In Vivo Monitoring of Inflammation After Cardiac and Cerebral Ischemia by Fluorine Magnetic Resonance Imaging

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Background—In this study, we developed and validated a new approach for in vivo visualization of inflammatory processes by magnetic resonance imaging using biochemically inert nanoemulsions of perfluorocarbons (PFCs).

Methods and Results—Local inflammation was provoked in 2 separate murine models of acute cardiac and cerebral ischemia, followed by intravenous injection of PFCs. Simultaneous acquisition of morphologically matching proton (1H) and fluorine (19F) images enabled an exact anatomic localization of PFCs after application. Repetitive 1H/19F magnetic resonance imaging at 9.4 T revealed a time-dependent infiltration of injected PFCs into the border zone of infarcted areas in both injury models, and histology demonstrated a colocalization of PFCs with cells of the monocyte/macrophage system. We regularly found the accumulation of PFCs in lymph nodes. Using rhodamine-labeled PFCs, we identified circulating monocytes/macrophages as the main cell fraction taking up injected nanoparticles.

Conclusions—PFCs can serve as a “positive” contrast agent for the detection of inflammation by magnetic resonance imaging, permitting a spatial resolution close to the anatomic 1H image and an excellent degree of specificity resulting from the lack of any 19F background. Because PFCs are nontoxic, this approach may have a broad application in the imaging and diagnosis of numerous inflammatory disease states. (Circulation. 2008;118:140-148.)

Key Words: inflammation ■ ischemia ■ magnetic resonance imaging ■ monocytes ■ macrophages ■ perfluorocarbons

Inflammation is associated with a large number of human diseases such as atherosclerosis, glomerulonephritis, inflammatory bowel disease, transplant rejection, neurodegenerative brain diseases, brain and spinal cord trauma, myocardi tis, and ischemic heart disease. Thus, the medical problem is vast and an exact diagnosis is often difficult. Accordingly, therapy frequently is limited to symptomatic treatment and the success of the prescribed therapy is difficult to assess. Although recent advances involve various imaging modalities such as positron emission tomography, computed tomography, magnetic resonance imaging (MRI), optical imaging, and ultrasound imaging,1-3 the visualization of inflammatory processes still poses a serious challenge, especially because in the initial phase the affected tissue does not exhibit specific physical properties that can be used to create contrast between inflamed and healthy regions.

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Among the different noninvasive imaging modalities capable of whole-body imaging such as positron emission tomography and single-photon emission computed tomography, MRI provides superior resolution and the potential to generate the required contrast to noninflamed areas by gadolinium enhancement. However, this attempt relies on the transient accumulation of intravascularly applied gadolinium contrast agent in the interstitial space because of enhanced endothelial permeability,6,5 which is a rather nonspecific phenomenon found to be associated with a variety of diseases. A more defined approach to delineate inflammatory areas from surrounding tissue is the tagging of infiltrating, immunocompetent cells with contrast agents.6,7 Noninvasive visualization of migrating cells by MRI has so far used predominantly superparamagnetic iron oxide particles, taking advantage of the high affinity of these species for the monocyte/macrophage system.6,9 Despite its excellent sensitivity, this attempt has the disadvantage that the particles are not detected directly. Local deposition results in regional magnetic field inhomogeneities and thus depletion of the MR signal. Consequently, anatomic proton (1H) MRIs often are difficult to interpret because it is not always clear whether dark areas are caused by these nanoparticles or by other inhomogeneities.
present, no method is available for a true positive MRI identification of infiltrating cells into inflamed tissue.

In this study, we demonstrate the feasibility and safety of imaging inflammation in mice with a “positive” contrast at high local resolution with fluorine MRI. The naturally occurring stable fluorine isotope $^{19}$F (100%) is MR active and exhibits a sensitivity close to the 1H nucleus.$^{10,11}$ Because of the lack of any $^{19}$F background in the body, observed signals originating from injected $^{19}$F-containing compounds exhibit an excellent degree of specificity. The merging of recorded $^{19}$F images with simultaneously acquired, morphologically matching 1H images enables an exact anatomic localization of fluorinated substances as “hot spots.”$^{12}$ In the present investigation, we used nanoparticles containing perfluorocarbons (PFCs), a family of compounds known to be biochemically inert. Some of the PFC members such as perfluorodecalin, perfluorotripropylamine, perfluorodichloroctane, and perfluorooctyl bromide (also known as perfluoron) were already used in patients as artificial blood substitutes.$^{13}$ However, we used perfluoro-15-crown-5 ether, a PFC in which all 20 fluorine nuclei are chemically and magnetically equivalent and thus exhibit superior properties for $^{19}$F MRI detection.$^{14}$ In contrast to previous studies using $^{19}$F MRI of PFCs to track injected stem/progenitor cells after ex vivo loading,$^{15,16}$ we applied emulsified PFCs systemically, resulting in an efficient and selective enrichment in circulating cells of the monocyte/macrophage system. This approach enabled us to monitor the infiltration of immunocompetent cells into inflammatory areas in an acceptable acquisition time with a spatial resolution close to the anatomic 1H image.

Methods

An expanded Methods section can be found in the online-only Data Supplement.

Preparation of the PFC Emulsion

Purified egg lecithin (E 80 S, 4% wt/wt, a generous gift from Lipoid, Ludwigshafen, Germany) was dispersed in isotonic phosphate buffer (10 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4) by magnetic stirring at room temperature for 30 minutes. When lissamine rhodamine B (rhodamine dihexadecanoic phosphatidylethanolamine, Molecular Probes, Leiden, the Netherlands) was used as a fluorescent lipid marker, a lipid mixture of lecithin and rhodamine dihexadecanoic phosphatidylethanolamine (99.5/0.5 mol/mol) was dissolved in ethanol, and the solvent was subsequently removed under reduced pressure at 35°C. The resulting lipid film was hydrated with buffer by gentle mixing and stirring. After addition of the perfluoro-15-crown-5 ether (10% wt/wt, Fluorochem Ltd, Glossop, UK), the dispersion was pretreated with a high-performance disperser (T18 basic ULTRA TURRAX, IKA Werke GmbH & Co KG, Staufen, Germany) at 14 000 rpm for 2 minutes. The resulting crude emulsion was high-pressure homogenized (70 MPa, 10 cycles, APV Gaulin Micron Laboratory 40, APV, Unna, Germany). The formed nanoemulsion was filtered through a 0.22-μm sterile filter unit (Milllex-GS, Millipore, Ireland) and stored until application at 6°C.

Animal Experiments

Animal experiments were performed in accordance with the national guidelines on animal care and were approved by the Bezirksregierung Düsseldorf. The male mice (C57BL/6; 20 to 25 g body weight; 10 to 12 weeks of age) used in this study were bred at the Tierversuchsanlage of Heinrich-Heine-Universität (Düsseldorf, Germany). They were fed a standard chow diet and received tap water ad libitum. In total, 60 mice were investigated: blood analysis and controls with PFC and saline injections, n = 30 and 10, respectively; myocardial infarction, n = 12; and cerebral ischemia, n = 8. Myocardial infarction was provoked by ligation of the left anterior descending coronary artery (LAD). In a separate experimental series, focal cerebral ischemia was induced by photothermosis (see the online-only Data Supplement for a complete description of both injury models). A detailed schematic of the experimental protocols applied to the different groups is shown in online-only Data Supplement Figure I.

