Monitoring of Monocyte Recruitment in Reperfused Myocardial Infarction With Intramyocardial Hemorrhage and Microvascular Obstruction by Combined Fluorine 19 and Proton Cardiac Magnetic Resonance Imaging

Yu-Xiang Ye, MD, PhD; Thomas C. Basse-Lüsebrink, PhD; Paula-Anahi Arias-Loza, PhD; Vladimir Kocoski, PhD; Thomas Kampf, MSc; Qiang Gan, PhD; Elisabeth Bauer, MSc; Stefanie Sparka, PhD; Xavier Helluy, PhD; Kai Hu, MD; Karl-Heinz Hiller, PhD; Valerie Boivin-Jahns, PhD; Peter M. Jakob, PhD; Roland Jahns, MD; Wolfgang R. Bauer, MD, PhD

Background—Monocytes and macrophages are indispensable in the healing process after myocardial infarction (MI); however, the spatiotemporal distribution of monocyte infiltration and its correlation to prognostic indicators of reperfused MI have not been well described.

Methods and Results—With combined fluorine 19/proton (1H) magnetic resonance imaging, we noninvasively visualized the spatiotemporal recruitment of monocytes in vivo in a rat model of reperfused MI. Blood monocytes were labeled by intravenous injection of 19F-perfluorocarbon emulsion 1 day after MI. The distribution patterns of monocyte infiltration were correlated to the presence of microvascular obstruction (MVO) and intramyocardial hemorrhage. In vivo, 19F/1H magnetic resonance imaging performed in series revealed that monocyte infiltration was spatially inhomogeneous in reperfused MI areas. In the absence of MVO, monocyte infiltration was more intense in MI regions with serious ischemia-reperfusion injuries, indicated by severe intramyocardial hemorrhage; however, monocyte recruitment was significantly impaired in MVO areas accompanied by severe intramyocardial hemorrhage. Compared with MI with isolated intramyocardial hemorrhage, MI with MVO resulted in significantly worse pump function of the left ventricle 28 days after MI.

Conclusions—Monocyte recruitment was inhomogeneous in reperfused MI tissue. It was highly reduced in MVO areas defined by magnetic resonance imaging. The impaired monocyte infiltration in MVO regions could be related to delayed healing and worse functional outcomes in the long term. Therefore, monocyte recruitment in MI with MVO could be a potential diagnostic and therapeutic target that could be monitored noninvasively and longitudinally by 19F/1H magnetic resonance imaging in vivo. (Circulation. 2013;128:1878-1888.)

Key Words: hemorrhage ● magnetic resonance imaging ● microcirculation ● monocytes ● myocardial infarction ● perfluorocarbons

The healing of myocardial infarction (MI) is a delicate inflammatory process in which monocytes and macrophages are central effectors and regulators. Optimal MI healing relies on a suitable degree of inflammation and its timely resolution.1 Reperfused MI tissue is notably heterogeneous; therefore, its repair presumably involves inhomogeneous infiltration of monocytes into tissues with varying degrees of ischemia-reperfusion injuries. As important markers of severe ischemia-reperfusion injury in myocardium, microvascular obstruction (MVO) and intramyocardial hemorrhage (IMH) occur in a sizable number of acute MI patients after reperfusion therapy and predict worse outcomes.2–6 They are also highly correlated to each other.7–10 However, the spatiotemporal distribution of monocytes in reperfused MI areas has not been well described. The mechanism of monocyte recruitment to MVO and IMH regions during the healing process and its effect on patient prognosis remain unclear.

Clinical Perspective on p 1888
Magnetic resonance imaging (MRI) is an ideal imaging modality to detect MVO and IMH. It is also capable of tracking cell migration in vivo. Most commonly labeled by iron oxide nanoparticles, targeted cells are recognized by the signal voids caused by magnetic susceptibility (T2*) effects on gradient echo magnetic resonance (MR) images. Recently, fluorine 19 (19F) MRI for cell tracking has attracted much interest because of its unambiguous detection and quantitative nature. Flögel et al demonstrated that 19F MRI can be used to visualize infiltration of circulating monocytes and macrophages into occluded MI tissue after intraarterial injections of 19F-perfluorocarbon (PFC) emulsion. This method may be particularly well suited for the tracking of labeled cells in reperfused MI areas, because the interpretation of iron oxide–labeled cells might be confounded by the T2* effect of IMH.

In the present study, the spatiotemporal recruitment of monocytes in reperfused MI tissue was noninvasively and unambiguously visualized by use of in vivo 19F MRI cell tracking in a rat model. It was registered to the distribution of IMH and MVO delineated by proton (1H) MRI to allow for investigation of monocyte infiltration in the heterogeneous tissue of reperfused MI during healing. The 19F MRI findings were confirmed by histology and immunohistochemistry. The functional and morphological outcomes under conditions of MVO and IMH were investigated with 1H MRI and histology.

**Methods**

Expanded methods and materials are provided in the online-only Data Supplement.

**19F-PFC Emulsion Formulation and Its Systematic Kinetics**

Adapted from a previously described protocol, PFC emulsion (20% vol/vol) was prepared with perfluoro-15-crown-5 ether (CE, Exfluar Research Corp, Round Rock, TX) and labeled by fluorescent DiD (Invitrogen, Darmstadt, Germany). Its particle size and mean lifetime in circulating blood were measured by dynamic light scattering and nuclear MR spectroscopy, respectively.

**Reperfused MI Model**

In accordance with the guidelines for research animal care of our institute and in accordance with the local ethics committee, reperfused MI was induced in Wistar rats (females; weight, 200±30 g; Charles River, Sulzfeld, Germany) by ligation of the left anterior coronary artery for 120 minutes, with subsequent removal of the knot via anterior sternotomy under isoflurane (Abbott GmbH, Wiesbaden, Germany) anesthesia. Sham-operated rats underwent the same procedure, except for arterial occlusion.

**19F/1H MRI**

In 16 MI and 3 sham-operated rats, the 19F emulsion was injected via the tail vein on day 1 after MI (650 µL/100 g body weight). In vivo 19F/1H MRI was performed longitudinally on days 3, 7, and 14 with a 7-T small-animal scanner (Bruker BioSpin GmbH, Reinstetten, Germany) using a 19F/1H double-resonant birdcage coil (Bruker BioSpin GmbH). We first performed multislice 1H cine imaging, which served as a localizer for 19F cell tracking. Next, 3 to 5 slices of 1H-T2*-weighted imaging (1H-T2*WI) were collected to detect IMH. After that, early- and late-enhancement MRI was performed after tail-vein injection of gadolinium (Gd)-DTPA (0.3 mmol/kg). MVO was determined by early enhancement (1–10 minutes). The infarcted area was delineated by late enhancement (after 10 minutes). Finally, 3-dimensional 19F images were acquired with the turbo spin echo sequence to cover the entire heart for cell tracking.

After the last in vivo MRI on day 14, hearts were excised and underwent ex vivo 19F/1H MRI before being frozen (−80°C) or fixed in formalin (4%). The hearts were carefully oriented to match the in vivo MRI. The absence of respiratory and cardiac motion in ex vivo imaging allows for precise colocalization of 19F and 1H images at high spatial resolution. For better visualization of the anatomy and IMH severity, both 1H-T2WI and 1H-T2*WI were acquired.

**1H MRI for Long-Term Follow-Up of MI With MVO and IMH**

To examine whether there were differential long-term outcomes in function and morphology, additional MI (n=12) and sham-operated (n=7) rats (without PFC emulsion injection) were assigned to a 28-day follow-up study. 1H MRI was acquired on days 3 and 28 after MI. Cine imaging, T2*WI, and early- and late-enhancement MRI were performed with parameters identical to those used previously in 19F/1H MRI.

**MR Data Postprocessing**

MR data postprocessing was performed with MATLAB (The MathWorks Inc, Natick, MA) and Paravision 4.0 (Bruker BioSpin GmbH). The 3-dimensional 19F MRI was reconstructed and interpolated to the same spatial resolution as the corresponding 1H MRI, and the images were superimposed for colocalization. The mean 19F signal intensity (normalized by an external PFC reference) in MI areas was calculated and compared.

**Histological Examination**

Hearts excised from the 19F-emulsion–injected rats (14 days after operation) were sliced at 1-mm intervals and carefully oriented to match the in vivo and ex vivo MRI. Hematoxylin-and-eosin staining was applied to observe pathological changes in the tissue. Perl’s Prussian blue (PB) staining identified IMH by detecting the presence of excessive iron. Monocytes/macrophages in MI tissue were recognized by immunohistochemistry staining with ED1 monoclonal antibody (AbD Serotec, Oxford, United Kingdom).

Hearts excised 28 days after MI (without PFC injection) were also sliced at 1-mm intervals. Replacement (in infarcted myocardium) and interstitial (in remote noninfarcted myocardium) fibrosis was quantified by bright-field microscopy of tissue stained with picrosirius red and expressed as the percentage of area covered by collagen fibers (collagen volume fraction). In the MI scar, the composition of thin and thick collagen fibers was analyzed quantitatively by color hues of picrosirius red staining illuminated by circularly polarized light. As the thickness of collagen fibers increases, the color hue evolves from green to yellow, orange, and red.

For the above-mentioned staining of tissue specimens, hematoxylin-and-eosin, picrosirius red, and ED1 immunohistochemistry were at 8-µm thickness. PB staining was at 30-µm thickness.

**Flow Cytometry**

To examine which cell populations were preferably labeled by the PFCònjugated PFC emulsion, whole blood leukocytes were analyzed by flow cytometry. On postoperative days 3, 7, and 14, blood samples were taken from MI rats (n=5) injected with PFC emulsion intravenously on day 1 after MI.

**Confocal Microscopy**

To determine whether the 19F signal in MI tissue indeed represented labeled monocytes/macrophages, fluorescent confocal microscopy was applied on frozen heart tissues collected 14 days after MI. Monocytes/macrophages were identified by immunohistochemistry with ED1 monoclonal antibody conjugated with fluorescein isothiocyanate. PFC emulsion was recognized by the fluorescent DiD label.

**Statistical Analysis**

Results are expressed as mean±SD. Statistical comparisons were examined by independent t test (between 2 groups) and 1-way or repeated-measures ANOVA (among ≥2 groups) when appropriate. P<0.05 was considered significant.
Results
PFC Emulsion, Its Kinetics, and Intracellular Loading of $^{19}$F

The average diameter of PFC emulsion was 228±6 nm. In circulating blood, the average mean lifetime of PFC was 12±2 hours. Most PFC was cleared from circulating blood by 48 hours after injection. Two days after PFC injection, the mean intracellular loading of $^{19}$F spins was 3.8×10$^{12}$ in blood monocytes (Figure I in the online-only Data Supplement).

Reperfused MI Model and Sham-Operated Animals

In the 14-day $^{19}$F/$^1$H MRI experiments, a total of 16 MI rats were characterized as MVO+IMH$^+$ (n=5), MVO+IMH$^-$ (n=10), and MVO-IMH$^-$ (n=1). In the 28-day follow-up experiments, an additional 12 MI rats were characterized as MVO+IMH$^+$ (n=5) and MVO+IMH$^-$ (n=7). IMH was confined within MI areas. All MVOs were detected on day 3, found in the core of MI regions, and accompanied by IMH. In 1 of those rats, MVO was not detectable 28 days after MI. In the remaining animals, MVO and IMH persisted until the end time point (day 14 or 28 after MI). The MVO area appeared smaller 28 days after MI. In the remainder of the present report, we refer to MVO+IMH$^+$ as “MVO” and MVO-IMH$^+$ as “isolated IMH.”

Flow Cytometry

On day 3, 32±8% of leukocytes from whole blood were labeled by PFC, as indicated by the fluorescent label DiD. Almost all the DiD$^+$ cells were CD11b$^+$, whereas most CD3$^+$ (T lymphocyte) and CD45RA(OX-33)$^+$ (B lymphocyte) cells were negative for DiD. The majority of CD11b$^+$ cells in blood remained labeled by PFC on day 7, although the amount of the internalized PFC decreased, as demonstrated by the drop in DiD mean fluorescence intensity. On day 14, DiD was undetectable in CD11b$^+$ cells in the blood (Figure 1).

In Vivo $^{19}$F/$^1$H MRI

The $^{19}$F signal was inhomogeneously distributed in reperfused MI areas. The spatial distribution of the $^{19}$F signal in MI tissue was similar on days 3, 7, and 14, with a tendency to shrink in size. In MI regions with isolated IMH, a more intense $^{19}$F signal was observed in the core of the MI area, with more severe IMH presenting a greater signal loss on T2*WI; a less intense $^{19}$F signal was found in regions of MI accompanied by less severe IMH; and a weak $^{19}$F signal appeared in nonhemorrhagic and peripheral MI areas. Adjusted to the baseline value of day 3, the normalized $^{19}$F signal intensity of MI areas with isolated IMH was highest on day 3 and decreased significantly afterward (Figure 2). In hearts with MVO, the $^{19}$F signal was much attenuated in MVO regions, where severe IMH was present. The normalized $^{19}$F signal in MVO areas was significantly lower than that in MI regions with isolated IMH on day 14 ($P=0.001$; Figure 3).

The contrast pattern of IMH could be highly dynamic. In 3 MI rats with isolated IMH, signal voids on $^1$H-T2*WI expanded dramatically in area and displayed stronger susceptibility effects from day 3 to day 7, whereas the $^{19}$F signal decreased but consistently and unambiguously represented the distribution of labeled monocytes/macrophages (Figure 4). No abnormality of contractile function or hyperintensity on Gd-DTPA late enhancement was found in sham-operated hearts, nor was the $^{19}$F signal.

Ex Vivo $^{19}$F/$^1$H MRI and Histological Examination

In hearts excised 14 days after MI, ex vivo and in vivo $^{19}$F/$^1$H MRI matched well in the distribution and intensity of $^{19}$F

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Figure 1. Flow cytometry of whole blood leukocytes (WBLs) after intravenous injection of DiD-labeled perfluorocarbon (PFC) emulsion in rats. WBLs from a control rat and a rat subjected to myocardial infarction and injected with DiD-labeled PFC emulsion were analyzed for DiD by flow cytometry. A, Dot plots show DiD vs fluorescein isothiocyanate (FITC) fluorescence on day 3. B, Gated on DiD$^+$ cells, histograms display specifically stained (blue) and nonstained (red) leukocytes from the same animal on day 3. Numbers indicate the percentage of DiD$^+$ expressing the specific cell marker. C, Gated on WBLs and specific cell markers, histograms show DiD$^+$ fluorescence from injected (blue) and noninjected control (red) rats on day 3. Numbers indicate the percentage of DiD$^+$ cells within the cell population analyzed. D, Mean fluorescence intensity (MFI) of DiD in CD11b$^+$ cells decreased gradually over time after injection on day 1 after myocardial infarction. Numbers indicate median values of MFI. a.u. indicates arbitrary units; and Max, maximum.
signal, as well as the morphology and severity of IMH. In hearts without MVO, higher $^{19}$F signal intensity was observed in regions with severe IMH that presented a strong signal loss on T2*WI. In comparison, ex vivo MRI of the hearts with MVO displayed a significantly attenuated $^{19}$F signal in MI regions that corresponded to the MVO areas delineated by in vivo early Gd-DTPA enhancement (MVO versus isolated IMH in normalized $^{19}$F signal intensity: 0.42±0.07 versus 0.91±0.19 arbitrary units, P<0.001). The ex vivo MRI also facilitated the correlation of histology with in vivo $^{19}$F/1H MRI (Figures 5A and 6A).

Histology and immunohistochemistry agreed well with ex vivo and in vivo $^{19}$F/1H MRI findings. Hematoxylin-and-eosin staining demonstrated postinfarction changes in MI areas derived from in vivo MRI. In MI zones, the localization and density of monocytes/macrophages detected by ED1 immunohistochemistry agreed well with the distribution and intensity of $^{19}$F signal on $^{19}$F/1H MRI, respectively. In IMH regions, the area and intensity of blue color on PB staining were consistent with the distribution and severity of signal voids on the corresponding T2* image, respectively (Figures 5B and 6B).

In hearts with isolated IMH, the highest monocyte/macrophage density was observed in the core of MI area, where a large amount of iron content (from severe IMH) was seen on PB staining of the neighboring tissue slice. A median density of monocytes/macrophages appeared in peripheral MI regions with less iron on PB staining, and only sparse monocytes/macrophages were present in peripheral MI areas without IMH (Figure 5B).

In hearts with MVO, ED1 immunohistochemistry showed that there were only sparse monocytes/macrophages in MI areas that corresponded to the MVO regions detected by in vivo early Gd-DTPA enhancement. These resembled islands that lacked monocytes/macrophages in infarcted

![Figure 2. Longitudinal observations on the spatiotemporal distribution of $^{19}$F signal in reperfused areas of myocardial infarction (MI) with isolated intramyocardial hemorrhage (IMH). Images in A and B were acquired on day 3. A: a, 1H-cine frame; b, $^{19}$F image; and c, $^{19}$F/1H superpositioned image of a and b. B: a–c, Multiecho T2*-weighted image (T2*WI); solid white line delineates areas of IMH. d, Early gadolinium (Gd) enhancement; dashed green line delineates hyperenhanced area during early enhancement. e, Late Gd enhancement; solid green line delineates hyperenhanced area during late enhancement. f, Parametric image relates the spatial distribution of IMH (solid white line), MI area (solid green line), and $^{19}$F signal. c, $^{19}$F/1H superpositioned image acquired on days 7 and 14. $^{19}$F signal was also seen in the liver and operation wound. d, Normalized $^{19}$F signal intensity relative to day 3 baseline value on days 3, 7, and 14. $^{19}$F signal is indicated by “hot” color. OPW indicates operation wound; and TE, echo time. Localized effusion.

![Figure 3. In vivo $^{19}$F/1H magnetic resonance image (MRI) of myocardial infarction (MI) with microvascular obstruction (MVO) on day 14. A, Early gadolinium (Gd) enhancement; dashed green line delineates hyperenhanced area during early enhancement, and solid blue line delineates MVO region. B, Late Gd enhancement; solid green line delineates hyperenhanced area during late enhancement. C, 1H-cine frame. D, $^{19}$F/1H superpositioned image. E–G, Multiecho T2*-weighted image (T2*WI); solid white line delineates areas of intramyocardial hemorrhage (IMH). H, Parametric image relates the spatial distribution of MVO (solid blue line), IMH (solid white line), the MI area (solid green line), and $^{19}$F signal. I, Chart shows normalized $^{19}$F signal intensity of MVO regions and isolated IMH areas on day 14. $^{19}$F signal is indicated by “hot” color. a.u. indicates arbitrary units; OPW, operation wound; and TE, echo time. Localized effusion.
myocardium. In the same region, an abundance of excessive iron content was evidenced by PB staining. In addition, spindle-shaped nuclei of (myo)fibroblasts appeared in these islands (Figure 6B).

**Long-Term Outcomes of MI With IMH and MVO**

On day 3 after MI, the MVO and isolated IMH groups had similar initial MI size, ejection fraction, end-diastolic volume, end-systolic volume, and LV mass. After 28 days, the MVO group demonstrated significantly lower ejection fraction (P=0.043) and worse recovery of pump function (Δ ejection fraction; P=0.004) than the isolated IMH group. The means of end-diastolic volume, end-systolic volume, and LV mass on day 28 were greater in the MVO group than in the isolated IMH group, although the difference was statistically insignificant (Table).

Within the MI area (containing replacement fibrosis), the collagen volume fraction was significantly higher in the MVO group than in the isolated IMH group (P=0.008). In the replacement fibrosis, the color hue analysis of picrosirius red staining illuminated by circularly polarized light revealed a significantly higher proportion of red fiber (P=0.003) and a significantly lower proportion of red fiber (P=0.006) in the MVO group than in the isolated IMH group. The percentages of yellow and green fibers were similar in the 2 groups. In the remote noninfarcted myocardium (containing interstitial fibrosis), the mean value of the collagen volume fraction was higher in the MVO group (3.67±1.86%) than in the isolated IMH group (1.95±0.21%), with a borderline probability value (P=0.069; Table).

**Fluorescent Confocal Microscopy**

Fluorescent confocal microscopy showed that DiD-labeled PFC emulsion was distributed intracellularly in monocytes/macrophages (ED1+) in MI tissue on day 14 after MI (Figure 7).

**Discussion**

In the present study, we demonstrate noninvasive in vivo imaging of monocyte recruitment in reperfused MI regions with IMH and MVO by 19F/1H MRI in rats. Importantly, monocyte infiltration was highly reduced in MVO regions, which was associated with worse LV functional outcome than MI with isolated IMH. Hybrid 19F/1H MRI offers the ability to simultaneously track monocyte infiltration in vivo noninvasively and determine the heterogeneous structure of reperfused MI tissue. Through the integration of these 2 observations, we could investigate monocyte distribution in various regions of reperfused MI, which may lead to a better understanding of the complex innate immune mechanisms of MI healing.

**Healing of Reperfused MI With MVO and IMH**

Reperfused MI tissue is heterogeneous because of variability in the extent of ischemia-reperfusion injury, susceptibility to ischemia, and inflammatory responses, as well as the presence of MVO, IMH, and other conditions. MVO and IMH occur in a sizable number of acute MI patients after primary percutaneous coronary intervention. MVO refers to hypoperfused myocardial tissue despite the patent epicardial coronary vessel and is caused by functional or anatomic changes of the coronary microcirculation. A large amount of clinical data have convincingly shown that MVO negates the benefit of primary percutaneous coronary intervention and strongly predicts early adverse LV remodeling and worse recovery of heart function in the long term, independent of MI size. MVO is also correlated with higher risks of early post-MI complications, heart failure, and mortality. IMH is a phenomenon in which red blood cells leak into the interstitium during reperfusion after the loss of capillary integrity. It indicates severe microvascular destruction and cardiomyocyte death and is associated with poor recovery of LV function and poor long-term clinical outcomes. However, MVO and IMH are highly correlated. The distinction between IMH across a spectrum of MVO is not yet elucidated. It is still debated whether IMH represents a marker of adverse ventricular remodeling beyond MI size, LV ejection fraction, and MVO.

Previous studies on MVO and IMH focused primarily on molecular and cellular mechanisms during the first several hours of ischemia-reperfusion and their association with long-term clinical outcomes. It is unclear whether the important prognostic values of MVO and IMH involve the healing process of MI. Adverse LV remodeling is determined not only by the MI size but also by the rate of MI healing and the material properties of MI segments. MVO and IMH are confined to necrotic myocardium; therefore, their initial tissue components are presumably similar. In the healing process, MI tissue undergoes dramatic changes in architecture, material composition, and...
Monocytes are a central effector in MI healing. They first remove cell debris and preexisting extracellular matrix, then form granulation tissue and promote angiogenesis in wounded myocardium. Finally, they stimulate synthesis and deposition of collagen to construct a compact scar. In both animal models and patients, delayed healing and worse outcomes have been observed when monocyte infiltration was impaired or unbalanced after acute MI. Therefore, it is important to investigate the spatiotemporal distribution of monocyte in reperfused MI tissue with MVO and IMH.

Monocytes/Macrophages Were Labeled by PFC and Unambiguously Detected by $^{19}$F MRI

In the present study, the majority of CD11b$^+$ cells in blood were labeled by PFC at least until postsurgical day 7, the time window during which monocytes are recruited from blood to MI zones most actively. Monocytes and neutrophils constituted the majority of the PFC-labeled CD11b$^+$ cells in blood in the present study. Given that most neutrophils infiltrate infarct areas during the first day after MI and that we injected PFC emulsion 1 day after operation, the PFC-labeled CD11b$^+$ cells being recruited to MI tissue should mostly be blood monocytes. In addition, using fluorescent confocal microscopy, we found that PFC emulsion was distributed intracellularly in monocytes/macrophages in the infarcted region. Therefore, the $^{19}$F signal that emerged from MI areas originated primarily from PFC-labeled monocytes/macrophages in the present study. Its intensity also corresponded well with the density of monocytes/macrophages shown by immunohistochemistry with ED1.
Spatial Distribution of Monocytes/Macrophages in Reperfused MI Zones: Highly Impaired Monocyte Recruitment in MVO Areas but Not in Isolated IMH Regions

In the present study, $^{19}$F/$^1$H MRI was used to visualize the distribution of monocytes in reperfused MI with IMH and MVO, allowing for correlation of the cellular innate immune response with the extent of ischemia-reperfusion injury. A higher $^{19}$F signal intensity was found in regions with severe IMH when MVO was absent. This relationship was consistent with the correlation between the density of monocytes/macrophages on ED1 immunohistochemistry and the blue intensity on PB staining. IMH indicates severe microvascular injury in reperfused MI areas. It is commonly believed that irreversible damage to the endothelium lags behind the death of cardiomyocytes. Therefore, the severity of IMH derived from the signal loss on T2*WI could suggest the extent of necrosis that needs to be cleared up by monocytes/macrophages. Under such circumstances, the observed intensity of monocyte/macrophage infiltration indicated by the $^{19}$F MRI signal appears to match the extent of injured tissue to be removed and repaired.

The present $^{19}$F/$^1$H MRI results revealed that monocyte infiltration was significantly impaired in MVO areas compared with hemorrhagic MI regions without MVO. The low $^{19}$F signal in MVO areas was not caused by the presence of IMH, because the strong magnetic susceptibility effects induced by IMH should not significantly influence the $^{19}$F signal intensity of the turbo spin echo sequence (online-only Data Supplement). This finding was also supported by the low density of monocytes/macrophages in MVO-corresponding areas on ED1 immunohistochemistry. Induced by severe ischemia-reperfusion injury, MVO was confined within necrotic regions and often accompanied by IMH. van Amerongen et al.21 and Nahrendorf et al.22 have shown that the depletion of monocytes shortly after myocardial injury caused delayed absorption of cell debris and subsequent attenuation in neovascularization, myofibroblast infiltration, and collagen deposition, which were associated with increased LV dilation and worse pump function in mice. Similarly, delayed necrosis removal and wound healing were observed in patients when corticosteroids, a potent anti-inflammatory agent, were administered during acute MI.16 Therefore, resembling the above scenarios, the lack of monocytes/macrophages in MVO regions could result in delayed healing and worse functional outcomes. It remains to be investigated whether the impaired infiltration of monocytes to MVO regions is merely attributable to the lack or insufficiency of tissue perfusion or also involves modulated inflammatory signaling. The differential cellular innate immune responses to MI with MVO and isolated IMH suggest that despite similar initial tissue composition (mainly necrosis), the healing processes of these 2 regions are unequal.

Dynamic Distribution Patterns of Monocytes/Macrophages in Reperfused MI Tissue Visualized by $^{19}$F Cellular MRI

$^{19}$F MRI revealed that the distribution of monocytes/macrophages was spatially similar on days 3, 7, and 14 in reperfused MI areas, while the LV was undergoing dramatic wall thinning and chamber enlargement. In contrast, Flögel et al.12 found that the $^{19}$F signal–positive regions increased in area over time during the first 6 days in infarct regions in an occluded MI model in mice. This might be attributable in part to the different infiltration patterns of inflammatory
cells in occluded and reperfused MI tissues; in the former, the front of immune cell infiltration starts at the borders of the infarct zone and progresses to the center, whereas in the latter, the invasion of inflammatory cells in the entire MI area may start immediately on successful tissue reperfusion.22,24 In addition, we found that 19F signal intensity was highest on day 3 and decreased gradually afterward. This observation is in agreement with the flow cytometry findings by Nahrendorf et al,22 in which the total number of monocytes quantified in tissue of mice with acute occluded MI was highest around day 3. However, the evolving intensity of the 19F signal in the present study could not be interpreted straightforwardly as the changing density of monocytes/macrophages in MI areas for the following reasons: (1) Mean fluorescence intensity of flow cytometry indicated that the intracellular loading of PFC emulsion decreased gradually from day 3 to day 7, even though most CD11b+ cells in blood were still labeled on day 7. Therefore, cells recruited later probably contributed less 19F signal than those recruited earlier. (2) Monocytes and their descendent macrophages are thought to undergo apoptosis after infiltrating MI tissue. The fate of the PFC in those apoptotic cells is unclear. Most likely, they were passed on to surrounding phagocytes in MI areas. It is also possible that a portion of PFC exited MI areas as their carrier cells migrated out of those regions. In contrast, Flögel et al12 found that the 19F signal intensity increased gradually during the first 6 days in infarct areas in a mouse model of occluded MI. This discrepancy might be the result of the different schemes of PFC administration (PFC emulsion was injected on both day 0 and day 4 in their study) and the different animal model (nonreperfused MI in their study).

### Table. Outcomes of MI With IMH and MVO

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MVO (n=5)</th>
<th>Isolated IMH (n=7)</th>
<th>Control (n=7)</th>
<th>P Value</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
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<td><strong>Day 3</strong></td>
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<td></td>
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<tr>
<td>MI size, %*</td>
<td>24.19±10.38</td>
<td>20.14±7.09</td>
<td>...</td>
<td>0.437</td>
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<tr>
<td>EF, %</td>
<td>40.49±7.61</td>
<td>44.29±8.23</td>
<td>69.29±3.04</td>
<td>&lt;0.001†</td>
<td>0.698</td>
<td>0.001†</td>
<td>&lt;0.001†</td>
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<tr>
<td>EDV, µL</td>
<td>394.19±84.90</td>
<td>337.18±71.45</td>
<td>195.43±59.35</td>
<td>&lt;0.001†</td>
<td>0.378</td>
<td>0.001†</td>
<td>0.005†</td>
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<tr>
<td>ESV, µL</td>
<td>238.94±78.01</td>
<td>192.11±67.76</td>
<td>61.14±23.77</td>
<td>&lt;0.001†</td>
<td>0.384</td>
<td>&lt;0.001†</td>
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<td>CO, mL/min</td>
<td>60.70±9.76</td>
<td>57.83±7.80</td>
<td>43.53±18.62</td>
<td>0.074</td>
<td>0.852</td>
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<td>LVM, mg</td>
<td>475.36±48.44</td>
<td>447.01±68.99</td>
<td>350.14±34.94</td>
<td>0.002†</td>
<td>0.690</td>
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<td><strong>Day 28</strong></td>
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<td>EF, %</td>
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<td>46.66±9.61</td>
<td>69.14±4.38</td>
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<td>EDV, µL</td>
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<td>0.328</td>
<td>0.086</td>
<td>0.119</td>
</tr>
<tr>
<td>ESV, µL</td>
<td>443.39±243.80</td>
<td>242.50±137.67</td>
<td>89.43±22.69</td>
<td>0.030†</td>
<td>0.295</td>
<td>0.066</td>
<td>0.058</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>83.51±17.08</td>
<td>73.42±14.40</td>
<td>65.97±15.44</td>
<td>0.186</td>
<td>0.521</td>
<td>0.162</td>
<td>0.648</td>
</tr>
<tr>
<td>LVM, mg</td>
<td>625.83±145.02</td>
<td>491.40±119.05</td>
<td>363.14±33.02</td>
<td>0.002†</td>
<td>0.103</td>
<td>0.002†</td>
<td>0.086</td>
</tr>
<tr>
<td><strong>Delta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔEF, %</td>
<td>-6.32±3.60</td>
<td>2.38±5.08</td>
<td>-0.14±2.27</td>
<td>0.005†</td>
<td>0.004†</td>
<td>0.037†</td>
<td>0.457</td>
</tr>
<tr>
<td>ΔEDV, µL</td>
<td>254.03±197.14</td>
<td>96.03±109.84</td>
<td>90.00±35.36</td>
<td>0.151</td>
<td>0.310</td>
<td>0.265</td>
<td>0.990</td>
</tr>
<tr>
<td>Δ ESV, µL</td>
<td>204.45±169.17</td>
<td>50.39±76.18</td>
<td>28.29±7.16</td>
<td>0.082</td>
<td>0.229</td>
<td>0.162</td>
<td>0.737</td>
</tr>
<tr>
<td>Δ CO, mL/min</td>
<td>22.81±15.05</td>
<td>15.59±18.80</td>
<td>22.44±16.06</td>
<td>0.690</td>
<td>0.750</td>
<td>0.999</td>
<td>0.733</td>
</tr>
<tr>
<td>Δ LVM, mg</td>
<td>150.47±113.92</td>
<td>44.39±103.28</td>
<td>13.00±18.07</td>
<td>0.075</td>
<td>0.279</td>
<td>0.111</td>
<td>0.720</td>
</tr>
</tbody>
</table>

Collagen volume fraction, %

| Noninfarcted | 3.67±1.86 | 1.95±0.21† | 1.14±0.21 | 0.022† | 0.069 | 0.027† | 0.586 |
| MI zone*     | 58.26±3.04 | 47.30±6.66† | ...       | 0.008† | ...   | ...   | ...   |

Hue of collagen fibers, %

| Red         | 2.92±2.69 | 15.05±7.05‡ | ...       | 0.006† | ...   | ...   | ...   |
| Orange      | 84.44±2.27 | 76.04±4.12‡ | ...       | 0.003† | ...   | ...   | ...   |
| Yellow      | 8.01±2.35  | 6.32±2.42‡   | ...       | 0.273  | ...   | ...   | ...   |
| Green       | 4.64±2.15  | 2.59±1.63‡   | ...       | 0.105  | ...   | ...   | ...   |

CO indicates cardiac output; EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; IMH, intramyocardial hemorrhage; LVM, left ventricular mass; MI, myocardial infarction; MVO, microvascular obstruction; P1, MVO vs isolated IMH; P2, MVO vs control; and P3, isolated IMH vs control.

*An independent t test was used to examine the significance of difference between the MVO and isolated IMH groups. For the rest of the parameters, 1-way ANOVA was applied to analyze the statistical difference among groups of MVO, isolated IMH, and control, followed by the Tukey-Kramer or Games-Howell post hoc test when appropriate.

†P<0.05.

‡n=6.

Adverse LV Remodeling Is Worse in Hearts With MVO Than in Those With Isolated IMH

In the present study, the MVO group showed worse pump function than the isolated IMH group 28 days after MI, even...
though the initial MI size and function were similar in the 2 groups. Advancing in parallel with healing, post-MI LV remodeling is a complex, dynamic, time-dependent process that involves differential alterations between the infarcted and noninfarcted myocardium. Early regional remodeling of the infarcted segments contributes significantly to global LV structural remodeling after MI. The prolonged exposure of extensive unhealed MI tissue to mechanical deformation forces could lead to greater infarct expansion and promote LV dilation in the MVO group. Furthermore, MVO hearts in the present study displayed extensive interstitial fibrosis ($P=0.069$). Fibrosis remote from the infarct site is considered “the major cause of ventricular remodeling” in ischemic cardiomyopathy. In the noninfarcted myocardium, chronic LV pressure overloading could stimulate interstitial fibrosis, which increases ventricular stiffness and impairs contractile function, resulting in progressive declines in ejection fraction. Therefore, the lack of monocytes/macrophages in MVO areas was possibly responsible for the worse remodeling and function of the MVO group in the long term.

Interestingly, the collagen volume fraction in the MI zones (replacement fibrosis) was significantly higher in the MVO group than in the isolated IMH group. This was sampled randomly in the entire MI region, which indicates the collective quality of scar formation. The higher collagen volume fraction in the scar appears to more strongly support ventricular morphology. However, the color hue analysis of collagen fiber composition showed that the proportion of red fiber (the thickest fiber) was significantly lower in the MVO group. This result might suggest that collagen fibers were less mature in the scar of MVO hearts on day 28.

**Monocyte Recruitment in MVO Areas: A Potential Diagnostic Marker and Therapeutic Target**

MVO negates the benefit of reperfusion therapy for acute MI. Various approaches have been explored to reverse MVO in clinical trials, but their efficacy is unclear. MVO regions are composed primarily of necrotic tissue; therefore, reversal of MVO is not expected to rescue viable myocardium but instead to optimize healing and achieve better long-term outcomes. The present results showed that the impaired infiltration of monocytes/macrophages in MVO regions was associated with worse functional outcomes in rats. Also in rats, Leor et al demonstrated that intramyocardial injection of activated human macrophages improved healing, remodeling, and function after occluded MI. Therefore, we believe that the promotion of monocyte recruitment into MVO regions might accelerate delayed wound healing and thus attenuate adverse LV remodeling, as well as improve function in the long term.

**Advantages of $^{19}$F MR Cell Tracking**

$^{19}$F MR cell tracking unambiguously detects labeled cells in heterogeneous reperfused MI tissue. Because the natural abundance of $^{19}$F in the living body is too small to be detected by MRI, the PFC-labeled cells could be specifically visualized by $^{19}$F MRI. In reperfused MI, IMH has often been detected by MRI when ischemia lasted for >2 hours before reperfusion. Garcia-Dorado et al have shown histologically that IMH could occur in reperfused MI areas after 45 minutes of ischemia. The $T2/T2*$ contrasts of IMH evolve dynamically, corresponding to the integrity of red blood cells, the oxygenation states of hemoglobin, and the progress of hemoglobin degradation. The $T2/T2*$ MR signal evolution of IMH might confound the interpretation of MR cell tracking in hemorrhagic MI regions when target cells are labeled by iron oxide particles. Advantageously, the $^{19}$F signal from the turbo spin echo MR sequence is not apparently influenced by the strong local proton $T2*$ effect (Figure II in the online-only Data Supplement) and is linear to the concentration of PFC (Figure III in the online-only Data Supplement). Therefore, $^{19}$F MRI allows for specific detection and easier quantification of labeled cells in IMH areas. In addition, $^{19}$F MRI preserves the full capability of $^{1}$H MRI for MI tissue characterization, whereas cell labeling by iron oxide particles may shorten the $T2$ and $T2*$ of background tissue and cloud the measurements of edema and IMH. $^{19}$F MRI also has potential for clinical translation. Some PFC emulsions are biologically inert, and their emulsions have been approved for clinical use. We estimated that 4000 to 5500 CD11b+ cells could be detected by $^{19}$F MRI (signal-to-noise ratio=3) in our setups (Figures I and III in the online-only Data Supplement). Human hearts span at least a 250-fold larger voxel volume (5x5x20 mm) than rat hearts (1x1x2 mm); therefore, it should be possible to image PFC-labeled cells in patients.

**Study Limitations**

Closely associated with IMH, MVO is a complex, time-sensitive phenomenon that remains to be fully understood. MVO in acute MI patients is complicated primarily by preexisting arteriosclerosis and distal microembolization of plaque and thrombus, which were not simulated in our animal model. Furthermore, the optimal time after reperfusion and the standardized medical imaging...
method or protocol for assessment of MVO and IMH have not been determined. Most often, IMH was found accompanied by MVO, although isolated IMH has been reported previously in some experimental and clinical studies. The relatively high incidence of isolated IMH in the present study could be caused by various factors: (1) MVO is a highly dynamic phenomenon. Its area could expand within the first 48 hours after reperfusion, and once established, MVO could resolve in either the early or late stages of reperfused MI. We cannot exclude that the isolated IMH in the present study was once accompanied by MVO, which resolved by the day 3 MRI. (2) The diffusion of Gd-DTPA from collateral microvasculature and the partial volume effects of MRI might make the detection of small MVOs more challenging in the hearts of small animals than in those of humans and large animals. (3) The high magnetic field and high spatial resolution in our MRI setup probably facilitated the sensitive detection of even small amounts of IMH. Although we demonstrated excellent colocation between T2* MRI–detected IMH and histopathology, unfortunately the present study did not histologically verify the presence of MRI-detected MVO.

Conclusions
We studied monocyte infiltration into reperfused MI zones with MVO and IMH by 19F/1H MRI and histology. Monocytes were recruited inhomogeneously in reperfused MI tissue. Their infiltration was highly attenuated in MVO regions. In the absence of MVO, more intense monocyte infiltration was observed in areas with severe IMH. In the long term, the MVO group exhibited worse heart function. The highly impaired monocyte infiltration in MVO regions could be related to delayed healing and worse functional outcomes. Therefore, monocyte recruitment in MI with MVO could be a potential diagnostic and therapeutic target that could be monitored non-invasively and longitudinally by 19F/1H MRI in vivo.

Acknowledgments
We thank Sabine Voll for assistance in experiments, Alexander Gotschy for statistical consultation, and Jon Bornholdt and Anagha Deshmah for proofreading.

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References
CLINICAL PERSPECTIVE

Timely restoration of obstructed coronary blood flow represents a pivotal component in the management of acute myocardial infarction (MI); however, microvascular obstruction (MVO) occurs in a sizable number of patients with acute MI. Because it deprives tissue of adequate perfusion in MI areas with severe ischemia-reperfusion injury, MVO negates the potential benefits of reperfusion therapy and strongly predicts worse long-term outcomes. In the present study, we visualized experimentally the spatiotemporal distribution of monocytes/macrophages in reperfused MI regions with MVO and intramyocardial hemorrhage in vivo noninvasively through the integration of fluorine-19 magnetic resonance imaging for cell tracking and proton magnetic resonance imaging for MI tissue characterization. Monocyte/macrophage infiltration was significantly impaired in MVO areas defined by magnetic resonance imaging, which was followed by worse pump function in the long term compared with the animals with intramyocardial hemorrhage but no MVO. Monocytes and macrophages are primary innate immune cells that orchestrate delicate MI healing. The attenuated monocyte/macrophage infiltration in MVO regions could result in delayed healing. Post-MI functional outcome is determined not only by MI size but also by the rate and quality of infarct healing. The prolonged exposure of extensive unhealed MI tissue to mechanical deformation forces could lead to greater infarct expansion and promote adverse ventricular remodeling. Therefore, enhanced recruitment of monocytes/macrophages focally in MVO regions may hold potential to improve long-term outcomes by accelerating MI healing. Moreover, ¹⁹F/¹H magnetic resonance imaging could be clinically translatable to noninvasively identify target patients and longitudinally monitor therapies that optimize MI healing by modulating monocyte/macrophage recruitment.
Monitoring of Monocyte Recruitment in Reperfused Myocardial Infarction With Intramyocardial Hemorrhage and Microvascular Obstruction by Combined Fluorine 19 and Proton Cardiac Magnetic Resonance Imaging


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SUPPLEMENTAL MATERIAL

Supplemental Methods

\textbf{\textsuperscript{19}F-PFC emulsion formulation and its systematic kinetics}

Adapted from a previously described protocol\textsuperscript{1}, \textsuperscript{19}F-perfluorocarbon (PFC) emulsion of 20\% v/v concentration was formulated by emulsifying perfluoro-15-crown-5 ether (CE) (Exflour Research Corp., Round Rock, TX, U.S.A.) in 3\% 3-sn-phosphatidylcholine (Sigma-Aldrich GmbH, Seelze, Germany) dispersed in phosphate-buffered saline (PBS). The emulsion was further homogenized by SLM Aminco French press with SA-073 minicell at 20,000 psi. (SLM Instrument, Rochester, U.S.A.). The lipid coatings of the emulsion particles were stained by fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, Vybrant® DiD cell-labeling solution, Invitrogen, Germany). The particle size was measured by dynamic light scattering (DLS) (Particle Analyzer FPAR-1000, Otsuka Electronics Co., Japan) and expressed as a mean of 5 measurements ± standard deviation (SD).

The mean lifetime (\(t_{\text{mean}}\)) of PFC emulsion in circulating blood was determined by measuring residual CE contents in blood samples of rats after intravenous (IV) injection of PFC emulsion with nuclear magnetic resonance (NMR) spectroscopy (using Bruker Avance 400, Bruker BioSpin, Rheinstetten, Germany) at 376.5 MHz. The dose was 650\(\mu\)l PFC emulsion/100g body weight. Samples of 100\(\mu\)L blood were taken from 3 female Wistar rats (250g, Charles River, Germany) via tail vein. The sampling time points were at 1, 2, 3.5, 5, 7, 9.5, 12, 26, 48 and 72
hours after PFC emulsion injection. Trifluoroacetic acid (TFA) (Sigma-Aldrich GmbH, Seelze, Germany) served as a reference in $^{19}$F-NMR measurements. The $t_{\text{mean}}$ of the PFC emulsion in circulating blood was derived by fitting the exponential equation:

$$S(t) = Ae^{-t/t_{\text{mean}}}$$  \hspace{1cm} (1)

to the measured $^{19}$F signal in blood ($S(t)$) and was expressed as a mean of 3 measurements ± SD.

Quantification of intracellular loading of $^{19}$F spins

The pilot studies of flow cytometry on blood samples suggested that PFC emulsion preferably labeled CD11b$^+$ cells in circulating blood (mainly monocytes and neutrophils). Therefore, we quantified the intracellular loading of $^{19}$F spins in CD11b$^+$ cells by NMR spectroscopy. The leukocytes were separated from pooled full blood of 3 rats (female, Wistar, 250±30g, Charles River, Germany) 2 days after IV injection of PFC emulsion (650µl/100g body weight). TFA (Sigma-Aldrich GmbH, Seelze, Germany) served as a reference in $^{19}$F-NMR measurements. The total number of the pooled leukocytes was calculated by counting the leukocytes in 20µl sample volume with a hemocytometer. The number of CD11b$^+$ cells was estimated by flow cytometry according to the proportion of CD11b$^+$ cells in all leukocytes in the blood sample. The mean intracellular loading of $^{19}$F spins in CD11b$^+$ cells was calculated by averaging the absolute number of $^{19}$F spins in the sample by the absolute number of CD11b$^+$ cells.

Flow Cytometry

On Days 3, 7 and 14 after myocardial infarction (MI), fresh whole blood samples for flow cytometric analysis were taken from 5 MI rats (400µl/animal) via tail vein. To analyze which cell populations were preferably labeled by DiD-labeled PFC emulsion, the following
monoclonal mouse anti-rat antibodies (mAbs) were used: anti-CD11b RPE (OX-42, mainly labeling monocyte, neutrophil, macrophage and dendritic cells) (AbD Serotec, Oxford, UK), anti-CD3 FITC (1F4, labeling T lymphocytes) (BD Pharmingen, San Diego, CA, U.S.A.) and anti-CD45RA FITC (OX-33, only labeling B lymphocytes) (BD Pharmingen, San Diego, CA, U.S.A.). Briefly, single-color staining was done in 50μl PBS/0.1% bovine serum albumin (BSA)/0.02% NaN3. Cells were incubated with the mAbs for 15 minutes at 4°C, washed twice in 200μl PBS/0.1% BSA/0.02% NaN3 and finally subjected to erythrolysis in 100μl PBS/0.1% BSA/0.25% saponine for 15 seconds. Following a final wash with 200 μl PBS/0.1% BSA/0.02% NaN3, the cells were subjected to analysis. Data were acquired by a LSRII flow cytometer (BD Biosciences) using the FACSDiva Software (BD Biosciences). The software FlowJo (TreeStar Inc., Ashland, OR, U.S.A.) was used to further analyze the acquired data.

**In vivo MRI**

Animals injected with PFC emulsion (16 infarcted and 3 sham-operated rats) were followed by $^{19}$F/$^1$H-MRI for 14 days after operation. *In vivo* $^{19}$F/$^1$H-MRI was acquired on post-operation Days 3, 7 and 14, at a 7-Tesla small animal scanner (Bruker BioSpin GmbH, Rheinstetten, Germany) using a $^{19}$F/$^1$H-double resonant birdcage coil (Bruker BioSpin GmbH, Rheinstetten, Germany). For $^{19}$F-MRI, a respiratory and cardiac triggered 3D turbo spin echo (TSE) MR sequence was applied with the following parameters: TR 750ms, TE 6.4ms, Turbo factor 2, number of averages 8, spatial resolution $1\times1\times2$mm. The image acquisition time was 20-30min, depending on the cardiac and respiratory frequencies. For $^1$H-MRI, multi-slice Cine MRI was acquired with the following parameters: 12 or 24 frames per RR interval, flip angle 30°, in-plane resolution $0.312\times0.312$mm, slice thickness 1mm. Microvascular obstruction (MVO) was identified by early
(1-10min) Gd-DTPA (0.3mmol/kg) enhancement using a segmented inversion recovery fast low angle shot (FLASH) sequence (TR 1000ms, TE 1.7ms and inversion time 450ms; in-plane resolution 0.312×0.312mm, slice thickness 1mm); intramyocardial hemorrhage (IMH) was detected by proton T2* weighted imaging (1H-T2*WI) with multi-echo Gradient Echo (GE) sequence (TR 150ms, TE ~3, 6, 9 and 12ms; in-plane resolution 0.156×0.312mm or 0.156×0.156mm, slice thickness 1mm); MI regions were determined by late Gd-DTPA enhancement (after 10min) using the same MR sequence and parameters as those for the early Gd-DTPA enhancement.

In animals without PFC emulsion injection (12 infarcted and 7 sham-operated rats), in vivo 1H-MRI was performed on post-operation Days 3 and 28, to examine the long-term functional outcome of MI with IMH and MVO. The initial MI size was determined by the total volume of hyper-enhanced myocardium on late Gd-DTPA enhancement on Day 3. The presence of MVO and IMH were examined by early Gd-DTPA enhancement and T2*WI, respectively, on Days 3 and 28 post-MI. In addition, multi-slice Cine images were acquired on the same days. The
epicardium and endocardium (excluding papillary muscles) were delineated on Cine MRI to extract the following left ventricular (LV) volumetric and functional parameters: end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), cardiac output (CO) and LV mass (LVM). Changes in these parameters over time (from Day 3 to Day 28) were also calculated (e.g. end-diastolic volume change \( \Delta \text{EDV} = \text{EDV}_{\text{Day28}} - \text{EDV}_{\text{Day3}} \)). The MR sequences and parameters for early/late enhancement, T2*WI and Cine were the same as previously stated.

**Ex vivo \(^{19}\text{F}/^{1}\text{H}-\text{MRI}\)**

*Ex vivo* \(^{19}\text{F}/^{1}\text{H}-\text{MRI}\) was only performed in hearts excised 14 days post-MI from animals with PFC emulsion injection. A home-built \(^{19}\text{F}/^{1}\text{H}\)-double resonant birdcage coil with 40mm diameter was used at the same MRI scanner as for the *in vivo* MRI. For *ex vivo* \(^{19}\text{F}-\text{MRI}\), a TSE pulse sequence was applied with the following MR parameters: TR 4000ms, TE 5.4ms, Turbo factor 20, number of averages 13, and spatial resolution of \(0.375\times0.375\times0.5\text{mm}\) and \(0.375\times0.375\times1\text{mm}\). For sensitive detection of IMH, 3D \(^1\text{H}-\text{T2*WI}\) was acquired with a multi-echo GE sequence (TR 150ms, TE ~3, 8, 13 and 18ms; spatial resolution 0.117×0.117×1mm).
For better visualization of the anatomy and IMH severity, $^1$H-T2WI was also acquired using a TSE sequence with the following MR parameters: TR 2500ms, effective TE 18ms, Turbo factor 4, in-plane resolution 0.117×0.117mm and slice thickness of 1mm and 0.5mm.

**Histological Examination**

Sections of myocardium underwent hematoxylin and eosin (H&E) staining for the determination of myocardial destruction and inflammatory cell infiltration. By a three-step staining procedure in combination with biotinylated secondary antibody anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA, USA), immunohistochemical staining (IHC) with mouse anti-rat ED1 monoclonal antibody (AbD Serotec, Oxford, UK) was applied to cryo-sections to detect monocytes/macrophages in MI areas. ED1 mAbs specifically recognize the antigen that is expressed predominantly on the lysosomal membrane of monocytes and macrophages in rats. The brown color was developed by the 3,3'-Diaminobenzidine (DAB) substrate KIT for peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) after anti-rat ED1 monoclonal antibody binding. The nuclei were counter-stained with haemalaun. The same treatments were applied to Paraffin fixed heart tissue to detect monocytes/macrophages in MI regions, in addition to antigen retrieval by heat before antibody incubation. IMH was histologically identified by Perl’s Prussian blue (PB) staining, which detects the presence of excessive iron. PB staining was performed on tissue sections with 30μm thickness; all other histology and immunohistochemistry were done on tissue sections with 8μm thickness.

In addition, picrosirius red (PSR) staining was performed on sections (8μm) of hearts excised 28 days post-MI, for quantitative examination of the collagen deposition (in the isolated IMH group,
PSR staining was done in six hearts, since one of the seven hearts was damaged before histological studies). The collagen volume fraction was expressed as the percentage of area occupied by collagen fibers (stained red) in photos from bright-field microscopy (×1000 magnification). Twelve to twenty-four micrographics were taken randomly in MI areas and remote non-infarcted myocardium (septum). The region of positive PSR staining (red) was segmented and its percentage area was calculated by using the software program of ImageJ² (NIH, U.S.A.). In brief, bright-field micrographics of PSR staining were separated into red, green and blue components. Then, a threshold was applied to segment the red-stained areas from the background. The areas without tissue were subtracted. Finally the collagen volume fraction was calculated from the segmented red-stained area divided by the area with tissue on the photograph.

In MI zones, the collagen fiber hue was analyzed by PSR staining illuminated by circularly polarized light. The color of collagen fibers depends on fiber thickness, when the tissue is stained by PSR and viewed with circularly polarized light. As the fiber thickness increases, the color of fibers evolves from green to yellow, orange and red³. We randomly took microcopies in MI regions under circularly polarized light (×400 magnification). The color hue was analyzed using ImageJ with a previously published method³,⁴. The proportion of the four colored fibers were calculated based on the following hue definitions: red 2-9 and 230-256, orange 10-38, yellow 39-51 and green 52-128.

Confocal microscopy

After fixation with acetone and blockage with PBS containing 10% horse serum, cryo-sections of MI hearts (20µm) were incubated with the mouse anti-rat ED1 monoclonal antibody (AbD
Serotec, Oxford, UK) overnight at 4°C. Then a biotinylated secondary antibody was added for 1 hour incubation, before incubation with streptavidin FITC and staining the nuclei with DAPI. Control sections were processed identically, with the exception of omitting the incubation with the primary antibody. After anti-rat ED1 immunohistochemical staining, cryo-sections of myocardium underwent serial scans with a thickness of 0.5 μm by a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany). A 40× HCX PL Apo objective was applied. Monocytes/macrophages were identified by the green fluorescent FITC (intracellular). The PFC emulsion was tagged by the near infrared fluorescent DiD.

19F signal impairment by susceptibility effects

When in proximity to PFC emulsion, degraded hemoglobin contents may shorten the spin–spin relaxation time (T2) of 19F and attenuate 19F signal intensity on 19F-TSE imaging. To quantitatively study the influence of magnetic susceptibility effects on the 19F-T2 and the signal intensity on 19F-T2WI, we created a scenario in which iron oxide micro-particles and PFC emulsion coincided within the same Kupffer cells in liver. Micrometer-sized iron particles (MPIO, 4mg Fe/kg) (Bangs Laboratories, Fishers, IN, U.S.A.) were injected intravenously in 3 rats (Wistar, female, 250±25g, Charles River, Germany). These animals have previously received IV injections of 650μl PFC emulsion/100g body weight. MPIOs are known to induce strong susceptibility effects, allowing for single-particle detection by 1H-T2*WI MRI5. We acquired in vivo 19F-T2WI (TSE) and 1H-T2*WI (multi-echo GE) on the same image plane of the liver in the same animal, before and after MPIO injection. These two MRI sequences were identical to those described in the previous section “In vivo MRI”.

