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JOURNAL OF THE AMERICAN HEART ASSOCIATION

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*Stroke* published online Mar 12, 2009;

DOI: 10.1161/STROKEAHA.108.531269

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214  
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ISSN: 1524-4628

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# Early-Stage Investigations of Ultrasmall Superparamagnetic Iron Oxide-Induced Signal Change After Permanent Middle Cerebral Artery Occlusion in Mice

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**Background and Purpose**—MR signal changes after intravenous ultrasmall superparamagnetic iron oxide (USPIO) injection are related to inflammatory cells at the subacute stages after focal cerebral injury. However, at the early stages, the interpretation of USPIO-related MR signal alterations remains controversial. Here, we compared MR signal changes after intravenous USPIO injection with the histological iron and macrophage distribution during the first 24 hours in a rodent model of acute stroke.

**Methods**—Multiparametric MRI at 7T and histological USPIO distribution were confronted from 6 to 24 hours after permanent middle cerebral artery occlusion in mice. Blood–brain barrier disruption was assessed using gadolinium MRI and immunoglobulin staining. Prussian blue staining was performed to depict the USPIO brain distribution. USPIO uptake by phagocytes was assessed by immunochemistry on brain tissue, peripheral blood cells, and monocyte cells derived from bone marrow culture.

**Results**—After USPIO injection, 4 areas of early signal change were observed on every MRI. In all these areas, iron particles were mostly free whether detected in the vascular and cerebrospinal fluid compartments or in the interstitium. Within the first 24 hours, USPIO-loaded cells were not detected in the blood of injured mice or in cultured monocytic cells incubated with USPIO at plasmatic concentration.

**Conclusions**—These results suggest that, in this model, early reproducible USPIO-related MR signal changes are mainly caused by passive diffusion of free USPIO after blood–brain barrier leakage and by intravascular trapping rather than by peripheral phagocyte infiltration. (*Stroke*. 2009;40:00-00.)

**Key Words:** brain ischemia ■ inflammation ■ magnetic resonance imaging  
■ ultrasmall superparamagnetic particles of iron oxide

Neuroinflammation is a crucial pathogenic mechanism in ischemic stroke.<sup>1</sup> Postischemic inflammation is dominated by cells of the mononuclear phagocyte system comprising resident microglia and bloodborne macrophages. Noninvasive monitoring of these cells may improve the understanding of the time course of postischemic brain inflammation. Accordingly, there is a real need for novel imaging techniques, specifically detecting inflammatory cells in vivo.

Ultrasmall superparamagnetic nanoparticles of iron oxide (USPIOs) are taken up by mononuclear phagocytic cells after intravenous injection and have been recently introduced as an MRI contrast agent marking macrophages/microglia in inflammatory lesions.<sup>2</sup> In various models of experimental brain ischemia, accumulation of iron oxide particles has been

observed in the infarct zone during the subacute stages of lesion development.<sup>3</sup> After 24 hours postischemia, most iron-related signal changes on MRI are indisputably paralleled by phagocyte-associated iron deposition detected on histology. Recent pilot studies have also used USPIO-enhanced MRI to assess neuroinflammation in human ischemic stroke.<sup>4,5</sup>

However, at the early stages after experimental focal cerebral ischemia, USPIO-related MRI signal alterations are difficult to interpret.<sup>6</sup> The exact route of USPIO uptake and brain distribution remains elusive, hampering the interpretation of USPIO-related signals. Three hypotheses have been proposed to explain the MRI signal changes observed after USPIO injection: (1) intravascular trapping of iron particles<sup>6,7</sup>; (2) USPIO uptake by phagocytes<sup>8</sup> on the assumption

Received July 9, 2008; final revision received October 26, 2008; accepted November 6, 2008.