PFC Injections

Mice were anesthetized with isoflurane (2.0%) with a home-built nose cone. A total volume of 100 μL (for fluorescence experiments) or up to 500 μL (for MRI) of the PFC emulsion was given intravenously through the tail vein at the time indicated in the different experiments.

MRI Studies

Data were recorded on a Bruker DRX 9.4-T wide-bore (89-mm) nuclear MR spectrometer (Bruker, Rheinstetten, Germany) operating at frequencies of 400.13 MHz for 1H and 376.46 MHz for $^{19}$F measurements. A Bruker microimaging unit (Mini 0.5) equipped with an actively shielded 57-mm gradient set was used, and images were taken from a 30-mm birdcage resonator tunable to 1H and $^{19}$F. After acquisition of the morphological 1H images, the resonator was tuned to $^{19}$F, and anatomically matching $^{19}$F images were recorded. For superimposing the images of both nuclei, the “hot iron” color lookup table (ParaVision, Bruker) was applied to $^{19}$F images.

Mice were anesthetized with 1.5% isoflurane and were kept at 37°C. For functional cardiac analysis, 1H images of murine hearts were acquired essentially as described$^{12}$ with an ECG- and respiratory-triggered fast-gradient-echo cine sequence (field of view [FOV], 30×30 mm2; matrix, 128×128; slice thickness, 1 mm). Corresponding $^{19}$F images were recorded from the same FOV using a multislice rapid acquisition with relaxation enhancement (RARE) sequence: RARE factor, 64; matrix, 64×64; slice thickness, 2 mm; averages, 256; acquisition time, 19.12 minutes. For fusion with $^{19}$F images, additional 1H data sets with a slice thickness of 2 mm were recorded. Brain images were acquired using multislice RARE sequences for both nuclei from a reduced FOV of 20×20 mm2 but otherwise unaltered geometry (see the online-only Data Supplement for a more detailed description of MRI setup, acquisition parameters, and quantification procedures).

Blood Analysis

Blood was obtained from the vena cava inferior at various times after injection of the PFC emulsion as indicated in the different experiments. Determination of serum markers of liver function was performed by the Central Laboratory of the University Hospital Düsseldorf using clinical routine protocols. In separate experiments, mononuclear cells were isolated from the blood samples by centrifugation over Histopaque density gradient (2.5-M L layers of both 1083 and 1119 [Sigma, Taufkirchen, Germany], 25 minutes, 700g at room temperature). Thereafter, the tube was immediately transferred into the nuclear MR spectrometer for MRI (see the online-only Data Supplement for details) or the mononuclear cells were collected from the interface of the layers and analyzed by fluorescence-activated cell sorter (see the next section).

Flow Cytometry

In preceding experiments with the murine macrophage cell line RAW 246.7 loaded in vitro with rhodamine-labeled PFCs (online-only Data Supplement Figure II), we confirmed that fluorescence...
of rhodamine bound to the coat of the PFC particles is detectable by fluorescence-activated cell sorter analysis (data not shown). Freshly prepared peripheral blood mononuclear cells were stained for flow cytometric analysis according to standard procedures (see the online-only Data Supplement for details). Cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and samples were gated on live cells based on forward and side scattering and by exclusion of propidium iodide–positive cells. For each sample, at least 10 000 live events were acquired and analyzed with the CellQuestPro software (Becton Dickinson, Franklin Lakes, NJ).

Immunohistochemistry
To avoid a dissociation of rhodamine label and markers of the initial PFC carrier as a result of downstream processes after infiltration, all organs analyzed by immunohistochemistry were excised 1 day after PFC injection. Slides were air dried, and red fluorescence images were recorded without further processing because of water solubility of rhodamine-labeled PFCs and the impossibility of adequate histological fixing of the nanoparticles. The sections selected for photographs were related to anatomic landmarks to ensure retrieval of the same area after immunohistochemistry. After processing for immunofluorescence of CD11b (see the online-only Data Supplement for a detailed description of protocols applied to heart and brain slices), cardiac and cerebral sections were again microscoped, making use of the anatomic landmarks defined in the previous session. Slides were viewed with an Olympus BX50 fluorescence microscope (Olympus, Hamburg, Germany) equipped with standard filter sets and using objectives without (before immunostaining) and with (after mounting) cover glass correction. We deliberately refrained from merging images taken before and after immunostaining because an exact overlay was hampered by unavoidable minute alterations of the dried histological slices during immunohistochemical incubation steps and subsequent mounting.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
PFC Infiltration Into the Heart After Infarction Assessed by In Vivo 19F MRI
Cardiac infarction was induced by ligation of the LAD, a procedure well known to be associated with an acute inflammatory response. Two hours after ligation, 500 μL of 10% perfluoro-15-crown-5 ether emulsion (average size, 130 nm; ζ potential, −31.3±1.5 mV) was applied via the tail vein (see the Methods section in the online-only Data Supplement for details on the PFC emulsion).

After surgery and application of the contrast agent, all animals (n=6) were imaged 5 times within 7 days. The infarcted area was localized by acquisition of fast-gradient-echo 1H cine movies via akinesis of the affected region within the left ventricle. Subsequently, anatomically matching 19F images were recorded for tracking of the injected PFCs. A typical example of consecutively recorded 1H and 19F images obtained 4 days after surgery (post OP) is illustrated in Figure 1A. The end-diastolic 1H image (Figure 1A, left) clearly shows the presence of ventricular dilatation and wall thinning within the infarcted area, and in the corresponding 19F image (Figure 1A, middle), a signal pattern matched the shape of the free left...

Figure 1. Infiltration of PFCs after myocardial infarction as detected by in vivo 19F MRI. A, Anatomically corresponding 1H and 19F images from the mouse thorax recorded 4 days after ligation of the LAD showing accumulation of 19F signal near the infarcted region (I) and at the location of surgery where the thorax was opened (T). PFCs were injected at day 0 (2 hours after infarction) via the tail vein. B, Sections of 1H images superimposed with the matching 19F images (red) acquired 1, 3, and 6 days after surgery (post OP) indicate a time-dependent infiltration of PFCs into injured areas of the heart and the adjacent region of the chest affected by thoracotomy. Note that at day 4, an additional bolus of PFCs had been injected to compensate for clearance of the particles from the bloodstream after 3 days (see text).
ventricular wall. Merging of these images (Figure 1A, right) confirms the localization of PFCs within the anterior, lateral, and posterior walls. In all animals studied, $^{19}$F signal also was detected in the adjacent chest tissue, where thoracotomy for LAD ligation was performed. Note that no background signal from other tissue is present. Repetitive measurements from day 1 after LAD ligation revealed a time-dependent accumulation of PFCs within the infarcted region as shown in a representative example in Figure 1B. End-diastolic $^1$H images acquired 1, 3, and 6 days after induction of myocardial infarction show the progressive left ventricular dilatation as a consequence of the insult. Merging with the matching $^{19}$F images (red) demonstrates the successive infiltration of PFCs into the affected area of the heart and the region of the chest injured by surgery. Detected $^{19}$F signals were restricted to the area near the infarcted region of the heart; at no time were infiltrating PFCs observed within the septum (see online-only Data Supplement Table I for individual data of all animals studied).