8
After *in vivo* MRI, livers were excised and fixed by 4% paraformaldehyde. We performed $^{19}$F-T2 mapping on these 3 livers with co-injections of MPIO and PFC emulsion. In addition, $^{19}$F-T2 maps were acquired in another 3 livers with only PFC emulsion injection (0.65ml/100g body weight). The T2 values of $^{19}$F in the livers were compared under the two conditions (in presence and absence of MPIO). For $^{19}$F-T2 maps, we applied multiple spin echo (MSE) sequence with TR 2100ms, TE 5ms and 150 echo images. The coil was the same one used in *ex vivo* $^{19}$F/$^{1}$H-MRI.

The T2* effects induced by MPIOs in livers and by IMH in myocardium were compared by the reduction in signal to noise ratio (SNR) on *in vivo* $^{1}$H-T2*WI. In livers, the decrease of SNR was determined by $(\text{SNR}_{\text{post}} - \text{SNR}_{\text{pre}}) / \text{SNR}_{\text{pre}}$, where $\text{SNR}_{\text{pre}}$ and $\text{SNR}_{\text{post}}$ stand for the SNR in the liver on $^{1}$H-T2*WI (TE=6ms) before and after MPIO injection, respectively. In MI hearts (n=6), the decrease of SNR was determined by $(\text{SNR}_{\text{IMH}} - \text{SNR}_{\text{remote}}) / \text{SNR}_{\text{remote}}$, where $\text{SNR}_{\text{IMH}}$ and $\text{SNR}_{\text{remote}}$ stand for the SNR in IMH areas and remote non-infarcted myocardium (septum) on *in vivo* $^{1}$H-T2*WI (TE=6ms), respectively. For $^{19}$F-MRI, the $^{19}$F signal intensity in the liver before and after MPIO injections was normalized by the $^{19}$F signal intensity of an external PFC reference.

The results were expressed as mean±SD and t-test was applied for the comparison between two groups. The difference was considered statistically significant, when $p<0.05$.

**Detection sensitivity and quantification of $^{19}$F-MRI**
To determine the detection limit of $^{19}$F-MRI and the correlation between the concentration of $^{19}$F atoms and the $^{19}$F SNR, a phantom experiment was performed on Eppendorf tubes containing PFC emulsion suspended in 1.5% agarose gel with the following concentrations of $^{19}$F atoms: 0.35, 0.7, 1.4, 2.1, 2.8, 3.5, 7.0 and 10.4 ($\times 10^{16}$) /voxel. The MRI scanner, the coil and the parameters of $^{19}$F- and $^1$H-MRI were identical to those used in \textit{in vivo} $^{19}$F/$^1$H-MRI, except for the higher spatial resolution of 0.11×0.11×1mm. The SNR of each concentration was calculated and expressed as mean±SD of 6 MRI scans. The concentration of $^{19}$F atoms versus its SNR value was plotted. The Pearson correlation coefficient was derived from the linear fit. SNR≥3 was considered as detectable. The limit of detection on PFC labeled blood monocytes was determined by converting the minimal detectable number of $^{19}$F atoms to the equivalent number of PFC-labeled blood monocytes two days after IV injection of PFC emulsion.
Supplemental Results

19F signal was not impaired by strong susceptibility effects

No significant reduction of 19F signal intensity has been observed when excessive iron oxide was present in liver Kupffer cells on in vivo MRI (p=0.52) (Supplemental Figure 2), even though the susceptibility effect was significantly stronger than that of IMH areas (changes of SNR: liver vs IMH = -66±17% vs -21±14%, p=0.004). Because the susceptibility effect depends on the micro-distribution of iron and PFC, 19F-T2 is likely maximally shortened when MPIO and PFC emulsion were in the same Kupffer cells. In hemorrhagic infarction only a portion of the paramagnetic hemoglobin-degraded products was internalized in monocytes/macrophages carrying PFC emulsion. The rest probably scatters in extracellular space. On ex vivo MRI of the liver, 19F-T2 mapping revealed around 60% shortening of 19F-T2 when MPIO was co-injected. Besides the moderate shortening of 19F-T2, the unaffected 19F signal intensity on 19F-TSE images was probably also a result of the short effective echo time applied during both in vivo (effective TE=6.4ms, Turbo factor=2) and ex vivo (effective TE=5.4ms, Turbo factor=20) MRI in our experiments. Therefore, the possibility of 19F signal reduction caused by the 1H-T2* effects in IMH areas could be excluded. In agreement with previous reports from other groups1,6,7, the 19F signal intensity on TSE MRI versus the 19F spin concentration was linear in our current data. As a result, in a first approximation the 19F signal intensity can represent the relative density of monocytes and macrophages in various tissue backgrounds, when the intracellular PFC loading is known.
Detection sensitivity and quantification of $^{19}$F-MRI

The minimal detectable concentration of $^{19}$F atoms was $1.4-2.1 \times 10^{16}$ $^{19}$F atoms/voxel, reaching the SNR of 2.8-4.3. This detection limit was equivalent to 3684-5526 labeled blood monocytes per voxel (two days after $^{19}$F-emulsion injection). The $^{19}$F signal was linearly correlated to the concentration of $^{19}$F atoms with the Pearson correlation coefficient of 0.99 (Supplemental Figure 3).
Supplemental Figures and Figure Legends

Supplemental Figure 1

(a) Residual 19F atoms in blood (μMol/μL) vs. Time after IV injection (hour)

(b) TFA

CD 11b+ cells labeled by PFC
(a): A representative clearance curve of PFC in circulating blood. The mean lifetime ($t_{\text{mean}}$) of PFC in blood was 12±2 hours. (b): NMR spectroscopy of leukocytes separated from full blood of rats, 2 days after IV injection of PFC emulsion. The peak at -92ppm was contributed by CD11b$^+$ cells labeled by PFC. The peak at -75.5ppm was the signal from the reference TFA.
$^{19}$F signal intensity on $^{19}$F-TSE image was not affected by strong local susceptibility effects. (a1) and (a2) are $^1$H-T2*WI and $^{19}$F-TSE images, respectively, before iron oxide injection. (b1): One hour after MPIO injection, there was dramatic signal loss in the liver on $^1$H-T2*WI. However, the $^{19}$F signal intensity of the liver (b2) was similar to that before MPIO injection (a2). The column chart shows the $^{19}$F signal intensity of the liver before (blue) and after (red) MPIO injection, normalized by the $^{19}$F signal intensity of an external PFC reference. No significant difference of $^{19}$F signal intensity was observed after MPIO injection (p=0.52).
Supplemental Figure 3

(a) and (b) show the distribution of labeled compounds labeled as g, h, a, b, d, e, c, and f. The numbers inside the circles indicate the concentration of the labeled compounds. The bottom image (b) shows a higher concentration of compounds g and h compared to a, b, d, and e.

(c) shows a scatter plot of the relationship between the SNR and \(^{18}\)F atom concentration (\(x10^{16}/\text{voxel}\)). The equation of the line is \(y = 1.4099x + 0.9137\) with an \(R^2 = 0.9948\).
The detection limit of $^{19}$F-MRI and the correlation between the $^{19}$F spin concentration and its $^{19}$F signal to noise ratio (SNR) determined by the phantom experiment. (a): $^1$H-MRI of the phantom containing Eppendorf tubes filled with PFC emulsion suspended in 1.5% agarose at different concentrations of $^{19}$F spins, where $a=0.35$, $b=0.7$, $c=1.4$, $d=2.1$, $e=2.8$, $f=3.5$, $g=7.0$ and $h=10.4 \times 10^{16}$ $^{19}$F spins/voxel. (b): color scaled $^{19}$F-MRI shows the corresponding signal intensity of the PFC emulsion suspensions and their SNR values are marked alongside. (c): The concentrations of $^{19}$F spins versus their mean SNR values are plotted. The standard deviation values were not shown on the plot because they are too small to be visible. The Pearson correlation coefficient was 0.9948.
Supplemental References