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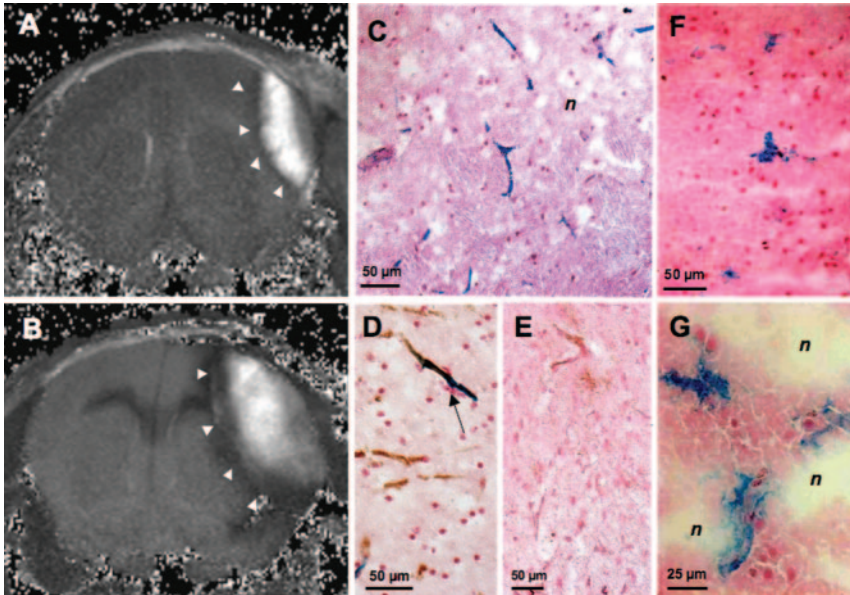
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*Stroke* is available at <http://stroke.ahajournals.org>

DOI: 10.1161/STROKEAHA.108.531269





**Figure 2.** MRI and immunohistochemistry in pMCAO+USPIO mice at 6 hours (top row) and 24 hours (bottom row) in Area I (perilesional area). The axial T2 map showed T2 decrease in the periphery of the lesion (arrowheads: A, at 6 hours; B, at 24 hours). Iron staining by Prussian blue (PB) revealed iron staining in capillaries in the border zone of the necrotic lesion (C). Double staining with PB and anti-CD31 for endothelial cells once again revealed iron staining in vessels (D, arrows). F4/80 immunostaining showed very few positive phagocytes at this early postpMCAO stage (E). In unfixed tissue, sparse spots of PB staining were also detected within the interstitium (F, at 6 hours; G, at 24 hours). n indicates necrotic tissue.

## Results

All animals survived the protocol.

### USPIO-MR Signals in Focal Cerebral Ischemia

Permanent middle cerebral artery occlusion resulted in a reproducible focal lesion in the ipsilateral dorsolateral cortex detected as an apparent diffusion coefficient decrease and heterogeneous hyperintense signal on T2-WIs on precontrast MRI at 4 hours post-pMCAO.

In all pMCAO+USPIO mice, 4 areas of consistent signal changes were observed on post-USPIO GRE and T2 imaging at 6 hours and 24 hours.

An early marked signal dropout was first detected in the border zone (Area I) of the lesion 1 hour postinjection and persisted 24 hours post-pMCAO (respectively, Figures 2A and 2B).

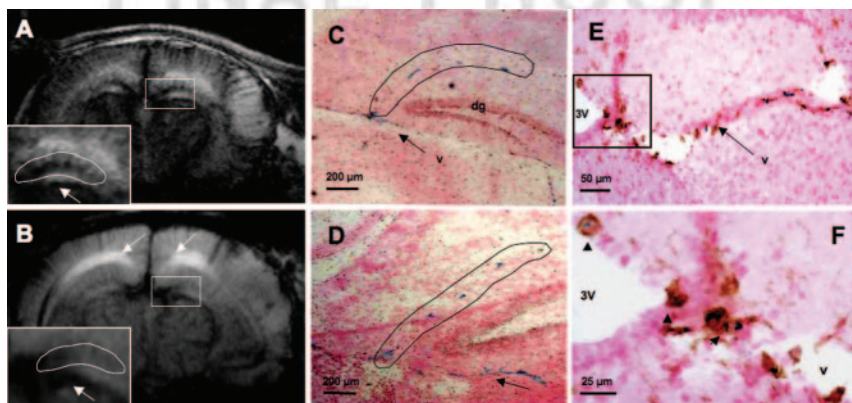
In the third ventricle and the 2 hippocampus regions (Area II), strongly contrasted hypointense signal lines appeared

early after USPIO injection (Figure 3A–B). The hypointensity of the upper dark line (circled on magnification of Figure 3A–B), which anatomically corresponded to the hippocampal lacunar molecular layer, decreased between Hours 6 and 24, unlike the other hypointense lines of this area, which corresponded anatomically to the velum (arrows on magnification of Figure 3A–B).

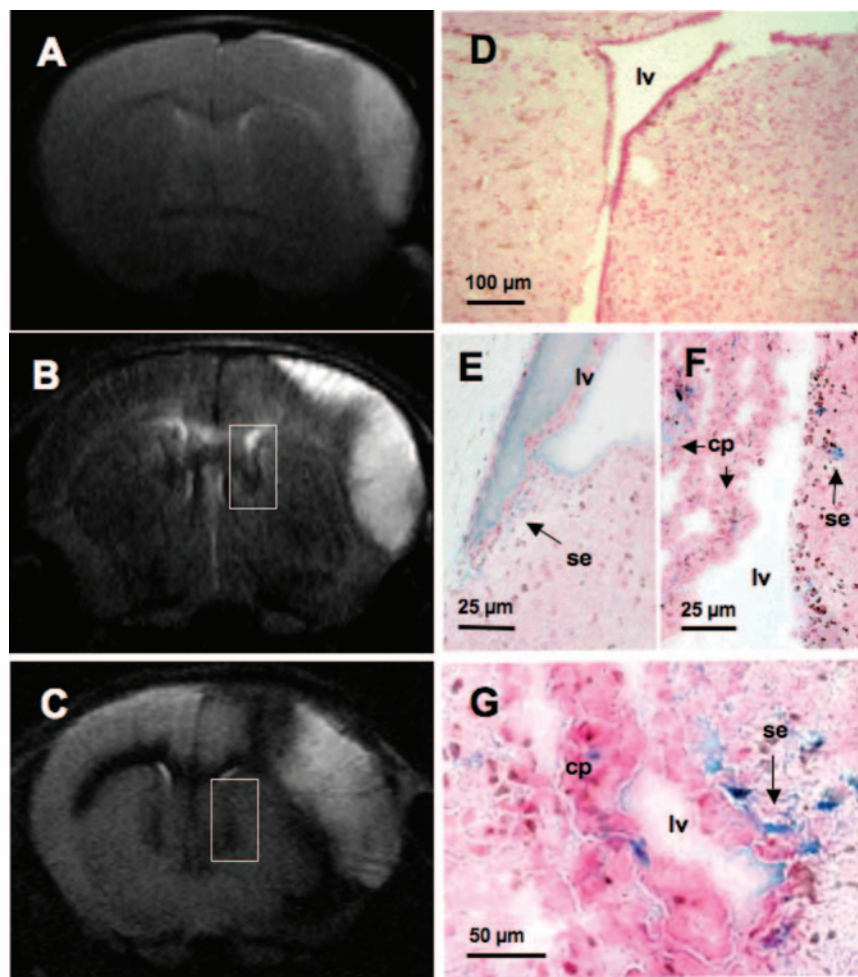
Along the lateral ventricles (Area III), signal loss was observed at 6 hours (Figure 4B) and 24 hours post-pMCAO (Figure 4C).

Finally, a hyperintense signal was observed in the corpus callosum (Area IV) on GRE T1-weighted imaging (Figure 3B, arrows) with a corresponding signal decrease on T2 maps (Figures 2B, 4C, and Figure 5I, arrows).

None of the described MR signal alterations were observed in control pMCAO mice not injected with USPIO. These control mice are presented on T1-WI (Figure 5D) and on T2-WI (Figures 4A and 5F–G). In nonoperated USPIO-



**Figure 3.** MRI and immunohistochemistry at 6 hours (top row) and 24 hours (bottom row) in pMCAO+USPIO mice in Area II (third ventricle and 2 hippocampi). The axial slice showed GRE signal loss in Area II (box: A, at 6 hours; B, at 24 hours) on magnification in the inset with corresponding arrow and circle on histology (C–D). Note the hypersignal (T1 enhancement) in the corpus callosum at 24 hours post-pMCAO (arrows) in B. PB revealed iron particles in vessels of the hippocampal lacunar molecular layer (circled in C–D; dg, dentate gyrus). Arrows in C and D show iron staining in the velum interpositum (v). Double staining with F4/80 and Prussian blue showed sparse USPIO-labeled macrophages in the periependymal zone of the third ventricle (E and on magnification [box] in F) and in the periependymal region of the third ventricle (3V).



**Figure 4.** MRI and immunohistochemistry at 6 hours (2 top rows) and 24 hours (bottom row) in pMCAO+USPIO mice in Area III (wall of the lateral ventricles). The axial T2-weighted slice before (A) and 1 hour (B) and 19 hours (C) after USPIO injection showed T2 decrease along the lateral ventricles (box). F4/80 immunostaining detected few phagocytes in this area (D). Prussian blue staining (E–G) on unfixed tissue revealed iron deposits in the lumen of the lateral ventricle (lv), in the subependymal region (arrow, se), and the choroid plexus (cp).

injected control mice, organized signal loss, anatomically corresponding to vessels, was seen on GRE imaging after injection at 6 hours and, to a lesser extent, at 24 hours.

#### **Histological Distribution of Iron and Phagocytes in Each Area of USPIO-MR Signal Change**

Iron particles were exclusively observed in the areas of reproducible MR signal change after USPIO injection and not in any other part of the brain.

##### **Area I: Perilesional Zone**

In the perilesional zone, iron staining was visualized exclusively within the blood vessels at 6 hours (Figure 2C) and 24 hours (Figure 2D) in all fixed brains. Vascular localization was furthermore demonstrated by double staining with anti-CD31 for endothelium visualization (Figure 2D). Very few F4/80+ phagocyte cells were present in the perilesional area at these early stages postischemia (Figure 2E) in line with a previous study.<sup>13</sup> In the current work, these F4/80+ phagocyte cells were always without iron particles. The unfixed procedure confirmed this vascular localization, but also showed some sparse interstitial Prussian blue staining (extracellular and extravascular; Figure 2F–G).

##### **Area II: Third Ventricle and Hippocampus Regions**

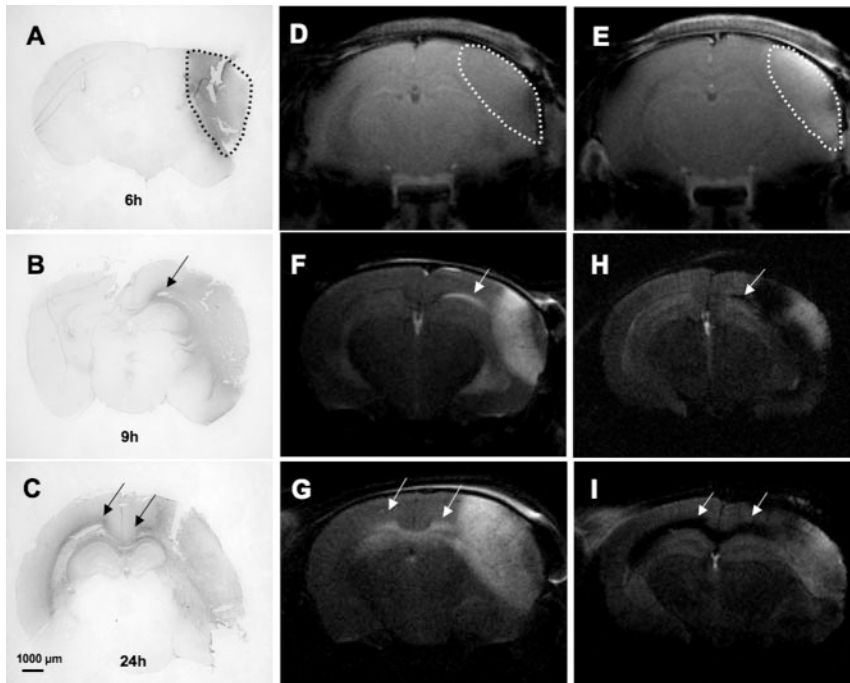
In this area, at 6 hours and 24 hours, iron staining was present in the vena (Figure 3C–D, arrows), which correspond to

prolongations of the pia matter in the brain bathed by cerebrospinal fluid (CSF) and containing vessels. Iron staining was also detected in the vessels of the lacunar molecular layers of the hippocampi (Figure 3C–D). The unfixed brain procedure was clearly better for visualizing free iron particles in this area (in 100% of brains versus 45% in fixed tissue). Using double staining with F4/80 antibody for phagocytes on fixed tissue, few iron-labeled macrophages were detected in the periventricular zone of the third ventricle (Figure 3E–F) or in the vena interposita (perivascular and meningeal phagocytes).

##### **Area III: Along the Lateral Ventricles**

In this area, iron particles were not observed in the lumen of the ventricle of fixed brains. In contrast, iron staining was detected in the ventricle and the choroid plexus of all unfixed brains (Figure 4E–F) at 6 hours post-pMCAO. In half of these unfixed brains, interstitial iron particles were also detected in the subependymal region (Figure 4E–F).

At 24 hours, iron staining of the choroid plexus remained intense, whereas lumen iron staining disappeared with both fixation procedures. In contrast, the subependymal diffusion increased at 24 hours. As already seen in the border zone of the lesion, very few F4/80+ phagocyte cells were present at these early postischemia stages (Figure 4D) and always without iron particles.



**Figure 5.** Correlation between early BBB disruption with IgG leakage (left column) and the spatiotemporal pattern of MR signal change (median and right column) in Area IV (corpus callosum [CC]). IgG leakage was initially confined to the lesion (circled in A, at 6 hours post-pMCAO), then to the ipsilateral CC (arrow in B), and finally spread to the whole CC (C). Early BBB breakdown was assessed by enhancement after DOTA-gadolinium (DOTA-Gd) injection on an axial GRE T1-weighted slice (D, before DOTA-Gd; E, after). Axial T2-weighted slice of pMCAO without USPIO mice showed a hyperintense signal in the lesion (vasogenic edema) and along the ipsilateral CC (arrow in F) at 6 hours and in the whole CC at 24 hours (G). The axial T2-weighted slice of pMCAO+USPIO mice showed USPIO-induced signal loss at the periphery of the lesion and in the ipsilateral CC (arrow in H) at 6 hours and in the whole CC at 24 hours (I).

#### **Area IV: Corpus Callosum**

Surprisingly, no iron staining was observed in the first 24 hours post-pMCAO whether on fixed or unfixed tissue despite a marked and reproducible iron-related MR signal. At 6 hours, there were some F4/80-positive cells in the ipsilateral corpus callosum. F4/80 immunostaining increased at 24 hours with marked phagocyte staining in the whole corpus callosum (data not shown), but without iron particles.

#### **Histological Distribution of USPIO and Phagocytes in Control Mice**

In nonoperated USPIO-injected mice, iron staining was observed in vessels and the vascular stroma of the choroid plexus on histology. There was no iron staining in the parenchyma, vessels, or CSF compartments (ventricles, vena) in noninjected mice. In pMCAO mice without USPIO injection, like in pMCAO+USPIO mice, very few F4/80+ phagocyte cells were present at these early postischemia stages in the border zone of the lesion. Likewise, there were only a few F4/80-positive cells in the ipsilateral corpus callosum at 6 hours in pMCAO mice with marked phagocyte staining in the whole corpus callosum at 24 hours.

#### **Assessment of Early BBB Disruption and Diffusion of Immunoglobulins**

To test the hypothesis of USPIO leakage through a damaged BBB, the relationships between USPIO-induced signal change on the one hand and presence of a vasogenic edema and BBB integrity on the other were assessed. MRI analysis after DOTA-gadolinium injection showed T1 enhancement of the lesion over time and allowed early BBB breakdown to be demonstrated in the whole lesion at 6 hours (Figure 5D–E) and also at 24 hours. On histology, IgG immunostaining was performed to visualize plasma molecules that had entered the brain through a disrupted BBB. IgG immunostaining in the

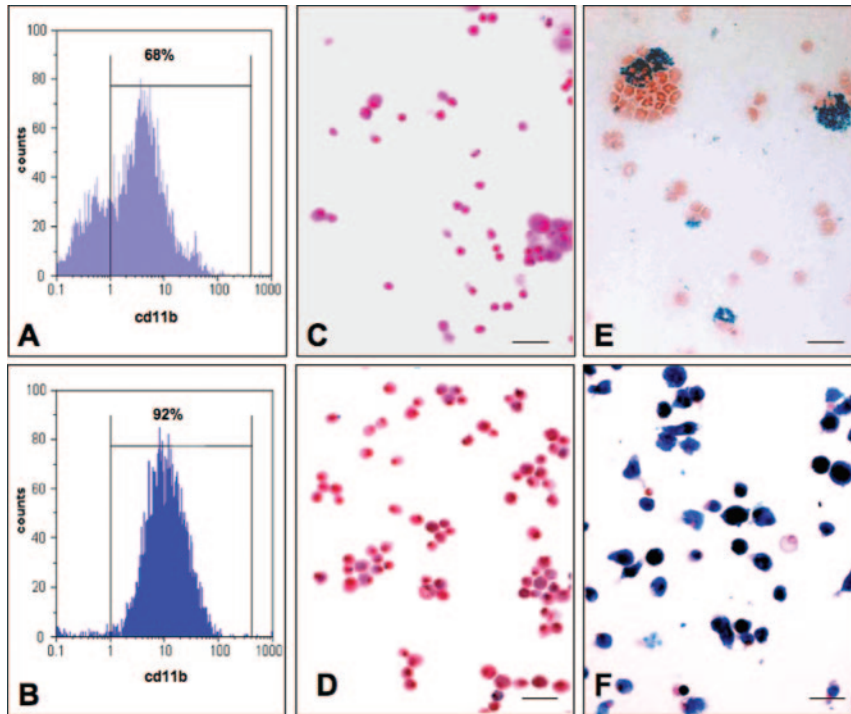
lesion (Figure 5A) and along the corpus callosum (Figure 5B–C) was colocalized with (1) vasogenic edema detected as hyperintensity on T2-weighted images at 6 hours (Figure 5F: lesion and ipsilateral corpus callosum, arrow) and 24 hours (Figure 5G: ipsilateral and contralateral corpus callosum, arrows) in pMCAO mice; and (2) T2 decrease after USPIO injection in pMCAO+USPIO mice (Figure 5H: perilesion and ipsilateral corpus callosum, arrow and Figure 5I: ipsilateral and contralateral corpus callosum, arrows).

#### **USPIO Not Detected in Blood Mononuclear Cells in the First 24 Hours Postinjury**

Prussian blue staining of peripheral blood mononuclear cell cytosols did not detect any iron-stained mononuclear cells in blood samples taken in the first 24 hours postinjury in injured mice (data not shown).

#### **Very Weak USPIO Uptake by BM-Derived Monocytic Cells or BM-Derived Macrophages**

Analyzing the ability of blood monocytes to ingest USPIO *in vitro* is difficult; specific culture conditions are required, including adherence to plastic, that alter monocyte functions.<sup>16</sup> Here, USPIO uptake capacity was compared in 2 myeloid cell populations: BM-derived monocytic cells, which can be considered as immediate precursors of blood monocytes, and BM-derived macrophages. BM culture phenotypes were assessed by fluorescence activated cell sorting analysis (Figure 6A–B). After 24 hours with USPIO, no USPIO uptake by monocytic cells was observed and very little if any by BM-derived macrophages (Figure 6C–D, respectively). In contrast, USPIO uptake was detectable after 48 hours of incubation (data not shown) and BM-derived macrophages and monocytic cells efficiently ingested anionic superparamagnetic nanoparticles (Figure 6E–F).



**Figure 6.** Assessment of USPIO uptake by BM-derived monocytic cells (top row) or BM-derived macrophages (BMDM; bottom row). Flow cytometry analysis of BM-derived cells showed that a great majority of cells (respectively, 68% of BM-derived monocytic cells in A and 92% of BMDM in B) expressed the pan-myeloid marker CD11b (the vertical bar indicates the threshold of fluorescence positivity as compared with control). No USPIO labeling was detected in BM-derived monocytic cells incubated 24 hours with USPIO as shown in C (bar=20  $\mu$ m). Weak USPIO uptake can be detected in sparse BMDM incubated with USPIO (D). As a positive control of phagocytic activity, BM-derived monocytic cells were incubated with anionic superparamagnetic nanoparticles (AMNP). E, Prussian blue staining showed that a subpopulation of monocytic cells had ingested nanoparticles. F, Almost all BMDM cells had ingested nanoparticles.

## Discussion

The present study observed consistent MRI signal changes after intravenous USPIO injection at 6 hours and 24 hours postinjury. Histological analysis showed that iron staining was mostly associated with the vascular and CSF compartments. Interestingly, at early time points, only brain phagocytes in contact with CSF had already phagocytized USPIO. USPIO-loaded cells were not detected in the blood of injured mice within the first 24 hours. In addition, cultured monocytic cells derived from BM cultures did not display phagocytic activity toward USPIO after 24 hours of incubation at plasmatic concentration. This suggests that, in this specific model of pMCAO, early USPIO enhancement in the brain is mainly caused by nonspecific mechanisms such as BBB leakage and intravascular trapping rather than by peripheral phagocyte infiltration.

It has been claimed that USPIO-enhanced MRI essentially reflects the recruitment of blood-circulating USPIO-loaded monocytes into inflamed tissue, thus demonstrating late-stage inflammation.<sup>4,8</sup> The present study shows that, at an early stage after experimental stroke, USPIO-induced MR alterations are not related to the recruitment of bloodborne USPIO-loaded macrophages. This result is in agreement with previous studies demonstrating that recruitment of bloodborne macrophages (derived from blood monocytes) was not observed during the early stages of experimental ischemia.<sup>17,18</sup> We also observed, in line with other work,<sup>11</sup> that USPIOs were poorly, if at all, phagocytized by BM-derived monocytic cells (precursors of blood monocytes) and BM-derived macrophages. These data suggest that the *in vivo* uptake of USPIO by mononuclear phagocytes depends notably on the intrinsic ability of each phagocyte population to ingest USPIO. Thus, blood monocytes, brain resident macrophages, infiltrating inflammatory macrophages, or parenchy-

mal microglia probably do not ingest USPIO equally efficiently or with the same kinetics. In addition, USPIO uptake may vary depending on the state of activation of these various phagocytic cells.<sup>19</sup> It cannot be ruled out that, during later stages, monocytes or a subpopulation of activated and/or inflammatory monocytes<sup>20</sup> acquire the ability to phagocytize USPIO. However, our results demonstrate that USPIO-induced MR alterations do not univocally reflect the entry of USPIO-loaded monocytes in the ischemic brain. They further suggest that, at early postischemia time points, USPIOs penetrate in the central nervous system as free particles, BBB disruption being attested by the presence of both gadolinium-enhanced lesions and immunoglobulin deposits. Accordingly, although detection of free USPIOs is probably hampered by standard histological analysis procedures (in vivo perfusion, fixatives, multiple baths), extracellular uptake of USPIO particles within the central nervous system parenchyma of ischemic brains was detectable. Because the interstitial fluid of brain parenchyma is drained into the CSF compartment, our data suggest that USPIOs may circulate as free particles from blood to brain parenchyma and from brain parenchyma to CSF. This hypothesis is further supported by the detection of USPIO in meningeal macrophages (comprising those residing in the velum) and periependymal microglia, 2 populations of brain-resident phagocytes that localize in CSF-bathed areas. The detection of USPIOs in perivascular macrophages is also in accordance with the presence of free USPIOs in the interstitial fluid. Indeed, perivascular macrophages, more than microglia, have been shown to quickly phagocytize antigens and particles circulating in the interstitial fluid of brain parenchyma.<sup>21</sup> Also, providing that free USPIO particles gain access to the CSF, it may be hypothesized that they subsequently penetrate periventricular parenchyma areas and are taken up by activated microglia. It is

noteworthy in this regard that, after the first 48 hours after experimental stroke, USPIOs are detected in the cytoplasm of microglial cells that localize in periventricular parenchymal areas.<sup>13</sup> Finally, free USPIO particles may also enter the CSF compartment through an altered blood/CSF barrier at the level of the choroid plexi.

A hyperintense signal was observed on T1-weighted GRE images in the corpus callosum on Day 1 in pMCAO+USPIO mice exclusively in line with a previous study.<sup>13</sup> However, surprisingly, we did not obtain any iron staining in this location. Failure to detect iron particles in the corpus callosum might be due to the well-documented lack of sensitivity of Prussian blue to interstitial USPIO (as opposed to iron compartmentalized in vessels or in cells).<sup>22,23</sup> There are indeed a number of indirect indications of the presence of free USPIOs in the corpus callosum: hyperintensity on T1-WI<sup>24</sup> and IgG immunostaining in spatiotemporal agreement with signal change on T2-WI after USPIO injection. Taken together, these data suggest that USPIO have diffused along with edematous fluid, probably after nonspecific leakage secondary to BBB disruption. This result further underlines the fact that free USPIOs cannot be definitely asserted to be absent in tissue simply on the grounds of lack of positive Prussian blue staining. Fluorescent iron oxide nanoparticles and/or electronic microscopy may help validate this hypothesis in the future.

Despite abundant evidence of an inflammatory response after stroke, anti-inflammatory treatment has so far failed in clinical trials.<sup>25</sup> In this context, noninvasive detection of inflammatory cells after brain ischemia could be helpful (1) to select patients who may benefit from anti-inflammatory treatment; and/or (2) to target the potential therapeutic time window. For this purpose, MRI coupled with USPIO, a contrast agent thought to be taken up by macrophages *in vivo*, appears a promising tool to provide noninvasive information about neuroinflammation. In early clinical studies,<sup>26,27</sup> no USPIO enhancement was observed 24 hours to 48 hours after USPIO injection or 2 to 4 days after stroke onset in most patients, whereas MR signal changes were consistently observed in studies performed at 5 to 6 days after stroke.<sup>4,5</sup> The heterogeneous patterns of USPIO enhancement between patients are probably critically dependent on the timing of contrast agent injection relative to stroke onset, but also on the time window of the subsequent MRI scans. Altogether our results demonstrate that further investigation will be necessary to analyze patients' MRIs with USPIO at early stages after stroke onset. In this perspective, the development of new MR strategies allowing unambiguous discrimination of free USPIOs versus USPIO-labeled cells as well as systematic investigation of potential pitfalls in MR data interpretation are clearly needed to validate the interest of the USPIO-enhanced MRI in larger cohorts.

### Acknowledgments

We thank Claire Corot of Guerbet for providing the USPIO and for fruitful discussions. We also thank Rhône-Alpes Genopole and Fondation Rhône-Alpes Futur, funded by the Réseau National des Genopoles.

### Sources of Funding

This research was supported in part by the Fondation pour la Recherche sur le Cerveau, by Cluster 11 of the Rhône-Alpes Region, by the European I-Know project (European commission, 6th Framework program), and by a grant from the Institut Fédératif des Neurosciences de Lyon (IFR19). It was also supported by an ANR INFLAM grant.

### Disclosures

None.

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