Although strong PFC signals were found in ex vivo $^{19}$F images of blood components (see below), in vivo signals from PFCs in the circulation were not detectable at all (eg, no signal within ventricular chambers; Figure 1). Even when $^{19}$F images were acquired immediately after injection, no $^{19}$F signal from the streaming blood could be observed because the pulse sequence used for $^{19}$F MRI (RARE) results in a signal void of flowing blood particles. Therefore, detected signals can be attributed unequivocally to accumulated PFCs in the tissue without contamination from $^{19}$F signals of circulating PFCs.

**Uptake and Transport of PFCs by Cells of the Monocyte/Macrophage System**

To characterize the mode by which PFCs can enter the injured heart tissue, murine blood samples were investigated ex vivo by $^{19}$F MRI after intravenous application of the emulsion. $^{19}$F images acquired after density gradient centrifugation of blood collected at different points after injection revealed a time-dependent accumulation of the $^{19}$F signal within the layer of the mononuclear cells (Figure 2). However, 3 days after injection, the PFCs were completely cleared from the bloodstream and were no longer detectable by $^{19}$F MRI.

To further specify the cell population containing the PFCs, experiments were performed using rhodamine-labeled PFCs. These experiments enabled us to trace the fluorescence label not only within the mononuclear blood cells by flow cytometry but also within the inflamed region by means of fluorescence microscopy of tissue sections.

After tail vein injection of fluorescently labeled PFCs and subsequent collection of blood samples, we analyzed the layer of mononuclear cells containing the PFCs as assessed by ex vivo $^{19}$F MRI (Figure 2). As shown in Figure 3A, 2 hours after injection of rhodamine-labeled PFCs, almost a
fifth of the mononuclear cells were found to be positive for rhodamine, with the large majority of the labeled cells (>80%) exhibiting the monocyte/macrophage marker CD11b (Figure 3B, top). Approximately half of this cell type was detected to be loaded with PFC particles (Figure 3C). The remaining rhodamine-positive cells were observed to be B cells (B220; Figure 3B, middle), with a marginal amount of T cells (<2%; CD3; Figure 3B, bottom). Control experiments in vitro with a murine macrophage cell line confirmed that the labeled PFCs are avidly taken up by macrophages (online-only Data Supplement Figure II).

The fate of rhodamine-labeled PFCs in cardiac tissue was investigated by histology. Microscopic survey images obtained from the same mouse shown in Figure 1A are displayed in Figure 4A. Micrographs show a pattern of rhodamine fluorescence that is similar to the signal distribution in the corresponding 19F MRI acquired immediately before organ excision (Figure 1A, right). The main fluorescence signals were located exclusively within the injured area. No rhodamine fluorescence was observed in the septum and necrotic areas, as confirmed by staining with triphenyltetrazolium chloride (data not shown).

Immunostaining of tissue sections for the monocyte/macrophage marker CD11b with FITC revealed some colocalization of fluorescence patterns for cells of the monocyte/macrophage system (green) and for rhodamine-labeled PFCs (red), as shown in Figure 4B. It should be noted, however, that technical reasons precluded a precise merge of the differently labeled sections. Because of the water solubility of the rhodamine-labeled PFCs, red fluorescence images had to be taken before immunohistochemistry for CD11b and required careful selection of anatomic landmarks to ensure retrieval of the same area.

**PFC Infiltration Into the Brain After Focal Cerebral Ischemia**

In another set of experiments, focal cerebral ischemia was chosen as an additional model of acute inflammation. After ischemia was induced by photothrombosis, all animals (n=4) were imaged at regular intervals up to 4 weeks after surgery. In RARE 1H images, the ischemic region appeared initially as a bright area (Figure 5A, top left), and the corresponding 19F images clearly show infiltration of PFCs into the border zone of the infarct, which was detected at the earliest at day 4 after photothrombosis. 19F signal also was transiently observed supracranially at the location of skin incision (Figure 5A, bottom left). Characteristic 1H and 19F images (Figure 5A) acquired from an individual mouse 7, 9, 12, and 19 days after focal cerebral ischemia was induced definitely show movement of the PFCs with the rim of the shrinking infarct over time (see online-only Data Supplement Table II for individual data of all animals studied).

To support the notion that PFCs were carried into the ischemic region by monocytes/macrophages, experiments with rhodamine-labeled PFCs were again conducted (n=4). Microscopic survey images after FITC immunostaining for CD11b exhibited a pattern of green fluorescence comparable to that observed for the 19F signal in the preceding MR experiment (Figure 5B). Furthermore, comparison of red and green fluorescence at large magnification indicated colocalization of PFCs and CD11b-positive cells (online-only Data Supplement Figure III).

**Detection Threshold and Absolute Quantification**

The sensitivity of our present approach can be estimated from Figure 2 by correlating the number of cells contained in the layer of the mononuclear cells with the signal-to-noise ratio in the corresponding areas of 19F images. Two days after PFC injection, the mean signal-to-noise ratio within this layer was determined to be 24 at a voxel size of 0.44 μL (FOV, 30×30 mm2; matrix, 64×64; slice thickness, 2 mm). The mononuclear cell layer contained 1.16×10⁶ cells distributed vertically over ~1 mm and horizontally over the inner diameter of the tube (14 mm as derived from axial 1H images), which results in a cell
number of $\approx 3300$ per $^{19}$F MR voxel within this layer. Assuming a minimal signal-to-noise ratio of 3 as the detection threshold, as little as $\approx 400$ cells are expected to be visible by MRI under these conditions. Taking into account that only a fraction of the mononuclear cells are loaded with PFCs (Figure 3), the detection limit may be even lower.

A similar conclusion was reached in a separate set of experiments in which RAW 264.7 macrophages were incubated ex vivo with PFCs under in vivo–like conditions and analyzed by $^{19}$F MRI after immobilization in agarose (for details, see the Methods section of the online-only Data Supplement). Stepwise dilution of PFC-loaded macrophages revealed that $< 200$ cells were detectable within a voxel of 0.44 $\mu$L (online-only Data Supplement Figure IV). By calibration of the absolute $^{19}$F signal intensities with PFC concentration standards ($R^2=0.99892$; online-only Data Supplement Figure V), the average PFC loading per cell was calculated to be 0.73$\pm$0.19 pmol (n=8). Assuming a similar uptake of PFCs in vivo, the number of PFC-containing cells within ischemic areas can be quantified by interpolation from $^{19}$F signal intensities of the affected regions (online-only Data Supplement Tables III and IV). A similar conclusion was reached in a separate set of experiments in which RAW 264.7 macrophages were incubated ex vivo with PFCs under in vivo–like conditions and analyzed by $^{19}$F MRI after immobilization in agarose (for details, see the Methods section of the online-only Data Supplement). Stepwise dilution of PFC-loaded macrophages revealed that $< 200$ cells were detectable within a voxel of 0.44 $\mu$L (online-only Data Supplement Figure IV). By calibration of the absolute $^{19}$F signal intensities with PFC concentration standards ($R^2=0.99892$; online-only Data Supplement Figure V), the average PFC loading per cell was calculated to be 0.73$\pm$0.19 pmol (n=8). Assuming a similar uptake of PFCs in vivo, the number of PFC-containing cells within ischemic areas can be quantified by interpolation from $^{19}$F signal intensities of the affected regions (online-only Data Supplement Tables III and IV).

