During cardiac ischemia and after reperfusion, platelets play an important role not only as a mediator of acute macrovessel closure but also by causing microvascular obstruction and inflammation in the ischemic/reperfused myocardium, both of which have a deleterious effect on myocardial damage. Platelet inhibition is a key factor in the management of acute myocardial infarction (AMI), and increasingly potent antiplatelet drugs targeting the platelet ADP receptor P2Y12 have improved patient outcome. In addition, the amount of platelets in the ischemic/reperfused myocardium has an important prognostic value. Studies have shown that a high degree of microvascular obstruction that

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myocardial damage. Inflammation and myocardial necrosis play important roles in ischemia/reperfusion injury after coronary artery occlusion and recanalization. The detection of inflammatory activity and the extent of myocardial necrosis itself are of great clinical and prognostic interest. We developed a dual, noninvasive imaging approach using molecular magnetic resonance imaging in an in vivo mouse model of myocardial ischemia and reperfusion.

**Methods and Results**—Ischemia/reperfusion injury was induced in 10-week-old C57BL/6N mice by temporary ligation of the left anterior descending coronary artery. Activated platelets were targeted with a contrast agent consisting of microparticles of iron oxide (MPIOs) conjugated to a single-chain antibody directed against a ligand-induced binding site (LIBS) on activated glycoprotein IIb/IIIa (LIBS-MPIOs). After injection and imaging of LIBS-MPIOs, late gadolinium enhancement was used to depict myocardial necrosis; these imaging experiments were also performed in P2Y12−/− mice. All imaging results were correlated to immunohistochemistry findings. Activated platelets were detectable by magnetic resonance imaging via a significant signal effect caused by LIBS-MPIOs in the area of left anterior descending coronary artery occlusion 2 hours after reperfusion. In parallel, late gadolinium enhancement identified the extent of myocardial necrosis. Immunohistochemistry confirmed that LIBS-MPIOs bound significantly to microthrombi in reperfused myocardium. Only background binding was found in P2Y12−/− mice.

**Conclusions**—Dual molecular imaging of myocardial ischemia/reperfusion injury allows characterization of platelet-driven inflammation by LIBS-MPIOs and myocardial necrosis by late gadolinium enhancement. This noninvasive imaging strategy is of clinical interest for both diagnostic and prognostic purposes and highlights the potential of molecular magnetic resonance imaging for characterizing ischemia/reperfusion injury. (Circulation. 2014;130:676-687.)
involves platelet microthrombi results in a poor outcome. In addition, the extent of myocardial necrosis is widely accepted to be an important prognostic factor, and cardiac magnetic resonance imaging (MRI) can be used to characterize wall motion and myocardial necrosis (late gadolinium enhancement [LGE]) after AMI.

Therefore, it would be highly desirable to characterize noninvasively the extent of platelet involvement in ischemia/reperfusion injury and the extent of myocardial necrosis. LGE specifically detects necrotic myocardial tissue, causing a signal increase in cardiac MRI. However, detection of platelets needs a targeted molecular imaging approach. We have previously developed a unique contrast agent that specifically detects activated platelets by targeting a ligand-induced binding site (LIBS) of the activated glycoprotein IIb/IIIa receptor using a single-chain antibody directed against these sites. This antibody was conjugated to microparticles of iron oxide (MPIOs), resulting in the unique activation-specific antiplatelet contrast agent (LIBS-MPIOs). These LIBS-MPIOs cause a signal decrease in T2*-weighted MRI at areas of platelet accumulation with an excellent sensitivity because the 1-µm MPIOs cause a magnetic field disturbance that far exceeds their own diameter. Using this approach, we were able to detect even small amounts of activated platelets in coronary and carotid artery thrombosis, plaque rupture, and cerebral inflammation in mice.

In this study, we induced ischemia/reperfusion injury in C57BL/6N mice by temporary ligation of the left anterior descending coronary artery and then performed molecular MRI of activated platelets by LIBS-MPIOs to characterize platelet accumulation contributing to microvascular obstruction and ischemia/reperfusion–associated inflammation. Furthermore, in a dual MRI approach, myocardial necrosis was directly assessed by LGE in the same animals. The central role of platelets in myocardial ischemia/reperfusion injury and the clinical relevance of the respective therapeutic receptor inhibition were confirmed in dual-contrast MRI and histology in P2Y12 knockout mice undergoing temporary left anterior descending coronary artery ligation.

**Methods**

**Contrast Agents**

**Platelet-Specific Contrast Agent (LIBS-MPIOs)**

The monoclonal "anti-LIBS"-antibody binds to LIBSs on the glycoprotein IIb/IIIa receptor only in its active conformation and demonstrates strong binding to ADP-activated platelets in the presence of fibrinogen. The cloning, generation, and production of the anti-LIBS single-chain antibody have previously been described in detail. To obtain a nonfunctional antibody for control purposes, exchange of the arginine in the RDD motif of the heavy-chain CDR3 region of a single-chain antibody was performed. Generation and purification of the antibody were performed as described elsewhere. For construction of the contrast agent, cobalt-functionalized autofluorescent MPIOs with a diameter of 1µm were conjugated to the histidine tag of the anti-LIBS/control single-chain antibody as described in the manufacturer’s protocol (Dynal Biotech, Oslo, Norway) and in previously published studies. Throughout this article, MPIOs conjugated to the anti-LIBS antibody are referred to as LIBS-MPIOs, and MPIOs conjugated to control antibody are called control-MPIOs. Injection of the LIBS-MPIO and control-MPIO contrast agent (each with 4×10⁸ particles in 50 µL saline) was via an 80-cm-long tube and the tail vein catheter with the animal positioned in the MR scanner. Flushing the tube with 100 µL saline ensured full injection.

