Noninvasive Assessment of Myocardial Inflammation by Cardiovascular Magnetic Resonance in a Rat Model of Experimental Autoimmune Myocarditis

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Background—Limited availability of noninvasive and biologically precise diagnostic tools poses a challenge for the evaluation and management of patients with myocarditis.

Methods and Results—The feasibility of cardiovascular magnetic resonance (CMR) imaging with magneto-fluorescent nanoparticles (MNPs) for detection of myocarditis and its effectiveness in discriminating inflammation grades were assessed in experimental autoimmune myocarditis (EAM) (n=65) and control (n=10) rats. After undergoing CMR, rats were administered with MNPs, followed by a second CMR 24 hours later. Head-to-head comparison of MNP-CMR with T2*-weighted, early and late gadolinium enhancement CMR was performed in additional EAM (n=10) and control (n=5) rats. Contrast-to-noise ratios were measured and compared between groups. Flow cytometry and microscopy demonstrated that infiltrating inflammatory cells engulfed MNPs, resulting in altered myocardial T2* effect. Changes in contrast-to-noise ratio between pre- and post-MNP CMR were significantly greater in EAM rats (1.08 ± 0.10 versus 0.48 ± 0.20; P < 0.001). In addition, contrast-to-noise ratio measurement in MNP-CMR clearly detected the extent of inflammation (P < 0.001) except for mild inflammation. Compared with conventional CMR, MNP-CMR provided better image contrast (CNR change 8% versus 46%; P < 0.001) and detectability of focal myocardial inflammation. Notably, MNP-CMR successfully tracked the evolution of myocardial inflammation in the same EAM rats.

Conclusions—Magneto-fluorescent nanoparticle CMR permitted effective visualization of myocardial inflammatory cellular infiltrates and distinction of the extent of inflammation compared with conventional CMR in a preclinical model of EAM. Magneto-fluorescent nanoparticle CMR performs best in EAM rats with at least moderate inflammatory response. (Circulation. 2012;125:2603-2612.)

Key Words: contrast media • inflammation • macrophage • magnetic resonance imaging • myocarditis

Myocarditis is defined as inflammation of myocardial tissue with characteristic inflammatory cell infiltration into myocardium.1,2 Clinically, myocarditis is a major cause of sudden death in young adults3 and is an important underlying cause of both dilated cardiomyopathy2 and arrhythmogenic right ventricular cardiomyopathy.4 Despite these serious consequences, there is no single confirmatory tool to diagnose myocarditis with absolute certainty, resulting in limited consensus on clinical practice guidelines for its evaluation and treatment.5,6

Clinical Perspective on p 2612

The introduction of endomyocardial biopsy combined with immunohistochemistry (IHC) and cardiovascular magnetic resonance (CMR) imaging has helped to overcome the major drawbacks of traditional diagnostic methodologies.7,8

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Whereas the invasive nature and poor standardization of endomyocardial biopsy have limited its widespread use, noninvasive CMR has emerged as a leading imaging tool in the diagnosis of myocarditis. However, myocardial inflammation, a critical pathogenic factor in myocarditis, cannot be directly visualized by CMR. T2*-weighted (T2W) imaging of tissue edema and myocardial early gadolinium enhancement (EGE) marks tissue hyperemia and capillary leak whereas myocardial late gadolinium enhancement (LGE) represents the late sequelae of inflammatory myocardial damage. Currently, CMR with or without gadolinium enhancement does not precisely identify inflammation in myocarditis. Thus, accurate noninvasive detection of inflammatory cellular infiltrates, as seen in viral myocarditis, is needed to drastically change the evaluation and management of clinical myocarditis.

Long-circulating iron oxide nanoparticles with MRI have been successfully used to image cellular inflammation in animal models with cardiac allograft rejection and autoimmune insulitis, as well as in patients with carotid artery stenosis and type 1 diabetes mellitus. Avid uptake of magneto-fluorescent nanoparticles (MNPs) by macrophages, facilitated by the vascular leakage that generally accompanies inflammation, can enable noninvasive visualization of tissue cellular infiltration.

On the basis of an in vitro feasibility study of MNP-MRI for the detection of inflammation, we investigated in vivo cellular CMR of macrophage-based inflammation in EAM rats, quantified cellular distribution of MNP-positive cells depending on inflammation grade, and compared the detectability of focal inflammation area in the myocardium of EAM rats between MNP-enhanced CMR and conventional CMR (T2W, EGE, and LGE) as a diagnosis of myocarditis. Magneto-fluorescent nanoparticle–enhanced CMR was also employed to noninvasively track the evolution of inflammation in myocarditis.