Control Experiments After PFC Injection

Without further intervention, at no time were $^{19}$F signals observed within the heart or the brain. However, $^{19}$F images showed a distinct signal in the spleen 1 day after injection of the PFC emulsion and a weaker signal in the liver that increased up to days 2 to 3, reaching an intensity similar to the signal from the spleen (online-only Data Supplement Figure VI). Interestingly, at the same time, additional signals regularly appeared in lymph nodes in the area of the upper thorax and the head and became clearly visible, as shown in Figure 6. The signals in the liver persisted for several months, but no adverse effects of the PFCs were observed in these animals, and serum markers of liver function were comparable to those of saline-treated animals (eg, the ratio of glutamic oxaloacetic transaminase to glutamic pyruvic transaminase was 2.53$\pm$1.01 [PFC, n=8] versus 2.26$\pm$0.57 [saline, n=7]).
**Discussion**

The present study describes a novel approach for visualizing local inflammatory processes by $^{19}$F MRI using in vivo tagging of circulating monocytes/macrophages with biochemically inert PFCs. Our results show that intravenous application of emulsified PFCs after local inflammation is provoked by acute cardiac or cerebral ischemia results in the accumulation of $^{19}$F-labeled cells within injured areas. Detection of infiltrating monocytes/macrophages by $^{19}$F MRI at a field strength of 9.4 T is feasible in the mouse at an acceptable acquisition time (20 minutes) with a resolution close to the anatomic $^1$H image. Therefore, PFCs can serve as a “positive” contrast agent for inflammatory processes (Figure 7), exhibiting a high degree of specificity because of the lack of any $^{19}$F background.

Compared with previous $^1$H MRI approaches for visualizing the infiltration of immunocompetent cells into inflamed areas by use of superparamagnetic iron oxide particles, the method presented here has the advantage of a direct positive detection of the tagging agent and therefore has the potential to work also in tissues that generally appear very dark in $^1$H MRI such as the lungs. Although techniques have recently been described to image superparamagnetic iron oxide particles with a bright contrast, the physical basis of detection is still the disturbance of the regional magnetic field by these particles. Therefore, it often remains difficult to unequivocally assign alterations in local contrast to accumulating superparamagnetic iron oxide particles. Furthermore, iron-based contrast agents are readily metabolized, whereas the fluorinated crown ether used in this study is biologically inert and cannot easily be degraded. The reason is the very stable C-F bond and the dense electron cloud of the fluorine atom, which results in a protective sheath. Experimentally, this provides the unique possibility for specifically and permanently labeling circulating monocytes/macrophages and following their fate within the body. It is of note that an absolute quantification of the observed signals is feasible (online-only Data Supplement Figures IV and V and Tables I through IV), which can be translated into the number of infiltrating immunocompetent cells.

Recent $^{19}$F MRI tracking studies of cells loaded ex vivo with PFCs and subsequently injected into mice either required long acquisition times (up to 3 hours) or were limited in spatial resolution (voxel size, 26 $\mu$L compared with 0.2 to 0.4 $\mu$L in the present work). In the latter investigation, the
limit of detection was reported to be ≈6000 labeled cells. The substantial higher sensitivity observed in our study is most likely due to the fact that the monocye/macrophage system in vivo more effectively takes up the injected PFCs compared with stem/progenitor cells incubated ex vivo. Labeling of ≈50% of the total monocye/macrophage cell population (Figure 3C) raises a question about function and integrity of the loaded cells. Previous studies revealed that perfluoro-15-crown-5 ether labeling had no significant effect on cell proliferation, function, or maturation.15,16 It seems likely that this also applies to the monocye/macrophage system because both the time course of accumulation and the localization of PFC-containing monocye/macrophages within ischemic areas are in good agreement with previous data on myocardial12,20 and cerebral infarction,6,9 suggesting unaltered infiltration kinetics and distribution of loaded cells. Furthermore, we did not observe any adverse effects on the animals after PFC injection, and no changes were noted in the release of liver enzymes, although this organ is a major site of PFC accumulation.

An interesting observation of this study was that lymph nodes are clearly delineated in 19F images. Although the bulk of PFCs were found in CD11b-positive cells, it should be noted that ≈20% of the injected particles were taken up by B cells (Figure 3B). However, it is difficult to decide whether the labeling of lymph nodes is due to trapping of labeled B cells or to the accumulation of PFCs in resident macrophages. Therefore, we cannot exclude the possibility that local PFC deposition also may occur via an alternative pathway; nanoparticles carried by the lymphatic flow to the sites of inflammation could have been taken up by immunocompetent cells already present at the sites of injury before PFC injection.

PFCs such as perflubron have been evaluated clinically as an artificial blood substitute. In these early studies, it was observed that perflubron is phagocytized by the reticuloendothelial system.22,23 In principle, perflubron should thus work as well as perfluoro-15-crown-5 ether, used in the present study, for 19F imaging of inflammatory processes. Perflubron has the additional advantage that it is readily cleared from the body through exhalation by the lungs within 1 week.24 Viewed from the MRI side, perflubron has a lower MRI sensitivity caused by signal splitting resulting from magnetically different 19F nuclei. However, this problem can be overcome by dedicated detection methods,25 the incorporation of gadolinium into the PFC droplets,26 or the preparation of emulsions with a higher PFC content. Furthermore, it should be noted that the voxel size in cardiac MR diagnostics at 3 T is in the range of 2 to 30 μL, whereas it was only 0.2 to 0.4 μL in our study at 9.4 T, which translates into a substantial sensitivity increase in the clinical setting.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Currently, neither a clinically useful method to assess local inflammatory processes associated with the risk of plaque rupture nor a robust imaging method that provides information about local activity of inflammation (which plays a crucial role in various cardiovascular disease states such as ischemia/reperfusion, myocarditis, transplant rejection, or stroke) is available. In the present study, we demonstrate in murine models of myocardial and cerebral ischemia that nanoemulsions of perfluorocarbons can be used to precisely visualize localized inflammatory processes as hot spots by simultaneous acquisition of morphologically matching proton (1H) and fluorine (19F) magnetic resonance images. Injected perfluorocarbons are phagocytized primarily by monocytes/macrophages, resulting in 19F magnetic resonance imaging intensity signals along the border of infarcted areas as a result of progressive infiltration of the labeled immunocompetent cells. Because of the lack of any 19F background in the body, observed signals are robust and exhibit an excellent degree of specificity. Perfluorocarbons are biologically inert and have been shown to be nontoxic in humans. Thus, 19F MRI has the potential to be clinically applicable as a new diagnostic modality not only for acute but also for chronic inflammatory processes such as plaques in atherosclerosis.
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ONLINE DATA SUPPLEMENT – EXPANDED MATERIAL AND METHODS

