl, M.W., and Nemoto, E.M. (1990). Early polymorphonuclear leukocyte accumulation correlates with the development of posttraumatic cerebral edema in rats. *J. Neurotrauma* 7, 207–217.
- Shapiro, E.M., Skrtic, S., Sharer, K., Hill, J.M., Dunbar, C.E., and Koretsky, A.P. (2004). MRI detection of single particles for cellular imaging. *Proc. Natl. Acad. Sci. USA* 101, 10901–10906.
- Shapiro, E.M., Skrtic, S., and Koretsky, A.P. (2005). Sizing it up: cellular MRI using micron-sized iron oxide particles. *Magn. Reson. Med.* 53, 329–338.
- Shapiro, E.M., Sharer, K., Skrtic, S., and Koretsky, A.P. (2006). In vivo detection of single cells by MRI. *Magn. Reson. Med.* 55, 242–249.
- Siglienti, I., Bendszus, M., Kleinschnitz, C., and Stoll, G. (2006). Cytokine profile of iron-laden macrophages: implications for cellular magnetic resonance imaging. *J. Neuroimmunol.* 173, 166–173.
- Sinz, E.H., Kochanek, P.M., Dixon, C.E., Clark, R.S., Carcillo, J.A., Schiding, J.K., Chen, M., Wisniewski, S.R., Carlos, T.M., Williams, D., DeKosky, S.T., Watkins, S.C., Marion, D.W., and Billiar, T.R. (1999). Inducible nitric oxide synthase is an endogenous neuroprotectant after traumatic brain injury in rats and mice. *J. Clin. Invest.* 104, 647–656.
- Sinz, E.H., Kochanek, P.M., Heyes, M.P., Wisniewski, S.R., Bell, M.J., Clark, R.S., DeKosky, S.T., Blight, A.R., and Marion, D.W. (1998). Quinolinic acid is increased in CSF and associated with mortality after traumatic brain injury in humans. *J. Cereb. Blood Flow Metab.* 18, 610–615.
- Smith, D.H., Soares, H.D., Pierce, J.S., Perlman, K.G., Saatman, K.E., Meaney, D.F., Dixon, C.E., and McIntosh, T.K. (1995). A model of parasagittal controlled cortical impact in the mouse: cognitive and histopathologic effects. *J. Neurotrauma* 12, 169–178.
- Soares, H.D., Hicks, R.R., Smith, D., and McIntosh, T.K. (1995). Inflammatory leukocytic recruitment and diffuse neuronal degeneration are separate pathological processes resulting from traumatic brain injury. *J. Neurosci.* 15, 8223–8233.
- Tanaka, R., Komine-Kobayashi, M., Mochizuki, H., Yamada, M., Furuya, T., Migita, M., Shimada, T., Mizuno, Y., and Urabe, T. (2003). Migration of enhanced green fluorescent protein expressing bone marrow-derived microglia/macrophage into the mouse brain following permanent focal ischemia. *Neuroscience* 117, 531–539.
- Thomale, U.W., Bender, M., Casalis, P., Rupprecht, S., Griebel, M., Neumann, K., Woiciechowsky, C., Unterberg, A.W., and Stover, J. (2007). Tacrolimus depresses local immune cell infiltration but fails to reduce cortical contusion volume in brain-injured rats. *Immunobiology* 212, 567–576.
- Weissleder, R., Elizondo, G., Wittenberg, J., Rabito, C.A., Bengel, H.H., and Josephson, L. (1990). Ultrasmall superparamagnetic iron oxide: characterization of a new class of contrast agents for MR imaging. *Radiology* 175, 489–493.
- Whalen, M.J., Carlos, T.M., Dixon, C.E., Schiding, J.K., Clark, R.S., Baum, E., Yan, H.Q., Marion, D.W., and Kochanek, P.M. (1999). Effect of traumatic brain injury in mice deficient in intercellular adhesion molecule-1: assessment of histopathologic and functional outcome. *J. Neurotrauma* 16, 299–309.
- Wu, Y.L., Ye, Q., Foley, L.M., Hitchens, T.K., Sato, K., Williams, J.B., and Ho, C. (2006). *In situ* labeling of immune cells with iron oxide particles: an approach to detect organ rejection by cellular MRI. *Proc. Natl. Acad. Sci. USA* 103, 1852–1857.
- Ye, Q., Wu, Y.L., Foley, L.M., Hitchens, T.K., Eytan, D.F., Shirwan, H., and Ho, C. (2008). Longitudinal tracking of recipient macrophages in a rat chronic cardiac allograft rejection model with non-invasive MRI using micrometer-sized paramagnetic iron oxide particles. *Circulation* 118, 149–156.
- Zhang, Z., Artelt, M., Burnet, M., Trautmann, K., and Schluesener, H.J. (2006). Early infiltration of CD8⁺ macrophages/microglia to lesions of rat traumatic brain injury. *Neuroscience* 141, 637–644.

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