**Methods**

**Induction of the EAM Rat Model**

Experimental autoimmune myocarditis was induced in 7-week-old male wild-type Lewis rats (n = 75) as described previously. Induction of the EAM rat model, fluorescence-activated cell-sorting analysis of inflammatory cellular infiltrates within EAM hearts, in vivo CMR protocol, histopathological examination, image analysis, MR image tracking of MNP-labeled macrophages in vivo, and summary of experiments are presented in detail in the online-only Data Supplement. The protocol was approved by the local institutional review committee on animal care (KBSI-AEC1009).

**Animal Experimental Protocol**

The feasibility of silica-coated MNPs incorporated with rhodamine-β-isothiocyanate (details in the online-only Data Supplement) in imaging myocardial inflammatory cellular infiltrates was first investigated using ex vivo histopathology methods in EAM rats on day 21 (21D) after immunization (n = 7). At 24 hours before sacrifice, MNPs (10 mg Fe/kg) were administered via tail vein. An additional group of wild-type Lewis rats without immunization (n = 5) was also injected with MNPs and served as controls. Next, frozen-sectioned EAM rat hearts underwent hematoxylin and eosin (H&E) and IHC stain for macrophages. Fluorescence microscopy (FM), confocal laser scanning microscopy, and transmission electron microscopy (TEM) were employed to detect the MNPs in tissues. Second, the in vivo performance of MNP-enhanced CMR was investigated to image myocardial inflammation. T2*-weighted MNP-CMR was performed 24 hours after tail vein injection of MNPs (10 mg Fe/kg) in 21D EAM (n = 16) and control (n = 5) rats. Third, we then directly compared MNP-CMR with LGE-CMR for its potential of clinical translatability as a tool to overcome the weakness of LGE-CMR. Experimental autoimmune myocarditis 21D rats (n = 4) first underwent LGE-CMR after gadolinium DTPA (Gd-DTPA); 0.2 mL/kg, Magnevist, Bayer Healthcare) administration. Two hours after LGE-CMR, MNPs were intravenously given to the same rats after verifying the washout of Gd-DTPA in the myocardium, followed by MNP-CMR 24 hours later. Cardiovascular MR images were correlated with H&E- and IHC-stained sections and corroborated by FM of the matched cryostat sections. Fourth, in another group of 21D EAM rats (n = 12), flow cytometry analyses of inflammatory cellular infiltrates in the myocardium were performed to determine how different inflammatory-cell populations contributed to the overall MNP accumulation within inflamed hearts. Fifth, the effectiveness of MNP-CMR in discriminating the different grades of myocardial inflammation was investigated in EAM rats of different postimmunization days from 12D to 21D (n = 24). Sixth, the distinctiveness of MNP-CMR was further verified by comparing with T2W-, EGE-, and LGE-CMR. Conventionally used CMR methods of T2W-, EGE-, and LGE-CMR were sequentially conducted along with MNP-CMR in the same rats of 15D to 17D EAM (n = 10) and control (n = 5), followed by IHC staining for confirmation of inflammation. Experimental autoimmune myocarditis rats underwent LGE- and T1W-CMR before Gd-DTPA administration, and then EGE- and LGE-CMR was performed followed by MNP-CMR. Seventh, serial MNP-CMR was conducted in order to assess dynamic evolution of myocardial inflammation, reflecting pathologic progress from mild to severe inflammation in the same rats. Magneto-fluorescent nanoparticles were intravenously injected at 15D and 20D after immunization, and MNP-CMR was obtained 24 hours later at 16D and 21D in the same rats (n = 3). Lastly, MNP-CMR was also conducted to serially image and track MNP-labeled macrophages in vivo. Magneto-fluorescent nanoparticle CMR images were obtained before MNP injection and 24, 72, and 120 hours after MNP injection in 18D EAM rats (n = 3).

**Fluorescence-Activated Cell-Sorting Analyses of Inflammatory Cellular Infiltrates and In Vivo Cardiac MRI**

After the process of mincing and digesting hearts, obtained cell suspensions were stained with PE-CD45RA, PE-CD3, PE-CD11b/c, PE-Granulocytes (BD Biosciences, NJ) and underwent flow cytometry analyses of inflammatory cellular infiltrates in the myocardium, reflecting pathological progress from mild to severe inflammation in the same rats. Magnetic-resonance imaging was performed with a gradient-echo T2*-weighted, spin-echo T2W and fat-saturated spin-echo T1W sequences by using a 4.7 T MRI system (BioSpec 47/40; Bruker, Ettlingen, