Magnetic labeling of primary murine monocytes using very small superparamagnetic iron oxide nanoparticles

Abstract
Due to their very small size, nanoparticles can interact with all cells in the central nervous system. One of the most promising nanoparticle subgroups are very small superparamagnetic iron oxide nanoparticles (VSOP) that are citrate coated for electrostatic stabilization. To determine their influence on murine blood-derived monocytes, which easily enter the injured central nervous system, we applied VSOP and carboxydextran-coated superparamagnetic iron oxide nanoparticles (Resovist). We assessed their impact on the viability, cytokine, and chemokine secretion, as well as iron uptake of murine blood-derived monocytes. We found that (1) the monocytes accumulated VSOP and Resovist, (2) this uptake seemed to be nanoparticle- and time-dependent, (3) the decrease of monocytes viability was treatment-related, (4) VSOP and Resovist incubation did not alter cytokine homeostasis, and (5) overall a 6-hour treatment with 0.75 mM VSOP-R1 was probably sufficient to effectively label monocytes for future experiments. Since homeostasis is not altered, it is safe to label blood-derived monocytes with VSOP. VSOP labeled monocytes can be used to study injured central nervous system sites further, for example with drug-carrying VSOP.

Key Words: CD11b, cytokine; Ferucarbotran; Mac1; MPS; MRI; Resovist; superparamagnetic iron oxide nanoparticles (SPIO); very small superparamagnetic iron oxide nanoparticles (VSOP); viability

Introduction
From the initial graft experiments (Billingham and Boswell, 1953) to the current rediscovery of the central nervous system (CNS) lymphatic vessels (Louveau et al., 2015b), the peculiarity of the CNS immune surveillance is an ongoing debate (Engelhardt et al., 2017), even though its unique character regarding the immune responses is undoubted (Louveau et al., 2015a). After understanding the cellular and molecular principles underlying the immune privileges of the CNS comprehensively, one can hope to find improved treatment options for neurological disorders such as multiple sclerosis (MS), Alzheimer’s disease, and stroke (Engelhardt et al., 2017). One major player involved in the CNS immune response are myelopoietic cells. Central myeloid cell populations in the CNS are parenchymal microglia and non-parenchymal macrophages, which can be sub-classified in perivascular macrophages, meningeal macrophages, macrophages of the choroid plexus, and blood-derived monocytes (Prinz et al., 2011). Non-parenchymal macrophages mediate immune responses at brain boundaries (Goldmann et al., 2016). The blood-derived monocyteic cells can be recruited to the CNS under pathophysiological as well as inflammatory conditions and contribute to brain pathology (Mammana et al., 2018). They infiltrate mechanical lesion sites along with zones of acute, anterograde (Wallerian) axonal degeneration and transform into microglia-like cells (Bechmann et al., 2005). In addition, monocytes play a crucial role in the onset of MS, where they are associated with axonal loss, astrogliosis, and neurodegeneration (Kuhlmann et al., 2002; Slavin et al., 2010). In contrast, TREM2-transduced myeloid precursors limited tissue destruction and promoted CNS repair (Takahashi et al., 2007) in experimental autoimmune encephalomyelitis, the animal model for MS. Thus monocytes play a dual role and are also able to modulate postlesional plasticity and can serve as cellular carriers to transport therapeutic substances or small molecular agents into the CNS by bypassing the blood-brain barrier, protecting the CNS (Abbott et al., 2010). Monocytes, which are transporting agents through the blood-brain barrier into the CNS are also used in various other promising animal studies, for example in Parkinson’s disease, where monocytes mediated transport of therapeutic nanozymes into the CNS plasma (Brynskikh et al., 2010). Another example of the importance of blood monocytes in diseases is their recruitment to the injured spinal cord where they promote recovery (Schwartz, 2010). Also, nanoparticle-loaded monocytes are attracted to epileptogenic brain tissue and could potentially be used for reducing the systemic dose of potentially toxic compounds (Han et al., 2019).

Since the first production of nanoparticles in the 1980s, their application potential concerning imaging and treatment of the CNS has increased significantly, for instance, in cancer therapy through magnetic hyperthermia or in vascular magnetic resonance imaging as contrast agents to visualize malformations. Especially superparamagnetic iron oxide nanoparticles (SPIO) were studied for a long time as contrast agents in magnetic resonance imaging (Rumenapp et al., 2012). Compared to conventional gadolinium-based contrast agents, they achieve a higher spatial resolution and therefore improve diagnostic accuracy (Shokrollahi, 2013). Many potential therapeutic applications for SPIO have been identified, including the treatment of brain tumors, MS, cerebral ischemia or stroke, or in vascular malformations. Especially superparamagnetic iron oxide nanoparticles (SPIO) have been found to be safe to label blood-derived monocytes with VSOP. VSOP labeled monocytes can be used to study injured central nervous system sites further, for example with drug-carrying VSOP.
First attempts have been made to elucidate the effects of nanoparticles on blood-derived monocytes to predict their influence, once the CNS has been reached (Tong et al., 2016). However, the incubation of monocytes with SPIOs might change their cellular integrity, differentiatio

Materials and Methods

Animals

Adult C57BL/6 female mice (Charles River, Sulzbach, Germany) at the age of 8 weeks were used. After general anaesthesia, blood was taken from the marginal vein of the ear. The samples were added to 20 g/ml of 4% PFA. Up to ten animals were kept in a cage with a constant 12-hour day/night rhythm, access to nest-building materials, a running wheel, and a supply of water and food ad libitum. Mice were bled and supplied by the Charité Institute of Radiology, Department of Neuroradiology, Charité, Berlin, Germany. All experiments were carried out in conformity with the European guidelines (2010/63/EU) for the laboratory animal welfare and use. All animals were killed with an overdose of anesthetics. Abbreviations: C57BL/6 = murine strain; CD11b = CD11b molecule; CNS = central nervous system; MPS = macrophages; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Nanoparticles

VSOP were synthesized and supplied by the Charité Institute of Radiology (Taupitz et al., 2000). We used two citrate-coated VSOP composed of monocrylaline iron oxide cores of magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃), which have been characterized in detail before (Neubert et al., 2012). We used VSOP-R1 (41.87 mg iron/L) and VSOP-R2 (83.74 mg iron/L). The hydrodynamic diameters of particles were 6.5 nm and 10.1 nm, respectively.

Isolation and treatment of peripheral monocytes

Murine monocytes were isolated as described in detail previously (Kaminski et al., 2012). Briefly, mice were sedated, and peripheral blood was collected after puncturing the right atrium. Accordingly, blood was diluted, overlaid with Histopaque-1077 (Biosera, Epinay-sur-Seine, France) described by Reimer and Balzer (2003) were used at particle concentrations of 0.75 mM. Mononuclear cell interface was washed and incubated in a suspension containing microbeads conjugated to rat anti-mouse cluster of differentiation molecule 11b (CD11b) monoclonal antibodies according to the supplier’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After that CD11b-positive cell fraction was gained using a magnetic-activated cell separation kit (Miltenyi Biotec), whereas the negative fraction was discarded. Subsequently, toxicity of the monocytes was quantified using a Neubauer chamber and Trypan Blue staining (Sigma-Aldrich). Cells were cultivated at a concentration of 1 × 10⁶–3 × 10⁷/mL in Roswell Park Memorial Institute (1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany) at 37°C in humidified 5% CO₂-enriched atmosphere for 1 day in vitro (DIV). VSOP-R1, -R2, and Resovist were added to the medium at a particle concentration of 0.75 mM (negative control) and 0.75 mM. Monocytic cytokine secretion was activated using Escherichia coli lipopolysaccharide (LPS) (Enzo Life Sciences, Inc., Farmingdale, NY, USA) at a concentration of 0.1 µg/mL SPIO, as well as LPS stimulation, were performed for 24 hours. Supernatants were collected after 1, 3, 6, 12, and 24 hours of incubation for cytokine assay. Monocytic cell death was induced by 5 minutes of 4% paraformaldehyde (PFA) (Merck KGaA, Darmstadt, Germany) treatment.