**In Vitro Static and Flow Chamber Adhesion Assay**

Static assays were performed with small Petri dishes coated with washed platelets. Platelets were activated with 20 µmol/L ADP and incubated with MPIOs. In vitro flow chamber adhesion assays were performed with collagen-coated glass capillaries. Microthrombi were formed by perfusion of whole blood into the capillary with a syringe pump (PhD 2000, Harapparatus). MPIOs were perfused through the capillary for 5 minutes. Both experiments were observed with an IX81 Olympus microscope (Olympus, Tokyo, Japan) and Cell®P 1692 (ANALyis Image Processing) software using differential interference contrast microscopy with a x20 objective. Binding was quantified with Image Pro Plus software.

**Gadolinium Application**

Multihance (gadoben acid dimeglumine; Bracco Suisse) was used as paramagnetic gadolinium-based contrast agent for the late-enhancement examination. A concentration of 0.4 mL/kg body weight was diluted in 50 µL saline and administered via the tail vein catheter as described above.

**Animals**

Eight- to 10-week-old C57BL/6N mice (WT) were obtained from Charles River (Munich, Germany). P2Y12 receptor–deficient (P2Y12−/−) mice were kindly provided by J. M. Boeynaems and B. Robaye (IRIBHM and Erasme Hospital, Université Libre de Bruxelles, Bruxelles, Belgium). P2Y12−/− mice were also on a C57BL/6N background, and all mice were housed in the local animal facility before the experiments. All experiments were conducted strictly according to the German animal protection law and in accordance with good animal practice as defined by the Federation of Laboratory Animal Science Associations (www.felasa.eu) and the national animal welfare body GV-SOLAS (www.gv-solas.de). The examinations undertaken in this study were approved by the federal authorities in Freiburg and the Institutional Review Board through animal experiment permission 35/9185.81/G-09/47.

**Surgical Procedures**

Mice were anesthetized with ketamine (125 mg/kg; Pfizer Pharmacia GmbH, Berlin, Germany), xylazine (6 mg/kg; Bayer Vital GmbH, Leverkusen, Germany), and 2% isoflurane (Abbott, Wiesbaden, Germany) and maintained at 37°C. After orotracheal intubation, mice were ventilated with a maximal end-inspiratory pressure of 10 cm H2O, at a respiratory rate of 110 breaths per minute, and an inspiration/expiration ratio of 1/1.5 with a small-animal respirator (TSE Systems, Bad Homburg, Germany). Left lateral thoracotomy in the third intercostal space was performed after right lateral positioning of the animal. The medial left anterior descending coronary artery was identified after pericardiotomy and ligated with an 8-0 Prolene suture. Complete ligation was confirmed by paling of the anterior wall of the left ventricle and ST-elevation in 3-lead ECG and removed after 50 minutes to allow reperfusion. The pneumothorax was evacuated, and the chest and skin were closed with a 6-0 Prolene suture. Analgesia was provided with buprenorphine (0.1 mg/kg sc), and mice were henceforth kept in individual clean cages. Throughout the procedure, oxygen saturation, respiratory rate, and heart rate were continually monitored with a MouseOX system (Starr Life Sciences, Oakmont, PA) and maintained within the normal ranges.

**MRI Protocols**

All MR experiments were performed on a dedicated small-animal MRI system (BioSpec70/20, Bruker Germany) run with A VANCE III electronics and Paravision 5.1 software and with the use of a 2-channel cryogenically cooled mouse head surface coil. The animals were placed head first in the supine position onto the cryo-coil animal bed supported by a cotton pad underneath the
spine to ensure that the mouse chest would fully fill the sensitive coil volume.

For animal monitoring and sequence triggering, neonatal ECG electrodes were attached to the left front and the right hind paws of the mice. In addition, a breathing sensor pad was placed beneath the animal, and the animal’s temperature was maintained by warm water–supported heating of the animal cradle. During MRI, anesthesia was maintained by slowly introducing isoflurane up to a maximum of 2 vol% in oxygen, stabilizing the animals at a breathing rate of ~70 breaths per minute.

The MRI protocols consisted of 3 parts. First, a retrospectively gated multislice IntraGate Fast Low Angle SHot (FLASH) pilot scan was performed to verify the animal position and to place the reference slice for transmitter gain adjustments of the cryo-coil. Planning of the final cardiac pseudo–short-axis slice position was performed with an ECG and respiration-triggered standard FLASH sequence (echo time/repetition time, 2.8 milliseconds/35 milliseconds), acquiring 3 parallel slices. On an axial image, 3 sagittal slices through the left ventricle were tilted toward the coronary plane until parallel to the septum. Perpendicular on these images, 3 further slices were planned parallel to the imaginary line connecting the middle of the basis and the apex of the left ventricle. Finally, on these pseudo–4-chamber-view images, the 3 left ventricular pseudo–short-axis slices were planned. For these, a respiration- and ECG-triggered FLASH sequence tailored to gain T2* and T1 contrast while maintaining sufficient signal to noise was used. To obtain images from the late diastolic phase, the trigger delay was chosen, deducted repetition time and an additional 20 milliseconds from the ECG gating–derived time period. Blood signal attenuation was achieved through a 7-mm saturation slice placed parallel to the imaging slices across the atrium. Images were acquired after precontrast agent application and a minimum of 5 scans lasting ~50 minutes after injection of LIBS-MPIOs/control-MPIOs to monitor platelet invasion. Subsequently, injection of MultiHance and an additional 4 to 5 scans followed for monitoring late enhancement through gadolinium uptake. With 4 averages, the sequence parameters included an echo time/repetition time of 2.8 milliseconds/35 milliseconds, a flip angle of 50°, a bandwidth of 81.5 kHz, a slice thickness of 0.6 mm, a field of view of 25x25 mm, and a matrix size of 256x256, resulting in a resolution of 100 μm in plane. To achieve the best signal-to-noise ratio, we refrained from obtaining cine-like movies with the need for repeated excitation pulses but focused on the recording of late diastolic single frames.

MRI Data Quantification

Gadolinium-Induced Late Enhancement for the Detection of Necrosis

The area of gadolinium uptake, representing the LGE and therefore necrotic myocardium, was marked manually in 3 representative sections with the use of ImageJ software (version 1.46, National Institutes of Health). A quotient of the area with LGE divided by the total size of the left ventricle was calculated for all 3 sections, and the average is given. The same methodology was used to quantify the size of areas with signal extinction after injection of LIBS-MPIOs.

LIBS-MPIO–Induced Signal Extinction for the Detection of Platelets

For MRI signal quantification, each slice of the heart was sectioned into 6 segments following a modified American Heart Association protocol using a Matlab-based (MathWorks Inc, Natick, MA) custom-made software. Cardiac segmentation was performed by such a segmentation tool by manually surrounding the left ventricular epicardial and endocardial walls, starting at the anterior crossing of right and left ventricles. In the customized software, this starting point initiated a segmentation of the left ventricle into 6 equiangular segments. A quotient of the mean signal intensities from the anterior and anterolateral segments in relation to the signal of both septal segments was calculated for each slice and every time point. Afterward, these quotients were normalized to the native preinjection scan. Finally, the mean of the 3 slices of the anterior and anterolateral segment was calculated.

Although volume application during the experimental procedures resulted in an increase in right ventricular volume, an impact on septal wall motion or signal behavior has not been observed in our studies, which allowed us to use the septum as a reference parameter when evaluating the signal change in the ischemic myocardial region.

Echocardiography

Ejection fraction was measured planimetrically in a midpapillary plane in a blinded evaluation of loops acquired with a 15G-7 Philips linear epicardial transducer on an iE33 ultrasound system (Philips, Germany).

Histology

Infarct Size

Hearts were excised; left anterior descending coronary artery ligation was re-established; and unaffected myocardium was perfused with Monolite blue. Tissue was sectioned freshly for triphenyltetrazolium chloride staining, and subsequent blinded analysis of infarct area in relation to the Monolite blue–negative area at risk was performed in 5 serial sections (midpapillary to apical).

Histology for Platelets, Neutrophils, and Necrosis

Myocardial tissue was embedded with optical coherence tomography (Sakura Finetek), cut into 10-μm-thick sections, and fixed with acetone. For staining of neutrophil granulocytes, a rat anti-mouse Ly-6G IgG2a antibody was used (No. 551459, BD Biosciences, Pharmingen, San Diego, CA), and for control purposes, a rat anti-mouse IgG2a isotype control (No. 559073, BD Biosciences) was used. Secondary staining was performed with a biotinylated rabbit anti-rat IgG (BA-4001, Vector, Burlingame, CA). Alkaline phosphatase and substrate kit (AK-5000 and SK-5200; Vector) and levamisol (X3021, DAKO, Hamburg, Germany) were used for detection. For staining of platelets, a rat anti-mouse CD41 antibody was used (GTX 76011, GeneTex, Irvine, CA), and for control purposes, an IgG1 isotype control was used (MCA1211, Serotec, Puchheim, Germany). Secondary staining was performed with a biotinylated rabbit anti-rat IgG (BA-4001, Vector). Alkaline phosphatase and substrate kit (AK-5000 & SK-5100; Vector) and levamisol (X3021, DAKO) were used for detection. Finally, samples were embedded with Kaiser glycerin gelatine (1092420100, Merck, Darmstadt, Germany) until adequate staining.

Platelets and platelet-neutrophil conjugates were counted in 2 representative pictures of ×20 magnification out of the ischemic area of 2 different CD41- or CD41/Gr1–stained slices. For counting MPIOs, the ischemic area of 10 representative CD41 stained slices was carefully examined.

For quantification of necrotic tissue, hematoxylin-eosin staining was performed with a standard protocol using a hematoxylin and eosin solution (Merck, Roth, Karlruhe, Germany) on frozen sections. The size of the necrotic myocardium in each animal was measured in 3 representative hematoxylin-eosin–stained sections at ×4 magnification, analogous to measuring the size of late-enhancement area. A quotient of the disrupted and therefore necrotic area divided by the total size of the left ventricle was calculated for all 3 sections and averaged.

Statistics

Statistical analysis was supported by GraphPad Prism (version 4.0, GraphPad Software Inc). For statistical analysis of 2 groups, a Mann-Whitney test was performed. For analysis of data from ≥2 groups, the Kruskal-Wallis test was used.

Comparisons of MRI signal intensities in the LIBS and control groups after MPIO application and the effect of gadolinium on myocardial signal within each group (all signal values before and after gadolinium injection) were undertaken with a 2-way repeated-measures ANOVA.

The accuracy of microscope-supported evaluation of MPIO and platelet counts was defined by error estimation. Correlations were
investigated by linear regression tests. Results are given as dot-plot graphs with means. Test results were considered significant at values of $P<0.05$.