Preparation and characterization of the PFC emulsion

**Preparation:** Purified egg lecithin (E 80 S, 4% wt/wt, a generous gift from Lipoid (Ludwigshafen, Germany)) was dispersed in isotonic phosphate buffer (10 mM phosphate, 150 mM NaCl, pH 7.4) by magnetic stirring at room temperature for 30 minutes. When Lissamine™ rhodamine B (rhodamine dihexadecanoic phosphatidylethanolamine, (rhodamine DHPE), Molecular Probes (Leiden, The Netherlands)) was used as a fluorescent lipid marker, a lipid mixture of lecithin and rhodamine DHPE (99.5/0.5 mol/mol) was dissolved in ethanol and the solvent was subsequently removed under reduced pressure at 35 °C, followed by evaporation under high vacuum. The resulting lipid film was hydrated with buffer by gentle mixing and stirring. After adding the perfluoro-15-crown-5 ether (10 % wt/wt, Fluorochem Ltd. (Glossop, UK)), the dispersion was pretreated with a high-performance disperser (T18 basic ULTRA TURRAX, IKA Werke GmbH & CO. KG, Staufen, Germany) at 14,000 rpm for 2 minutes. The resulting crude emulsion was high pressure homogenized (70 MPa, 10 cycles, APV Gaulin Micron Lab 40, APV, Unna, Germany). The formed nanoemulsion was filtered through a 0.22 µm sterile filter unit (Millex-GS, Millipore, Ireland) and stored until application at 6 °C.

**Size determination by photon correlation spectroscopy (PCS):** Emulsion samples were diluted with freshly particle-free filtered isotonic phosphate buffer (0.2 µm Minisart, Sartorius AG, Göttingen, Germany) in order to achieve an appropriate counting rate. All samples were placed into the sample holder of a Zetamaster S (Malvern Instruments GmbH, Herrenberg, Germany) 10 minutes before starting the measurement in order to allow equilibration to thermostated 25 °C. Three cycles of each ten runs were performed for data collection. Data were calculated by cumulant analysis.

ODS – 1
**Zeta potential measurement:** The zeta potential was measured at 25 °C by laser doppler anemometry using a Zetamaster S (Malvern Instruments GmbH, Herrenberg, Germany). Samples were diluted (1:100) either with particle-free filtered water (type I reagent grade water, Simplicity UV, Millipore GmbH, Schwalbach, Germany) or with particle-free filtered isotonic phosphate buffer to the desired concentration prior to the measurement. An electric field of 30 V/cm was applied to the sample cell. The zeta potential was recorded as the average of at least ten runs measured at the stationary level.

**Cryotransmission electron microscopy (cryoTEM):** Emulsions were diluted with water (type I reagent grade water) in order to obtain a total lipid concentration of 4-6 mM. Sample preparation and measurement were carried out as described elsewhere.\(^1\,^2\) In brief: after placing a drop of the sample on a grid (Quantifoil S7/2 Cu 400 mesh, holey carbon films, Quantifoil Micro Tools, Jena, Germany), most of the liquid was carefully removed with blotting paper leaving a thin film stretched over the holes. The samples were immediately shock-frozen by dipping into liquid ethane, and after removing the remaining ethane samples were transferred to the microscope using a special holder. The transmission electron microscope was a Leo Omega 912 (Leo, Oberkochen, Germany). Examinations were carried out at low temperatures of approximately 100 K. Zero-loss filtered images were taken by a SSCCD camera (Proscan HSC 2, Oxford Instruments, Abingdon, USA) under low dose conditions, i.e., using the minimal dose focusing device.

**Characterization:** After preparation all PFC nanoemulsions showed an average size around 130 nm (polydispersity index 0.28) as measured by PCS. Upon storage at 6 °C over a period of 10 weeks no changes in vesicle size were found (data not shown). Additional information on the emulsion drops was obtained by cryoTEM. The emulsion droplets show a spherical
shape (online Data Supplement Fig. VII) and some lipid vesicles coexist with the nanoemulsion droplets (not shown). The size distribution of the droplets correlates well with the data found with PCS. After storage at 6 °C for 10 weeks no structural alterations were found (data not shown). Zeta potentials of the PFC nanoemulsions in water and in buffer were determined as $\zeta = -31.3 \pm 1.5 \text{ mV}$ and $-5.5 \pm 1.2 \text{ mV}$, respectively. Over a period of 10 weeks no changes in zeta potential were found (data not shown).

**Animal experiments**

Animal experiments were performed in accordance with the national guidelines on animal care and were approved by the Bezirksregierung Düsseldorf. The male mice (C57BL/6, 20-25 g body weight, 10-12 weeks of age) used in this study were bred at the Tierversuchsanlage of Heinrich-Heine-Universität, Düsseldorf, Germany. They were fed with a standard chow diet and received tap water *ad libitum*. A total number of 60 mice has been investigated: blood analysis and controls with PFC/saline injections, 30/10; myocardial infarction, 12; cerebral ischemia; 8. Myocardial infarction was provoked by ligation of the left anterior descending coronary artery (LAD). In a separate experimental series focal cerebral ischemia was induced by photothrombosis (see below for a description of both injury models). A detailed scheme of the experimental protocols applied to the different groups is shown in Fig. I of the online Data supplement.

**Myocardial infarction:** Mice were intubated and anesthetized by mechanical ventilation with isoflurane (2.0%) at a rate of 150 strokes/min and a tidal volume of 250-300 μl. Each animal was placed in a supine position with paws taped to an electrocardiogram (ECG) board (lead II) to measure S-T segment elevations during myocardial infarction. The chest was opened with a lateral cut along the left side of the sternum. Subsequently, the pericardium was gently
dissected to allow visualization of coronary artery anatomy. Ligation was proceeded with an 8-0 polypropylene suture with a tapered needle passed underneath the LAD, 1–3 mm from the tip of the left auricle. The success of infarction was verified microscopically by the absence of blood flow in the epicardium as well as significant elevations of S-T segment. The chest was then closed with 6-0 polypropylene suture with one layer through the muscle and a second layer through the skin and subcutaneous material.

**Focal brain ischemia:** For the induction of cortical photothrombosis mice were anesthetized with enflurane in a 2:1 N₂O/O₂ atmosphere, and a fiber optic bundle coupled to a cold light source (Schott EL 1500, Mainz, Germany) was centered 2.5 mm posterior and 2.5 mm laterally from bregma. After intraperitoneal injection of 1 mg Rose Bengal (Sigma) the brain was illuminated through the intact skull for 15 minutes as previously described.³

**PFC injections:** Mice were anesthetized with isoflurane (2.0%) using a home-built nose cone. A total volume of 100 µl (for fluorescence experiments) or up to 500 µl (for MRI) of the PFC emulsion was given intravenously through the tail vein at the time indicated in the different experiments.

**Magnetic resonance imaging**

Data were recorded on a Bruker DRX 9.4 Tesla Wide Bore (89 mm) NMR spectrometer operating at frequencies of 400.13 MHz for ¹H and 376.46 MHz for ¹⁹F measurements. Experiments were performed using ParaVision 4 (Bruker) as operating software. A Bruker microimaging unit (Mini 0.5) equipped with an actively shielded 57-mm gradient set (capable of 200 mT/m maximum gradient strength and 110 µs rise time at 100% gradient switching) was used. Images were taken from a 30-mm birdcage resonator tunable to ¹H and ¹⁹F with
optimal sensitivity for $^{19}\text{F}$. After acquisition of the morphological $^1\text{H}$ images, the resonator was tuned to $^{19}\text{F}$ and anatomically matching $^{19}\text{F}$ images were recorded. For superimposing the images of both nuclei, the “hot iron” colour lookup table provided by ParaVision was applied to $^{19}\text{F}$ images. $^{19}\text{F}$ and $^1\text{H}$ MR images were subsequently merged by using Adobe Photoshop (Adobe Systems). The validity of the fusion procedure was confirmed by merging $^1\text{H}$ and $^{19}\text{F}$ MR images from geometrically defined PFC phantoms (containing 90% H$_2$O and 10% perfluoro-15-crown-5 ether) from which exactly matching $^1\text{H}$ and $^{19}\text{F}$ images were obtained.

Mice were anesthetized with 1.5% isoflurane in a water-saturated gas mixture of 20% oxygen in nitrogen applied at a rate of 75 ml/min by manually restraining the animal and placing its head in a home-build nose cone. The frontpaws and the left hindpaw were attached to ECG electrodes (Klear-Trace, CAS Medical Systems, Branford). In order to minimize antenna artefacts ECG wires were shielded and arranged in loops within the probe head. Respiration was monitored from a pneumatic pillow positioned at the animal’s back. Vital functions were acquired by a M1025 system (SA Instruments, Stony Brook) and used to synchronize data acquisition with cardiac and respiratory motion. Throughout the experiment, mice were respiring spontaneously at a rate of approximately 100 min$^{-1}$ and were kept at 37 °C.

**Cardiac imaging:** For functional analysis $^1\text{H}$ images of murine hearts were acquired using an ECG- and respiratory-triggered fast gradient echo cine sequence essentially as described$^{4,5}$. A flip angle of 15°, echo time (TE) of 1.8 ms, and a repetition time (TR) of about 4 ms were used. The pixel size after zero filling was 117×117 µm$^2$ (field of view (FOV) 30×30 mm$^2$, matrix 128×128, slice thickness (ST) 1 mm, acquisition time per slice for one cine sequence 1-2 min). $^{19}\text{F}$ images were recorded using a multislice RARE sequence (8 slices, RARE factor 64, FOV 30×30 mm$^2$, matrix 64×64 resulting in a pixel size after zero filling of 234×234 µm$^2$,}
ST 2 mm, TR 4.5 s, TE 3.38 ms, 256 averages, acquisition time, 19.12 min). No cardiac or respiratory gating was applied for $^{19}$F MRI. For fusion with $^{19}$F images additional $^1$H datasets with a ST of 2 mm were recorded.

**Brain imaging:** Images were acquired using multislice RARE sequences for both nuclei from a FOV of $20 \times 20 \text{ mm}^2$. $^1$H: 16 slices, RARE factor 16, matrix 256$\times$192 resulting in a pixel size after zero filling of $78 \times 78 \mu\text{m}^2$, ST 1 mm, TR 5 s, TE $5.83 \text{ ms}$, acquisition time 1 min. $^{19}$F: 8 slices, RARE factor 64, matrix 64$\times$64 resulting in a pixel size after zero filling of $156 \times 156 \mu\text{m}^2$, ST 2 mm, TR 4.5 s, TE $3.64 \text{ ms}$, 512 averages, acquisition time, 38.24 min. For fusion with $^{19}$F images additional $^1$H datasets with a ST of 2 mm were recorded.

The full experimental protocol for cardiac and brain MR studies, respectively, including both $^1$H and $^{19}$F imaging took around one hour and was well tolerated by all mice which recovered from anesthesia within 1-2 min after removal of the nose cone.

**Volumetric analysis of inflamed areas:** Anatomical matching multislice $^1$H and $^{19}$F MR data sets were used to quantify inflamed myocardial and cerebral regions, respectively. Affected volumes were calculated from $^{19}$F images by planimetric analysis of PFC signals, multiplication with the slice thickness, and summation over all slices. In order to correct for differences in organ size obtained values were related to total heart and brain volumes, respectively, which were assessed from the corresponding $^1$H images. $^{19}$F signal-to-noise ratios were determined from PFC signals originating from the myocardium and the brain only, and were averaged over the entire region containing PFCs. Signals derived from liver, chest, skull, lymph nodes or other tissues were not included into the calculation.
**Ex vivo blood imaging:** Immediately after separation of the blood components by centrifugation over Histopaque density gradient (see below), the tube was fixed in a home-build adapter and inserted into the resonator. To cover the tube over its entire size two overlapping coronal $^1$H and $^{19}$F image sets with a FOV of $30 \times 30$ mm$^2$ were acquired. The same parameters as given in the section for cardiac imaging were used with the exception that $^1$H images were recorded with a non-triggered gradient echo sequence (TE, 2.4 ms; TR 150 ms). The overlapping images were subsequently merged by using Adobe Photoshop (Adobe Systems).

**Analysis of PFC-treated macrophages and absolute quantification:** RAW 264.7 macrophages (~$10^6$) were incubated for 2, 4, 6, and 24 h with PFCs in complete medium (2 ml DMEM supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 50 µmol/l 2-mercaptoethanol, and 500 µl PFC emulsion). Thereafter, cells were washed with PBS, collected in 750 µl of medium, and counted for exact determination of the cell number per µl. In additional experiments, cells collected after PFC incubation for 6 h were diluted stepwise to assess the $^{19}$F MR detection threshold of PFC-loaded cells. To prevent settling of the cells during MR data acquisition and to disperse cells within the sample as homogenous as possible, they were immobilized in 200-µl microfuge tubes by gently mixing of cell suspensions (100 µl) with a 2% agarose solution (100 µl) on ice. For absolute quantification of the PFC content within cells, concentration standards were prepared by dilution of the PFC stock emulsion. For sake of comparison, standards were also immobilized in agarose.

Tubes were placed in a home-build adapter capable to be loaded with up to seven samples (see online Data Supplement Fig. IV) and inserted into the resonator. Subsequently, $^1$H and $^{19}$F MR images were acquired with the same parameters as in the *in vivo* heart experiments.
(i.e. a true voxel size of 0.44 µl for $^{19}$F MR images). Within each sample the mean absolute $^{19}$F signal intensity per voxel was determined using the region-of-interest (ROI) tool of ParaVision. The known amount of PFC per voxel from the concentration standards was used to calibrate $^{19}$F signal intensities (online Data Supplement, Fig. V). This correlation was used to calculate the average PFC-loading per cell under consideration of the $^{19}$F signal intensity per voxel and the corresponding number of cells per voxel for each macrophage sample.

**Blood analysis**

Blood was obtained from the *vena cava inferior* at various times after injection of the PFC emulsion as indicated in the different experiments. Determination of serum markers of liver function was performed by the Central Laboratory of the University Hospital Düsseldorf using clinical routine protocols. In separate experiments, mononuclear cells were isolated from the blood samples by centrifugation over Histopaque density gradient (2.5 ml layers of both 1083 and 1119 (Sigma), 25 min, 700 g at room temperature). Thereafter, the tube was either immediately transferred into the NMR spectrometer for MRI (see above) or the mononuclear cells were collected from the interface of the layers and analyzed by FACS (see next section).