Immunohistochemistry, Prussian blue, and viability staining

Monocytes were cultivated for 1 DIV with and without SPIO treatment on poly-lysine (Sigma-Aldrich) coated glass slides. Then specimens were fixed with 4% PFA (Merck KGaA). Nuclear Fast Red (NFR) (Carl Roth, Karlsruhe, Germany) and Prussian blue staining procedures, embedding with Immuno-Mount (Thermo Fisher Scientific) as well as coverslipping were performed as described before (Perl and Good, 1992; Tyson et al., 2009).

For assessing morphologic characteristics, monocytes were stained after incubation with rat anti-mouse CD11b monoclonal antibody (mAb) overnight at 4°C (1:1000, RRID: AB_2829357; Leinco Technologies, St. Louis, MO, USA), goat anti-rat Alexa Fluor 568 mAb for 2 hours at room temperature (1:500, RRID: AB_2534121; thermo Fisher Scientific), and 4’,6-diamidino-2-phenylindole (DAPI) (Carl Roth) followed by embedding using Immuno-Mount (Thermo Fisher Scientific) and coverslipping.

To analyze the total number as well as the amount of viable and dead cells, monocytes were stained using a cell viability imaging kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer’s protocol. Brieﬂy, 50 µL of dye mixture was added to 200 µL of the medium of each specimen followed by 30 minutes of incubation at 37°C in a humidified 5% CO₂-enriched atmosphere.

Microscopy and live imaging

All immunofluorescence and bright-field images of fixed and stained monocytes were taken using an Olympus BX51 microscope equipped with narrow-band filters (Olympus, Hamburg, Germany) as well as a digital camera and Magnafire 2.18 software (Intas, Göttingen, Germany). Adjustment of brightness, background, and contrast was accomplished via ImageJ version 1.52 (NIH, Bethesda, MD, USA) (Schneider et al., 2012).

After 24 hours of incubation with and without SPIO or SPIO treatment, long shots of unfixed monocytes were taken for live imaging by the use of an Olympus IX81 microscope equipped with a heated measuring chamber (37°C) with humidified CO₂-enriched atmosphere, an F-View II digital camera and Xcellence software (all from Olympus). Contrast modifications and image acquisition were performed for all specimens. In total, 15 specimens (n = 3) were analyzed which were obtained from one experimental setup using 2 × 10⁶ cells per well (three samples for untreated negative control, three samples for 0.75 mM VSOP-R1, three samples for 0.75 mM VSOP-R2, three samples for 0.75 mM Resovist, and three samples for 4% PFA). For this, three images of individual sectors were taken in each well for every staining. Data collection, as well as background, brightness, and contrast modifications, were performed using ImageJ. The amount of vital (calcein acetoxymethyl ester (Calcein-AM)-positive) and dead (propidium iodide (PI)-positive) monocytes was calculated as a ratio in % compared to the total counted cell number (Hoechst 33342-positive). In total, 34 mice were used for microscopic analysis.

Lactate dehydrogenase assay

For assessing monocytes’ integrity, determination of lactate dehydrogenase (LDH) activity in cell culture supernatants was performed using a CytoToxicity Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the manufacturer’s protocol.

Cytokine and chemokine evaluation

The quantity of C-X-C motif chemokine (CXCL1)/KC, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, interferon-gamma induced protein; 10 kDa (IP-10), monocyte chemoattractant protein (MCP)-1 plus-3, macrophage inflammatory protein-alpha, and beta (MIP-1α/b) as well as tumor necrosis factor (TNF)-α in monocyte cell culture supernatants was verified with a bead-based multiple analyte immunoassay (FlowCytomix) in accordance with the manufacturer’s manual (ebioscience, San Diego, CA, USA). All measurements were performed using double determinations of every specimen. Analysis and data evaluation were carried out by the use of a FACSCanto II flow cytometer equipped with FACS Diva software (both from BD Biosciences, Franklin Lakes, NJ, USA) as well as eBioscience FlowCytomix software. Statistical analysis of live and dead (calcein acetoxymethyl ester (Calcein-AM)-positive) and dead (propidium iodide (PI)-positive) monocytes was calculated as a ratio in % compared to the total counted cell number (Hoechst 33342-positive). In total, 34 mice were used for microscopic analysis.

MP5 for iron quantification

After 1, 3, 6, 12, and 24 hours of incubation with or without 0.75 mM VSOP-R1, 0.75 mM VSOP-R2, and 0.75 mM Resovist, monocytes specimens (1 × 10⁶ cells in each treatment) were performed using double determinations of every specimen. Analysis and data evaluation were carried out by the use of a FACSCanto II flow cytometer equipped with FACS Diva software (both from BD Biosciences, Franklin Lakes, NJ, USA) as well as eBioscience FlowCytomix software. Statistical analysis of live and dead (calcein acetoxymethyl ester (Calcein-AM)-positive) and dead (propidium iodide (PI)-positive) monocytes was calculated as a ratio in % compared to the total counted cell number (Hoechst 33342-positive). In total, 34 mice were used for microscopic analysis.

Ultraviolet-visible spectroscopy

VSOP-R1, VSOP-R2, and Resovist were diluted in distilled water at a particle concentration of 3 mM. Absorption was measured in a wavelength range from 420 nm to 600 nm using a Cary 100 Scan photometer equipped with Cary WinUV software (both from Agilent Technologies, Mulgrave, Australia) and 10 mm quartz cuvettes (Hellma, Muelheim, Germany).

Statistical analysis

Data are presented as mean ± standard deviation (SD). All statistical analyses were performed with GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Results

The vitality of untreated monocytes after incubation

After DIV, monocytes were visualized by immunofluorescence staining to verify an intact cellular structure. Using CD11b and DAPI labelling (Additional Figure 1), we were able to prove maintained monocytic characteristics (e.g., a kidney-shaped nucleus) in course of the experimental setup (Figure 1A). In
addition, a viability assay was performed after 1 DIV to analyze the amount of dying or dead monocytes that have lost their membrane integrity. Exemplary images of untreated monocytes stained with Hoechst 33342, PI, and Calcein-AM are shown in Figure 1B first row. We quantified the negative control and found an average percentage of ~77.9% vital monocytes (Figure 1C).

Decrease of monocyte viability is treatment-dependent

The influence of VSOP-R1, -R2, and Resovist on murine monocytes’ vitality was tested over a 1DIV incubation period at a concentration of 0.75 mM each. Representative images of VSOP-R1 treated cells are shown in Figure 1B row 2, for VSOP-R2 in Figure 1B row 3 as well as for Resovist in Figure 1B row 4. Cells were stained using 4% PFA treatment which served as the positive control (Figure 1B last row 5). While the total cell number (Hoechst 33342 staining) is more or less constant in all specimens depicted (Figure 1B), an increase of PI-positive (dying or dead) monocytes, is visible, compared to the negative untreated control. These results are in line with the statistical analysis, representing the calculated ratio in % of vital (Figure 1C) and dead monocytes in proportion to the total cell number (Figure 1C). Here, we observed a significant vitality loss of ~28.9% for VSOP-R3 incubated samples. In contrast, VSOP-R2 treatment intensified cell death and resulted in a viability reduction of ~34.8%, whereas Resovist application decreased monocyte vitality by ~35.0%. In comparison, PFA incubation maximized cell death and reduced the average cell vitality substantially to a minimum of ~7.7%.

We have measured the LDH activity for VSOP-R1, VSOP-R2, Resovist, and control (Figure 1E). Only in the VSOP-R2 treatment group was a statistically significant increase in LDH activity detected (P < 0.05) compared to the untreated control.

Monocytes accumulate VSOP and Resovist

After 1 DIV, monocytes were labeled to verify their VSOP and Resovist uptake (Figure 2A-D). For this purpose, cells were incubated with and without 0.75 mM VSOP-R1, -R2, and Resovist. Subsequently, we used NFR and PB iron staining to visualize the cellular structure and iron deposits respectively and demonstrated the non-existence of iron around or within cell bodies of the untreated control (Figure 2A). In contrast, we confirmed the presence of iron in monocytic specimens treated with VSOP-R1 (Figure 2B), VSOP-R2 (Figure 2C), and Resovist (Figure 2D).

VSOP treatment does not alter monocyctic cytokine secretion

During nanoparticle treatment, we determined the monocyctic cytokine homeostasis with regards to stimulating, as well as, pro- and anti-inflammatory molecules in specimen supernatants. From 1 to 24 hours of incubation, we were able to verify the release of CXCL1/KC, IL-6, IL-10, MIP-1α, and TNF-α utilizing our experimental setup (Figure 2E-J). The statistical evaluation proved no changes in VSOP-R1, VSOP-R2, or Resovist-treated samples compared with the negative control. Compared to all treatment groups and time points, a significant effect (P < 0.01) was exclusively detectable for MIP-1α secretion after 24 hours of incubation in the LPS-stimulated samples (Figure 2H). In addition, neither LPS nor nanoparticle incubation activated a GM-CSF, MCP-1, and MCP-3 release (data not depicted). Also, a relatively high SD for all measuring points has to be noted.

Monocyte uptake seems to be nanoparticle- and time-dependent

Mononuclear cell uptake was analyzed between 2 and 24 hours of incubation via MPS. The quantified iron content of the untreated negative control served as zero value and was subtracted from each measurement shown in Figure 2K. We attributed the general iron basis level to the magnetic nanoparticles conjugated antibodies applied in terms of cell separation. MPS analysis confirmed the uptake of VSOP-R1, -R2, and Resovist. We found the highest uptake in VSOP-R1 treated samples with a constant increase during incubation. Iron uptake in VSOP-R1 absorption stagnated after 6 hours and was approximately reduced by 40–50% compared to VSOP-R1. Resovist uptake was detectable at all time points measured. However, compared to VSOP-R1 and VSOP-R2, it remained at a consistently low level. We excluded statistical analysis due to the small amount of data required and have added this information as a proof of principle.

Ultraviolet-visible spectroscopy shows differences in the SPIO absorption properties

We identified three divergent absorption spectra that decreased with increasing wavelength and gradually converged (Additional Figure 1B). While VSOP-R1 and VSOP-R2 showed only minor differences, a significantly higher absorption was detectable for Resovist.

Discussion

Nanoparticles have become an integral part of our everyday life, for example, in agriculture, cosmetics, and food production, material engineering or in the pharmaceutical industry. Due to their small size, they facilitate an increased chemical reactivity and bioavailability that correlates with their high surface-to-volume ratio (Kessler, 2011; Contado, 2015). However, the benefits and risks of applying nanoparticles which probably penetrate various organs, tissues, or cells in their systemic circulation have to be considered carefully. The safety of nanoparticles penetrating the CNS has to be ensured. For this reason, detailed information about the nanoparticle-dependent release, translocation, elimination, metabolism, induced toxicity, or labeling-efﬁcacy of blood-derived monocytes are mandatory and should be collected for further conclusions.
In this study, we analyzed the influence of Resovist, VSOP-R1, and -R2 on the viability, cytokine, and chemokine homeostasis as well as the iron uptake of murine blood-derived monocytes. We have already been able to prove that the separation method implemented ensures a monocytes purification > 75% of particles within 6.5–10.1 nm. (D) Monocytes incubated with 0.75 mM Resovist treated with 0.75 mM VSOP-R1 (hydrodynamic diameter of 6.1 nm with 75% of particles within 4.1–7.5 nm) were almost covered with PB-positive iron particles (blue arrows). (E) Monocytes incubated with 0.75 mM VSOP-R2 (hydrodynamic diameter of 8.7 nm with 75% of particles within 6.5–10.1 nm). (D) Monocytes incubated with 0.75 mM Resovist (SPION). (E–K) Monocytic cytokine secretion measured between 1 h and 24 h of treatment with and without different SPION indicates no nanoparticle-induced effects (two-way analysis of variance and Tukey’s post hoc test: error bars represent standard deviation (SD); the number of double determinations for all graphs: n = 2; **P < 0.01). (E) CXCL/KC. (F) IL-6. (G) IP-10. (H) MIP-1α. (I) MIP-1β. (J) TNF-α. (K) MPS iron quantification after 1–24 h treatment with and without different SPIO indicates no nanoparticle-induced effects (two-way analysis of variance and Tukey’s post hoc test: error bars represent SD; the number of double determinations: n = 1). CXCL/KC: c-x-c-motif ligand 1; DIV: day in vitro; IL: interleukin; IF: interferon gamma-induced protein; LPS: lipopolysaccharide; MIP: macrophage inflammatory protein; MPS: magnetic particle spectroscopy; Neg: negative; NFR: Nuclear Fast Red; PB: Prussian Blue; SD: standard deviation; SPION: superparamagnetic iron oxide nanoparticles; TNF: tumor necrosis factor; VSOP: very small superparamagnetic iron oxide particles.

In our last experiment, we characterized the uptake of VSOP-R1, -R2, and Resovist. Based on the images received, we suspect both extracellular and intracellular iron deposits for all treatment groups. To clarify nanoparticle attachment or uptake in detail, further investigations using ultrathin sections electron micrographs (facilitating higher magnification and resolution) would be beneficial. Using electron microscopy, Resovist was detected especially in THP-1 intracellular vesicles. In contrast, VSOP were confirmed in aggregates on the cell surface, in vesicles and in distributed internal structures. Additionally, VSOP showed a high extracellular binding affinity for glycosaminoglycans plus apoptotic debris and membrane vesicles (Ludwig et al., 2013). Interestingly, recently published data prove a VSOP binding to the THP-1 glycolyca that occurs within seconds after being applied to a cell suspension (Poller et al., 2020).

Monocytes circulate in the body to recognize foreign structures to destroy them by phagocytosis and initiation of the acquired immune response. This is induced by antigen presentation and the release of activating molecules like cytokines. For this reason, analysis of their secretion allows conclusions to be drawn about the biocompatibility of a substance. Cytokines, including chemokines (such as CXCL1/KC, GM-CSF, IP-10, MIP-1α, MIP-1β, MCP-1, MCP-3 IL-6, and TNF-α) which we have focused on in this study, are small proteins that mediate signaling and cell interaction during inflammatory processes. Among other things, they are not only responsible for leukocytes recruitment, positioning, accumulation as well as induction of phagocytosis and production of reactive oxygen species but also for cell differentiation and survival (Mohamed et al., 1997; Baggioni, 1998; Laurenzi et al., 2001; Streit et al., 2001; Ramesh et al., 2013; De Feo et al., 2017). Pathological overexpression of cytokine is linked with severe degenerative diseases including MS, Parkinson’s disease as well as human immunodeficiency virus-associated dementia (Ramesh et al., 2013). Nonetheless, there is evidence that cytokines can also have a healing effect (Kremlev et al., 2004).

As shown in our secretion assay, we found a time- but not a treatment-dependent increase of cytokine production, confirming a monocytic immune competence. Nanoparticles had therefore no impact and their results were comparable to THP-1 control. Both Resovist and Resovist significantly elevated cell death. Admittedly, values of LPS-dependent increase of cytokine production, confirming a monocytic immune competence. Nanoparticles had therefore no impact and their results were comparable to THP-1 control. Both Resovist and Resovist significantly elevated cell death. Admittedly, values of LPS-dependent increase of cytokine production, confirming a monocytic immune competence. Nano...
quantification method which can provide information about the content, binding character, internalization, and biodegradation of a substance based on its magnetic properties (Ludwig et al., 2013; Poller et al., 2016, 2018). This new MPS analysis process offers several advantages compared with destructive techniques such as colorimetric phosphonate measurement (Ludwig et al., 2013). As shown, the output seems to be nanoparticle- and time-dependent and overall consistent with the results gained by Ludwig et al. (2013). The iron content of VSOP-R1 treated samples constantly increased during incubation and was the highest altogether. In contrast, VSOP-R2 accumulation stagnated after 6 h and possibly reached a saturation level. Furthermore, Resovist was detectable but its quantity was more or less consistently low, potentially indicating a reduced monocyte attraction or responsiveness. We, therefore, believe that our MPS analysis with 0.75 mM VSOP-R1 is preferable to effectively label monocytes for future experiments. Our data also partly corresponds with study outcomes analyzing the effects of VSOP and Resovist on THP-1. The latter findings are, in our point of view, encouraging and expanding the knowledge of magnetically labeling monocytes for in vivo applications prospectively.

We are conscious that all results show a relatively high standard deviation that is related to the reduced number of tests. Additionally, the data presented is inadequate to fully understand the nanoparticle uptake mechanisms of murine-derived monocytes in detail. In our opinion, however, we have taken important steps to gain deeper insights into the magnetic labeling of primary immune cells.

Working with murine monocytes is challenging as their count in whole blood is minimal and their processing yield is correspondingly low due to the complexity in enrichment. It is essential nevertheless, as their metabolism and function differs immensely from equivalent tumor cell lines. One might argue that monocytes can be easily isolated in large quantities from auffy coat via surface marker CD14. However, if magnetic labeling is aimed at magnetic resonance imaging tracking of migrating living cells in vivo, then animal experimentation using mice or similar organisms and application of species-specific cells is the only safe option.

Optimized isolation and labeling strategy are mandatory, further experiments are required to clarify questions regarding nanoparticle accumulation, phagocytosis or their degradation. Subsequent tests should achieve a higher study significance. We are convinced that the results shown here represent a small contribution to the characterization of SPIO for future trials. Based on our findings, we will continue to work with VSOP-R1 instead of VSOP-R2 or Resovist in a concentration of 0.75 mM or less to try and reduce the incubation time to the minimum.

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Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Additional file: Additional Figure 1: Monocyte morphology and SPIO absorption.

References
Abdott NJ, Patahadeeva AA, Dolman DE, Yoof SR, Bleydi DJ (2010) Structure and function of the blood-brain barrier. Neurobiol Dis 37:12-25.
Allen AJ, Hesse BW (2015) Engineered nanomaterials and type I allergic hypersensitivity reactions. Front Immunol 6:222.
Bagglin MJ (1998) Chemokines and leukocyte traffic. Nature 392:565-568.
Bevers MM, Goldman RD, Skovranek S, Budzienki E, Simonis C, Fabisch-Pirker U, Nitsch R, Pircher I (2005) Circulating monocyte cells infiltrate layers of aorto-aneurinal atherosclerosis where they differentiate into macrophages. FASEB J 19:647-659.
Billingswell BM, Boswell T (1953) Studies on the problem of corneal homografts. Proc R Soc Lond B Biol Sci 141:392-396.
Brynjolfsson E, Oviedo Y, Mosley R, Li U, Boska MD, Klyachko NL, Kabovav AB, Hendlmann GE, Batraev EV (2010) Macrophage delivery of therapeutic nanomolecules in a murine model of Parkinson's disease. J Neurochem 113:931-942.
Contado C (2015) Nanomaterials in consumer products: a challenging analytical problem. Front Chem 3:140-35.
De Feo D, Merlino A, Brambilla E, Ottoni L, Laterza C, Miron R, Srivinasa S, Farina C, Garcia Manteiga JM, Butte E, Baricagli M, Corri G, Grett M, Martin G (2017) Neural precursor cell-secreted TGF-beta2 redirects inflammatory monocyte-derived cells in CNS autoimmunity. J Clin Invest 127:3837-3953.
Engelhardt B, Vajkoczy P, Wolfer RO (2017) The mouse thymus and immune privilege in the CNS. Nat Immunol 18:123-131.
Feldmann M, Pedersen M, Ringvoll S, Burger C, Lim (2011) Chondroitin gene expression is affected by very small iron oxide particles-labeling in long-term in vitro MRI tracking. J Mag Reson Imaging 33:1206-1213.
Göttmann T, Wieghefer P, Jordi MD, Prutek F, Hageneier N, Frenzel K, Armani S, Staszewski O, Kierdorf K, Krauger M, Locatelli G, Hochgenzer H, Zeller R, Epelman S, Geiimann F, Priller J, Ross FM, Behcme I, Krempien M, Enderlein M, Lenmink RS, Stamm C, Matzka R, Lehmann U, Kuchler F, Bitsch A, Schuchardt J, Bruck W (2002) Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain 125:2202-2212.
Lauring MA, Arndt C, Ross M, Göttmann T, Bitsch A, Schuchardt J, Bruck W (2001) Magnetic resonance imaging tracking of migrating living cells: a method for functional studies of mononuclear cells in CNS microd transplants. Eur Surg Res 33:267-274.
Kовалenko M, Tysiak E, Asbach P, Aktas O, Waiczies H, Smyth M, Schnorr J, Taupitz M, Engelhardt B, Vajkoczy P, Weller RO (2017) The movers and shapers in immune privilege of the CNS. Nature 555:337-341.
Kuhlmann T, Lingfeld L, Bisch W, Schuchardt J, Bruck W (2002) Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain 125:2202-2212.
Lauring MA, Arndt C, Ross M, Göttmann T, Bitsch A, Schuchardt J, Bruck W (2001) Magnetic resonance imaging tracking of migrating living cells: a method for functional studies of mononuclear cells in CNS microd transplants. Eur Surg Res 33:267-274.
Kovalenko M, Tysiak E, Asbach P, Aktas O, Waiczies H, Smyth M, Schnorr J, Taupitz M, Engelhardt B, Vajkoczy P, Weller RO (2017) The movers and shapers in immune privilege of the CNS. Nature 555:337-341.
Kuhlmann T, Lingfeld L, Bisch W, Schuchardt J, Bruck W (2002) Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain 125:2202-2212.
Lauring MA, Arndt C, Ross M, Göttmann T, Bitsch A, Schuchardt J, Bruck W (2001) Magnetic resonance imaging tracking of migrating living cells: a method for functional studies of mononuclear cells in CNS microd transplants. Eur Surg Res 33:267-274.
Additional Figure 1 Monocyte morphology and SPIO absorption.

(A) Fluorescence image of two isolated peripheral monocytes after 1 day in vitro showing characteristic kidney-shaped nuclei (light blue arrows, DAPI staining) as well as ubiquitous CD11b-receptor expression (red arrows, CD11b Alexa Fluor 568 labeling). Scale bar: 15 µm. (B) UV/Vis spectroscopy of VSOP-R1 (hydrodynamic diameter of 6.1 nm with 75% of particles within 4.1–7.5 nm), VSOP-R2 (hydrodynamic diameter of 8.7 nm with 75% of particles within 6.5–10.1 nm), and Resovist reflect differences in the SPIO absorption characteristics. CD11b: Cluster of differentiation molecule 11b; DAPI: 4',6-Diamidino-2-phenylindole; SPIO: superparamagnetic iron oxide nanoparticles; UV/Vis: ultraviolet-visible; VSOP: very small superparamagnetic iron oxide particles.