Results
Attachment of LIBS-MPIOs to Activated Platelets and Microthrombi In Vitro
Binding of LIBS-MPIOs to platelets was confirmed by static and flow chamber adhesion experiments. On an activated platelet monolayer, attachment of LIBS-MPIOs was significantly greater compared with control-MPIOs ($P<0.001$; Figure 1A). Similar results were obtained for flow chamber experiments with microaggregates/thrombi in which the number of LIBS-MPIOs attached was significantly higher than control-MPIOs ($P<0.01$; Figure 1B and Movies I and II in the online-only Data Supplement).

Platelet-MPIO Infiltration Is Highest 2 Hours After Reperfusion
For estimation of the optimal time point of maximal platelet accumulation in the ischemic/reperfused myocardium and hence for molecular imaging, platelet aggregates with bound LIBS-MPIOs were quantified after reperfusion (10 minutes and 2, 4, 6, 8, 10, and 12 hours). As depicted in Figure 1C, the maximum amount of LIBS-MPIO platelet aggregates was found 2 hours after reperfusion, and this was significantly different compared with all other time points ($P<0.05$). A typical image of an LIBS-MPIO platelet aggregate is shown in the inset. Platelets are stained in red; MPIOs appear as a round, brown structure highlighted by arrows.

LIBS-MPIO and Gadolinium Allow Imaging of Platelets and Necrosis
MRI was performed as described in the Methods section. A representative image of a short-axis image from the midventricular areas is depicted in Figure 2. For animals with LIBS-MPIO (Figure 2A) and control-MPIO (Figure 2B) injection, a baseline scan before contrast agent application is shown in the first column on the left. After contrast agent application, a continuous signal decrease can be observed in ischemic areas of animals with LIBS-MPIO injection (red arrows). This signal decrease is the typical susceptibility artifact induced by MPIOs and therefore depicts areas of contrast agent binding. No such signal effect is visible in animals with control-MPIO injection. At 37 minutes, gadolinium was injected to image the LGE for the detection of myocardial necrosis. The typical gadolinium-induced bright signal can be observed for both animal groups, indicating that myocardial necrosis was evident in these animals.

Molecular MRI of Myocardial Injury
LGE was quantified in all animals to prove comparable amounts of necrosis between animals injected with LIBS-MPIOs and control-MPIOs using representative sections of basal, medial, and apical myocardial regions in pseudo–short-axis.

![Figure 1](image)

**Figure 1.** Static and flow chamber adhesion assays showing strong binding of ligand-induced binding site (LIBS)-microparticles of iron oxide (MPIOs) on activated platelets. **A**, Representative microscopy images of MPIO attachment under static conditions. Attachment of LIBS-MPIOs was significantly higher than for nontargeted control-MPIOs (MPIOs exemplarily depicted by arrows). **B**, Representative microscopy images of flow chamber adhesion assays demonstrating that more LIBS-MPIOs attach to the microthrombus under flow compared with control-MPIOs. **C**, Time point assessment for optimal myocardial platelet imaging. For staining of platelets, a rat anti-mouse CD41 antibody was used, which then was detected by a biotinylated rabbit anti-rat IgG. Red staining was obtained by alkaline phosphate with a corresponding substrate kit. Platelet aggregates with bound LIBS-MPIOs were quantified at different time points after reperfusion (10 minutes and 2, 4, 6, 8, 10, and 12 hours). Maximum amounts of absolute LIBS-MPIO platelet aggregates were found 2 hours after reperfusion, with significant differences compared with all other time points ($P<0.05$). A representative image of an LIBS-MPIO platelet aggregate is shown in the inset (platelets, red; MPIOs, round brown structure; see arrows; ×100 magnification).
images. The LGE area of the left ventricle (expressed as percent) is demonstrated in Figure 3A, which showed no significant differences between the 2 groups injected with either platelet-targeted or nontargeted MPIOs. For further characterization of the inflammatory myocardial process, simultaneous staining for platelets (CD41) and neutrophils (Gr1) was

Figure 2. Dual imaging with ligand-induced binding site (LIBS)–microparticles of iron oxide (MPIOs) and gadolinium (Gd). Representative sections of a short-axis image from the medial ventricular areas are depicted. For animals injected with LIBS-MPIOs (A, top) and control-MPIOs (B, bottom), baseline scans are shown on the left. After contrast agent injection, a continuous signal decrease can be seen in ischemic areas of animals with LIBS-MPIO injection (red arrows) as the typical susceptibility artifact induced by MPIOs. No signal effect is visible in animals with control-MPIO injection. After imaging for 37 minutes, gadolinium was injected to observe the late gadolinium enhancement for detection of myocardial necrosis, which is present in both treatment groups (yellow arrow).