**Flow cytometry**

In preceding experiments with the murine macrophage cell line RAW 246.7 loaded *in vitro* with rhodamine-labelled PFCs (online Data Supplement Fig. II) we confirmed that fluorescence of rhodamine bound to the coat of the PFC particles is detectable by FACS analysis (data not shown).
Freshly prepared peripheral blood mononuclear cells (PBMC) were stained for flow cytometric analysis according to standard procedures: To prevent non-specific binding of primary antibodies, cells were first incubated with anti-FcγII/IIIR mAb 2.4G2 (BD PharMingen) in ice-cold PBS containing 2% fetal bovine serum and 0.05% sodium azide. Subsequently, cells were incubated for 30 min on ice with the following biotinylated primary monoclonal antibodies (mAb): anti-CD11b mAb M1/70.15, anti-B220 RA3-6B2, anti-CD3 145-2C11 or isotype-matched control antibodies (all from BD PharMingen). After washing, samples were incubated for 30 min with FITC-conjugated streptavidin (BD PharMingen). After another wash, propidium iodide (1 µg/sample) was added. Cells were immediately analyzed on a FACScalibur flow cytometer (Becton Dickinson). Samples were gated on live cells based on forward as well as side scattering and by exclusion of propidium iodide-positive cells. For each sample at least 10,000 live events were acquired and analyzed with the CellQuestPro software.

**Immunohistochemistry**

Frozen sections (8 µm) were cut from organs of animals which received rhodamine-labelled PFCs at the points in time indicated in the different experiments. To avoid a dissociation of rhodamine label and markers of the initial PFC carrier due to downstream processes after infiltration, all organs analyzed by immunohistochemistry were excised 1 day after injection. Slides were air dried and red fluorescence images were recorded without further processing because of water solubility of rhodamine-labelled PFCs and the impossibility of adequate histologic fixing of the nanoparticles. The sections selected for photographs were related to anatomical landmarks in order to ensure retrieval of the same area after immunohistochemistry. Subsequently, slides were fixed and incubated with mAb against mouse
CD11b (for details see below). After processing for immunofluorescence of CD11b, heart and brain sections were again microscoped making use of the anatomical landmarks defined in the previous session. Slides were viewed with an Olympus BX50 fluorescence microscope equipped with standard filter sets and using objectives without (before immunostaining) and with (after mounting) cover glass correction. Images were captured with an analogue video camera driven by the analySIS B software. We deliberately refrained from a merging of images taken before and after immunostaining, since an exact overlay was hampered by unavoidable minute alterations of the dried histologic slides during immunohistochemic incubation steps and subsequent mounting.

For overview of the ischemic brain region (Fig. 5B) two overlapping fluorescence images were taken at 40-fold magnification and subsequently merged by using Adobe Photoshop (Adobe Systems). Rhodamine fluorescence images of the entire heart (Fig. 4A) were acquired at a magnification of 12.5 with a 12-bit CCD monochrome camera (courtesy of Olympus).

**Heart sections** were fixed for 10 min in Zamboni’s fixative and rinsed thrice with PBS. Subsequently, slides were transferred for 10 min into blocking solution (10% normal goat serum (Linaris) in PBS containing 0.1% saponin). Incubation with the primary Ab (rat anti mouse CD11b (Integrin alpha M; MAC-1), clone M1/70 (Dianova), 1:100, 2% normal goat serum in PBS with 0.1% saponin) was done overnight at 4 °C. After three washing steps with PBS containing 0.1% saponin, sections were incubated for 4 h protected from light with the secondary Ab (pure goat anti rat FITC-conjugated (Jackson Immunoresearch), 1:200, 2% normal goat serum in PBS with 0.1% saponin). Thereafter, slides were washed 4 times and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene Oregon).
**Brain slides** were fixed for 10 min at 4 °C in paraformaldehyde (PFA) and rinsed thrice with acetone. Thereafter sections were washed with PBS and exposed for 30 min to 0.3% H2O2 in PBS. After another washing step, blocking was performed for 30 min with 3% normal goat serum in PBS. Subsequently, incubation with the primary Ab against CD11b (MAC-1, clone MCA-74G, 1:50 in PBS) was done overnight at 4 °C in a moist chamber. After washing with PBS the secondary Ab (Alexa Fluor 488 goat anti-rat IgG, 1:100 in PBS) was applied for 60 min to the exclusion of light. Sections were thoroughly rinsed and mounted in Vectashield Mounting Medium (Vector H-1000).

**Immunofluorescence analysis of macrophages treated with rhodamine-labelled PFCs**

RAW 264.7 macrophages (0.5·10^6) were seeded onto glas coverslips in 2.5 ml complete medium (DMEM supplemented with 10 % fetal bovine serum, 2 mmol/l glutamine and 50 µmol/l 2-mercaptoethanol). After overnight incubation at 37 °C, rhodamine-labelled PFCs (10 µl/well, final dilution 1:250) were added, and macrophages were incubated for further 2 h. Cells were washed with PBS and fixed with 3% PFA for 20 min at room temperature. Nuclei were stained with DAPI (400 µg/ml) and coverslips were mounted with fluoromount. Cells were analyzed on a Nikon Eclipse TE 200 inverted microscope equipped for epifluorescence. Pictures were taken with a digital video camera driven by the LUCIA software. Red and blue fluorescence was recorded separately, and images were merged using Adobe Photoshop (Adobe Systems).
REFERENCES


Online Data Supplement – Figure I

Scheme of the experimental protocols applied to the different groups

**Myocardial ischemia (n=12)**

- LAD ligation
- Daily $^1$H/$^{19}$F MRI scans
- PFC injection
- Immunohistology (n=6)
- Histology (n=6)

**Cerebral ischemia (n=8)**

- Photothrombosis
- Daily $^1$H/$^{19}$F MRI scans
- Weekly $^1$H/$^{19}$F MRI scans
- PFC injection
- Immunohistology (n=4)
- Histology (n=4)

**Control experiments (n=30)**

- PFC injection
- Daily $^1$H/$^{19}$F MRI scans
- Weekly $^1$H/$^{19}$F MRI scans
- Monthly $^1$H/$^{19}$F MRI scans
- Blood analysis (n=3 per point in time, total n=21)
  - $^1$H/$^{19}$F MRI, flow cytometry, blood count
- Blood analysis (n=3 per point in time, total n=9)
  - blood count
Uptake of PFCs by RAW 264.7 macrophages

Cells were incubated for 2 h with rhodamine-labelled PFC emulsion (dilution 1:250). The scale bar represents 10 µm.
Colocalization of rhodamine-labelled PFCs and monocytes/macrophages in the brain after focal cerebral ischemia.

Anatomically matching sections from a mouse brain 7 days after photothrombosis before (top) and after processing for immunofluorescence of CD11b (bottom). Red fluorescence images had to be acquired before immunohistochemistry, because of water solubility of the rhodamine-labelled PFCs. Thus, areas selected for photographs were referenced to anatomical landmarks. PFCs were injected at day 6 after surgery via the tail vein. The scale bar represents 50 µm.
Detection threshold of PFC-loaded macrophages

Matching $^1$H and $^{19}$F MR images of RAW 264.7 macrophages incubated for 2, 4, 6, and 24 h with PFCs. Cells were subsequently immobilized in agarose and placed in 200-µl microfuge tubes (FOV 30x30 mm$^2$, slice thickness 2 mm, voxel size $^{19}$F 0.44 µl). 6-h samples were diluted stepwise to determine the minimal amount of cells detectable. Images were acquired using the same acquisition parameters as in the in vivo experiments.

Cell counts (cells per voxel): 2h, 478; 4h, 467; 6h, 530; 24 h, 365

Std = concentration standard: 0.75 nmol PFC per voxel
**Online Data Supplement – Figure V**

**$^{19}$F MRI signal intensity vs. PFC concentration**

Signals were obtained from PFC concentration standards using the same acquisition parameters as in the *in vivo* experiments.

![Graph showing the relationship between signal intensity and nmol PFC per voxel with $R^2 = 0.99892$.](image-url)

$R^2 = 0.99892$
Online Data Supplement – Figure VI

PFC uptake by spleen and liver under control conditions

Anatomical matching $^1$H and $^{19}$F MR images of the same animal 1, 2, and 3 days after PFC injection (FOV 30x30 mm$^2$).
Cryotransmission electron micrograph of PFC emulsion
The emulsion of PFC and lecithin mixture in PBS was prepared by high pressure homogenization (scale bar, 200 nm).
Table I: Reproducibility of detected tissue areas and signal-to-noise ratios in $^{19}$F MR images of the heart after myocardial infarction.

<table>
<thead>
<tr>
<th>Animal</th>
<th>MC volume with PFCs [µl]</th>
<th>Total MC volume ($^1$H) [µl]</th>
<th>Inflamed MC proportion [%]</th>
<th>$^{19}$F S/N ratio (MC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>47.8</td>
<td>152.5</td>
<td>31.4</td>
<td>7.5</td>
</tr>
<tr>
<td>#2</td>
<td>50.2</td>
<td>155.5</td>
<td>32.3</td>
<td>10.8</td>
</tr>
<tr>
<td>#3</td>
<td>42.2</td>
<td>167.8</td>
<td>25.2</td>
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<td>#4</td>
<td>20.5</td>
<td>100.4</td>
<td>20.5</td>
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<td>17.5</td>
<td>91.9</td>
<td>19.1</td>
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<td>#6</td>
<td>29.6</td>
<td>102.3</td>
<td>28.9</td>
<td>6.8</td>
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<tr>
<td>Mean ± SD</td>
<td></td>
<td>26.2 ± 5.1</td>
<td>8.8 ± 2.9</td>
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</table>

Myocardial (MC) regions were quantified from multislice $^1$H and $^{19}$F MR data sets acquired 7 days after LAD ligation. MC volumes affected by inflammation were calculated from $^{19}$F images by planimetric analysis of PFC signals within the left ventricle, multiplication with the slice thickness, and summation over all slices. The obtained values were related to the total myocardial volume as assessed from the corresponding $^1$H images to correct for differences in heart size. $^{19}$F signal-to-noise (S/N) ratios were determined from PFC signals originating from the myocardium only. S/N ratios were averaged over the entire MC region containing PFCs as given in column 2. Signals derived from liver, chest or other tissues were not included into the calculation.
Table II: Reproducibility of detected tissue areas and signal-to-noise ratios in $^{19}$F MR images of the brain after focal cerebral ischemia.

<table>
<thead>
<tr>
<th>Animal</th>
<th>CB volume with PFCs ($[^\mu l]$)</th>
<th>Total CB volume ($^1$H) $[^\mu l]$</th>
<th>Inflamed CB proportion [%]</th>
<th>$^{19}$F S/NR ratio (CB)</th>
</tr>
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<tr>
<td>#1</td>
<td>15.0</td>
<td>491.1</td>
<td>3.06</td>
<td>5.5</td>
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<tr>
<td>#2</td>
<td>14.3</td>
<td>510.4</td>
<td>2.79</td>
<td>7.5</td>
</tr>
<tr>
<td>#3</td>
<td>21.4</td>
<td>504.5</td>
<td>4.23</td>
<td>6.8</td>
</tr>
<tr>
<td>#4</td>
<td>17.6</td>
<td>520.3</td>
<td>3.38</td>
<td>7.1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>3.34 ± 0.54</td>
<td>6.7 ± 0.8</td>
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</table>

Cerebral (CB) regions were quantified from multislice $^1$H and $^{19}$F MR data sets acquired 8 days after photothrombosis. CB volumes affected by inflammation were calculated from $^{19}$F images by planimetric analysis of PFC signals within the brain, multiplication with the slice thickness, and summation over all slices. The obtained values were related to the total cerebral volume as assessed from the corresponding $^1$H images to correct for differences in brain size. $^{19}$F signal-to-noise (S/N) ratios were determined from PFC signals originating from the brain only. S/N ratios were averaged over the entire CB region containing PFCs as given in column 2. Signals derived from skull, lymph nodes or other tissues were not included into the calculation.
Table III: Quantification of PFC content after myocardial infarction within the ischemic region of the heart for selected points in time (values are means ± SD, n = 6). Amount of labelled cells were calculated on base of an average loading of 0.73 pmol PFC per cell (see Result section for details).

<table>
<thead>
<tr>
<th>Point in time</th>
<th>Intensity [a.u.] per voxel</th>
<th>nmol PFC per µl</th>
<th>Labelled cells per µl</th>
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<tbody>
<tr>
<td>1 d post OP</td>
<td>5.95 ± 1.24</td>
<td>1.03 ± 0.21</td>
<td>1400 ± 292</td>
</tr>
<tr>
<td>3 d post OP</td>
<td>7.18 ± 1.87</td>
<td>1.34 ± 0.35</td>
<td>1819 ± 471</td>
</tr>
<tr>
<td>6 d post OP</td>
<td>9.05 ± 2.54</td>
<td>1.80 ± 0.50</td>
<td>2450 ± 687</td>
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</table>
Table IV: Quantification of PFC content after cortical photothrombosis within the ischemic region of the brain for selected points in time (values are means ± SD, n = 4). Amount of labelled cells were calculated on base of an average loading of 0.73 pmol PFC per cell (see Result section for details). Note that $^{19}$F voxel size in brain images was approximately 2-fold smaller than in heart images (0.2 vs. 0.44 µl; see Method section for details).

<table>
<thead>
<tr>
<th>Point in time</th>
<th>Intensity [a.u.] per voxel</th>
<th>nmol PFC per µl</th>
<th>Labelled cells per µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 d post OP</td>
<td>2.98 ± 0.62</td>
<td>1.47 ± 0.31</td>
<td>1956 ± 419</td>
</tr>
<tr>
<td>12 d post OP</td>
<td>3.06 ± 0.55</td>
<td>1.56 ± 0.28</td>
<td>2130 ± 383</td>
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<tr>
<td>15 d post OP</td>
<td>2.84 ± 0.65</td>
<td>1.29 ± 0.30</td>
<td>1761 ± 405</td>
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<tr>
<td>19 d post OP</td>
<td>1.84 ± 0.31</td>
<td>0.91 ± 0.12</td>
<td>1234 ± 210</td>
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</tbody>
</table>