Figure 3. Quantification of myocardial necrosis, inflammation, and platelet accumulation. Late gadolinium enhancement (LGE) was not influenced by the injection of ligand-induced binding site (LIBS)–microparticles of iron oxide (MPIOs) and control-MPIOs (A). Simultaneous staining for platelets (CD41, red) and neutrophils (Gr1, black) shows platelet-neutrophil conjugates (B, ×20 magnification; C, negative control omitting primary antibody; D, ×100 magnification, with an example of bound MPIOs [arrows]) with comparable amounts of such conjugates in both groups (E). For quantification and characterization of platelet accumulation, F depicts a representative myocardial section with platelet aggregates stained by anti-CD41 immunohistochemistry and direct visualization of bound MPIOs (G, arrows). No platelet accumulation can be observed in nonischemic myocardium such as the septal wall (H). Microthrombi are equally distributed in both groups (I), whereas MPIO binding was significantly higher in animals injected with LIBS-MPIOs than with control-MPIOs (J). A significant correlation between the amount of bound LIBS-MPIOs and microthrombi in ischemic areas is evident (K). IHC indicates immunohistochemistry.
Molecular MRI Noninvasively Characterizes Platelet Accumulation and Myocardial Necrosis

The effect of MPIO-induced signal decrease was quantified as described in Methods. Signal quantification was pooled for the 2 segments (anterior and anterolateral) representing the areas of ischemia (Figure 4A, red arrows). After LIBS-MPIO injection, a significant signal decrease, as is typical for an MPIO-induced effect, can be observed (Figure 4B, red line; \( P<0.02 \), LIBS-MPIOs versus control-MPIOs), suggesting effective binding of LIBS-MPIOs to activated platelets. After gadolinium injection, the signal in LIBS-MPIO–injected animals increased above baseline, representing the gadolinium-induced signal increase in necrotic myocardium (\( P<0.003 \) for mean values after contrast agent versus after gadolinium).

Animals injected with control-MPIOs showed no MPIO-induced signal decrease and no significant increase after injection of gadolinium in a comparison of the mean values of all time points before and after injection of gadolinium.

**P2Y₁₂ Knockout Mice Demonstrate Reduced Platelet and Neutrophil Accumulation, Which Is Reflected in Molecular MRI**

Additional experiments were performed in P2Y₁₂\(^{-/-}\)mice to study the extent of myocardial ischemia/reperfusion injury and platelet accumulation in a setting with reliable platelet inhibition. After injection of LIBS-MPIOs, no signal increase was observed in these mice, and a similar effect was seen in animals injected with control-MPIOs (Figure 5A and 5B). Furthermore, the presence of myocardial necrosis was confirmed after the injection of gadolinium in both groups (Figure 5A and 5B, right, yellow arrows). The extent of LGE was significantly lower in P2Y₁₂\(^{-/-}\)mice, as quantified by MRI (\( P<0.005 \); Figure 6A). As a consequence of the reduction in platelet accumulation in P2Y₁₂\(^{-/-}\)mice, no significant difference in the MRI signal after MPIO injection was observed between control-MPIOs (green line) and LIBS-MPIOs (yellow line) in these animals (Figure 6B). When the mean values of all time points before and after the injection of gadolinium were compared, there was no significant difference for LIBS-MPIO–injected animals or control-MPIO–injected animals.

To confirm the observed reduction in LGE signal, we also assessed infarct size in histology and echocardiography. Infarct size was significantly smaller in P2Y₁₂\(^{-/-}\)mice than in the WT controls (Figure 6C, left ventricular ejection fraction demonstrated a trend toward better preservation in P2Y₁₂\(^{-/-}\)mice (Figure 6D) compared with WT.

To support these findings, immunohistochemistry was performed in all P2Y₁₂\(^{-/-}\)mice. Indeed, only minor platelet infiltration was detected in ischemic areas (CD41 staining, Figure 7A), and infiltration with platelet-neutrophil conjugates decreased (CD41/Gr1 staining, Figure 7B). Quantification of platelets in WT versus P2Y₁₂\(^{-/-}\)mice confirmed that there is significantly less platelet accumulation in P2Y₁₂\(^{-/-}\)mice (\( P<0.0005 \); Figure 7C), and the amount of bound MPIOs was significantly reduced to the level of WT.
mice in all P2Y<sub>12</sub><sup>−/−</sup> mice (P<0.01 versus WT with LIBS-MPIOs; Figure 7D). When the results of the P2Y<sub>12</sub><sup>−/−</sup> mice are added to the results from WT animals, the correlation between bound MPIOs and the presence of platelets remains highly significant (P=0.0001; Figure 7E), and the amount of platelet-neutrophil conjugates was reduced in P2Y<sub>12</sub><sup>−/−</sup> animals (P<0.005; Figure 7F). In summary, the results from immuno-histochemistry confirm a decrease in platelet and neutrophil accumulation in P2Y<sub>12</sub><sup>−/−</sup> mice, which corresponds well to the MRI findings in these animals.

As expected, the extent of myocardial necrosis was not significantly different between LIBS-MPIO- and control-MPIO-injected mice, whereas the extent of myocardial necrosis was less in P2Y<sub>12</sub><sup>−/−</sup> mice (Figure 8A and 8B). The size of the area with an LIBS-MPIO-induced effect in MRI correlated well with the size of the LGE area of the left ventricle (P<0.01; Figure 8C) and with the area of necrosis in histology (P<0.05; Figure 8D). Together with the data obtained with the P2Y<sub>12</sub><sup>−/−</sup> mice, the area of necrosis correlated well with the LGE area (P<0.0001; Figure 8E).

**Discussion**

In this study, we were able to noninvasively characterize ischemia/reperfusion injury after temporary coronary artery ligation in mice. We established a unique dual-contrast MRI approach that allows on one hand the detection of necrosis in myocardium subjected to ischemia/reperfusion using the late-enhancement effect of gadolinium-enhanced MRI. On the other hand, using a unique molecular contrast agent specifically targeted to activated platelets, we were able to detect platelet accumulation as a marker of microvascular obstruction and inflammation in mouse myocardium. Immunohistochemical analysis demonstrated an excellent correlation of infarct size and platelet accumulation with the MRI findings, showing the
potential of this noninvasive approach. Furthermore, using P2Y\textsubscript{12}\textsuperscript{−/−} mice as a control providing reliable platelet inhibition and reflecting therapeutic intervention, we were able to confirm the feasibility of using activated platelets as targets for imaging, the central role of platelets in myocardial ischemia/reperfusion injury, and the potential benefits of imaging the effects of therapeutic inhibition of platelets, in particular of the P2Y\textsubscript{12} receptor.

Figure 7. Quantification of platelet accumulation and inflammation in P2Y\textsubscript{12}\textsuperscript{−/−} mice. Only minimal platelet accumulation was found in ischemic/reperfused areas (A, anti-CD41 staining), and the number of platelet-neutrophil-aggregates decreased (B, CD41/Gr1 stain). Comparison of wild-type mice (WT) and P2Y\textsubscript{12}\textsuperscript{−/−} mice confirms a significantly smaller number of microthrombi (C), and the number of bound microparticles of iron oxide (MPIOs) was significantly reduced in all P2Y\textsubscript{12}\textsuperscript{−/−} mice, toward the level of WT mice (D). When the data obtained with the P2Y\textsubscript{12}\textsuperscript{−/−} mice are added to the data obtained with the WT animals, the correlation between bound MPIOs and microthrombi remains highly significant (E; orange, P2Y\textsubscript{12}\textsuperscript{−/−} animals), whereas the amount of platelet-neutrophil aggregates was reduced in P2Y\textsubscript{12}\textsuperscript{−/−} animals (F). IHC indicates immunohistochemistry; LIBS, ligand-induced binding site.

Figure 8. Quantification and correlation of myocardial necrosis, ligand-induced binding site (LIBS)–microparticle of iron oxide (MPIO), and late gadolinium enhancement (LGE) effects. In hematoxylin-eosin staining, the proportion of myocardial necrosis was not significantly different between LIBS-MPIO– and control-MPIO–injected animals, whereas the myocardial necrosis was lower in P2Y\textsubscript{12}\textsuperscript{−/−} mice (A and B). The extent of the area with an LIBS-MPIO–induced effect in magnetic resonance imaging correlated well with the extent of the LGE area of the left ventricle (C) and with the area of necrosis in histology (D). When these data are pooled with the results from the P2Y\textsubscript{12}\textsuperscript{−/−} mice, the area of necrosis correlated well with the LGE area (E). WT indicates wild-type.
With the increasing reduction in mortality resulting from AMI, assessing the risk of developing adverse cardiovascular events and deciding between revascularization and medical treatment have become major challenges. \(^{28,29}\) Left ventricular ejection fraction (eg, \(\leq 30\%\)) is currently one of the major means for stratifying risk, for example, for predicting the development of sudden cardiac death. \(^{30}\) However, a considerable number of patients with left ventricular ejection fraction \(>30\%\) still suffer sudden cardiac death, and the consequence of classifying a high number of patients falsely as being at high risk is exerting pressure on our healthcare systems. \(^{30}\)

Various methods have been assessed for delivering a patient-specific risk stratification and thus ultimately providing personalized medicine. Among these are advanced technologies such as computed tomography and MRI, for example, dual-energy and multidetector computed tomography, that, especially in combination with functionalized contrast reagents, have the potential to improve the contrast between diseased and normal myocardium. However, the lower contrast sensitivity and the required radiation are challenging limitations compared with MRI. \(^{31}\)

MRI after MI is increasingly seen as a leading imaging modality for risk assessment and personalized therapeutic decision making. For example, so far, LGE in MRI is the strongest predictor of mortality and major cardiac adverse events compared with clinical characteristics, coronary angiographic assessment, and left ventricular echocardiographic parameters. \(^{31,32}\) Recently, another new MRI method allowing detection of fibrosis by T1-weighted imaging has been shown to be a promising risk classifier. \(^{30,33}\) The molecular MRI of activated platelets may represent an additional method that warrants further testing in regard to its potential use for risk prediction in patients after MI. In contrast to LGE and T1-weighted MRI, imaging of activated platelets detects a process that directly drives inflammation and may thus be a better reflection of ischemia/reperfusion injury, MRI offers the advantage of compare these methods head to head in the same patient.

Positron emission tomography (PET) has superior sensitivity, and, especially in combination with the capability of the CT and MRI to exactly localize PET signals, it is highly attractive for the assessment of cardiac ischemia and viability. \(^{28,34}\) \(^{18}\)F-fluoro-2-deoxy-D-glucose (\(^{18}\)F-FDG) can be used as a marker of inflammation in MI. However, ischemia induces a shift toward glycolysis in cardiac cells, which can result in an \(^{18}\)F-FDG signal that is not inflammation specific. \(^{35}\) Other molecular markers in PET, which are increasingly available, could be used compared with LIBS-MPIOs to determine the functional role of platelets in cardiac ischemia/reperfusion. Nevertheless, if a functionally predictive molecular imaging approach directed against activated platelets can be developed for application in humans, MRI would be preferable over PET because it is nonradioactive, independent of cyclotron access, cheaper, and more broadly available.

Interestingly, although there is a strong correlation between the overall area of LIBS-MPIO signal and LGE signal, the 2 imaging techniques do not represent the same ventricular area. LIBS-MPIO signals and histological localization are found to be partially outside the necrotic myocardium. This may reflect the inflammatory reaction of ischemia/reperfusion injury, which is in accordance with recent findings that platelets play a pathogenic role in this pathological process and are a therapeutic target and that their involvement determines the rate of complications in mice such as ventricular rupture. \(^{6,36}\)

Overall, the combined LIBS-MPIO/LGE imaging could provide important clinical information delineating the area at risk and inflammation, in addition to the area of necrosis as determined by LGE imaging. Whether the combination of these 2 imaging methods allows a clinically relevant risk prediction of adverse cardiovascular events in patients after MI remains to be determined.

The area of LIBS-MPIO signal correlated well with the area of histological platelet accumulation. In addition, the area of infarct as measured in histology and by LGE correlated well with the overall LIBS-MPIO signal. However, the area of platelet accumulation does not have to be restricted to ischemic myocardium, as was similarly shown for matrix metalloproteinase activity as a marker of inflammation in the nonischemic myocardium. \(^{37}\) The area of postischemic inflammation detectable by platelet-targeted imaging might be larger and might have a prognostic value on its own. However, the clinical value of the LIPS-MPIO signal remains to be determined. The increasingly available combination of MRI and PET will provide the opportunity to compare MR platelet accumulation data with PET inflammation data such as those obtained by \(^{18}\)F-FDG. \(^{28}\)

P2Y\(_{12}\) receptor blockers are known to prevent atherothrombosis. However, recent data indicate that they can also reduce ischemia/reperfusion injury in MI, which is suggested to be mediated by reduced platelet and neutrophil accumulation. \(^{25,37}\) Molecular MRI accurately reflects reduced platelet accumulation and platelet-neutrophil complex deposition, as well as a reduction in myocardial damage in the histological assessment of P2Y\(_{12}\) receptor-deficient mice. Overall, these data suggest that molecular MRI of activated platelets may represent a novel method to assess the extent of functional inhibition achieved by various antiplatelet regimens.

Platelets also play a pivotal role in the context of microvascular obstruction after MI. \(^7\) The angiographically observed no-reflow phenomenon after opening of occluded vessels by percutaneous coronary intervention is a critical phenomenon, with such patients having an increased risk for congestive heart failure, rhythm disturbances, or death. \(^{38,40}\) So far, there is no consensus on the detection or characterization of a microvascular obstruction, eg, by single-photon-emission computed tomography or contrast-enhanced echocardiography. Currently available techniques have limitations in sensitivity and specificity, which includes LGE in MRI that tends to underestimate the subsequent scar formation. \(^5\) Therapeutic approaches to reduce microvascular obstruction by using glycoprotein IIb/IIIa inhibitors positively influenced myocardial flow and infarct size in a dog model, \(^{42}\) thereby indicating that platelets play a causative role in microvascular obstruction. Platelets may accumulate either intravascularly via adhesion to inflamed endothelium in the form of occluding microthrombi or extravascularly through the leaking of ruptured microves- sels. \(^7\) Overall, imaging of activated platelets may provide a measure of microvascular obstruction and thus potentially represents a tool for the prognosis of outcome in MI patients.
In addition, MRI of activated platelets is attractive as a direct parameter of successful (or unsuccessful) reperfusion of the microcirculation.

A major strength of our imaging approach is the platelet-targeted contrast agent itself. The LIBS-MPIO contrast agent allows the detection of activated platelets with a unique level of sensitivity and specificity. LIBS-MPIOs have already been used in a number of studies by our group and have allowed the detection of coronary and carotid thrombosis or cerebrovascular inflammation in mice. The LIBS antibody also binds to human platelets, also in an activation-specific manner, which is an important step toward the translation of this promising technology to application in humans. The presence of LIBS-MPIOs in ischemic/reperfused heart correlates well with the histological presence of platelets in the heart. This is an important prerequisite for noninvasive characterization of pathologies by MRI.

The hypointense contrast effect generated by MPIOs necessitated precontrast and postcontrast agent imaging and therefore a single MRI sequence throughout the experiment. Such an imaging protocol has to provide good T2* and T1 contrast in 1 scan. In addition, it has to allow a reasonable time resolution at sufficient signal-to-noise ratios. The applied ECG-triggered FLASH sequence provides this balance. Moreover, during the protocol setup, it proved to be superior to the retrospectively triggered IntraGate method. A translation of the recently described cardiac mouse imaging approach with IntraGate was not transferable to our scientific question because of irregular heart rates resulting from the induced myocardial injury.

Limitations
The major limitation of the present study is the difficulty of directly using the described MPIOs in patients. MPIOs are covered with polystyrene, which has potential toxicity in humans. Work on the development of human-compatible contrast-bearing nanoparticles such as liposomes or dendrimers, allowing conjugation with antibodies or peptide mimetics, is ongoing, and we are optimistic that there will be a compatible formulation in the near future. The anti-LIBS antibody itself is a single-chain antibody with a small molecular weight of \( \approx 25 \) kDa, which should not cause significant immunoreactivity. Importantly, during all of the experiments performed for this study, no animals died with signs of toxicity or showed symptoms suggesting an allergic reaction.

A possible translation into human application also has to take into account the larger size of the examined species and the magnetic field strength typically used in humans, mostly 1.5 or 3 T. However, this reduced absolute image resolution should be overcompensated for by the increase in object size, resulting in an improved image representation: The larger vessel sizes in humans clearly outweigh the reduced achievable resolution, resulting in more precise scans of the object. In the case of an equal slice thickness, the area of an image pixel would increase by roughly a factor of 100 coming from mice (in plane 0.1x0.1 mm) to humans (1x1 mm), whereas the cross section of the coronary artery will increase by a factor of \( \approx 300 \) (diameter: mouse, \( \approx 175 \) \( \mu \text{m} \); human, 3 mm). In fact, in a previous in vitro MRI study, we have demonstrated the general feasibility of detecting LIBS-MPIOs at a clinically relevant field strength of 3 T.

An intrinsic limitation of MRI is its sensitivity and hence the contrast generated via targeted agents. In particular, a negative contrast agent generated by MPIOs demands a comparison of precontrast and postcontrast images and therefore has longer scan times than, for example, PET. However, the “noninvasiveness” of MRI counterweights this limitation, and progress in research of on the development of MRI contrast agent holds promise for the clinical use of molecular MRI in patients.

A restriction to image quality specific to this study was the use of ECG-gated image acquisition. The nonconstant repetition time leads to a compromised steady state of longitudinal magnetization. Occasionally, the resultant image artifacts did not compromise image evaluation. However, an unclearly defined steady state hinders the selection of an optimal flip angle and thus full optimization of the signal intensity achieved. One possible solution to this is continuous excitation with data acquisition and phase step increment triggered through ECG. This option, allowing a defined Ernst angle excitation, is part of our current work.

Conclusions
By using a unique dual imaging approach of MRI in mice, we were able to image the extent of myocardial necrosis via LGE and platelet accumulation via a contrast agent that selectively targets activated platelets, which contribute to ischemia/reperfusion-associated inflammation and potential obstruction of the microcirculation. Findings in immunohistochemistry and MRI correlated very well, providing a robust basis for noninvasive characterization of these pathologies. Furthermore, \( \text{P2Y}_{12} \) knockout in mice confirmed the role of platelets in myocardial ischemia/reperfusion injury and the specificity of our molecular MRI approach. Overall, this pilot study on MRI of activated platelets in MI justifies further testing to establish the role of platelets in ischemia/reperfusion injury and to assess the effect of antiplatelet therapy and its use as an indicator of the prognosis of ventricular remodeling and adverse cardiovascular events.

Acknowledgments
We thank Dr Jennifer Rivera for careful revision of this manuscript. We also are grateful to Dr Pierre Levan and Rebecca Ramb, Dipl Math, for statistical advice.

Sources of Funding
This study was financed by DFG grants MU2727/3-1, MU2727/6-1 (both to Drs von Elverfeldt and von zur Muhlen) and HE6382/1-1 (Dr Heidt). Drs Duerschmied and von Elverfeldt were supported by grant F/09/12 of the German Heart Research Foundation. Dr Maier was supported by a grant of the German Heart Foundation, Dr Wang by the National Health and Medical Research Council of Australia, and Dr Peter by a Future Fellowship of the Australian Research Council.

Disclosures
None.

References


Clinical Perspective

Inflammation and myocardial necrosis play important roles in ischemia/reperfusion injury in patients with myocardial infarction and successful medical or interventional recanalization. The detection of inflammatory activity and the extent of myocardial necrosis itself are of great clinical and prognostic interest. In this study, we noninvasively characterize ischemia/reperfusion injury after temporary coronary artery ligation in mice. We established a unique dual-contrast magnetic resonance imaging approach that allows on one hand the detection of necrosis in myocardium using the late-enhancement effect of gadolinium-enhanced magnetic resonance imaging. On the other hand, using a unique molecular contrast agent specifically targeted to activated platelets, we were able to detect platelet accumulation as a marker of microvascular obstruction and inflammation in mouse myocardium. Immunohistochemical analyses demonstrated an excellent correlation of infarct size and platelet accumulation with the magnetic resonance imaging findings, showing the potential of this noninvasive approach. Furthermore, using P2Y12−/− mice as a control providing reliable platelet inhibition and reflecting therapeutic intervention, we were able to describe the feasibility of activated platelets as targets for imaging, to confirm the central role of platelets in myocardial ischemia/reperfusion injury, and to newly present the potential of imaging the effects of therapeutic inhibition of platelets. Overall, this pilot study on magnetic resonance imaging of activated platelets in myocardial infarction justifies further testing to establish the role of platelets in ischemia/reperfusion injury and to assess the effect of antiplatelet therapy and its use as indicator of prognosis of ventricular remodeling and adverse cardiovascular events.
Dual-Contrast Molecular Imaging Allows Noninvasive Characterization of Myocardial Ischemia/Reperfusion Injury After Coronary Vessel Occlusion in Mice by Magnetic Resonance Imaging

Dominik von Elverfeldt, Alexander Maier, Daniel Duerschmied, Moritz Braig, Thilo Witsch, Xiaowei Wang, Maximilian Mauler, Irene Neudorfer, Marius Menza, Marco Idzko, Andreas Zirlik, Timo Heidt, Peter Bronsert, Christoph Bode, Karlheinz Peter and Constantin von zur Muhlen

_Circulation_. 2014;130:676-687; originally published online June 20, 2014; doi: 10.1161/CIRCULATIONAHA.113.008157

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

MOVIE LEGEND

Movie 1

*In vitro* flow chamber adhesion assay showing minimal binding of control-MPIOs to microthrombi. In vitro flow chamber adhesion assays were performed using collagen-coated glass capillaries. Microthrombi were formed by perfusion of whole blood. This video was recorded over a period of 5 min.

Movie 2

*In vitro* flow chamber adhesion assay showing strong binding of LIBS-MPIOs to microthrombi. In vitro flow chamber adhesion assays were performed using collagen-coated glass capillaries. Microthrombi were formed by perfusion of whole blood. This video was recorded over a period of 5 min.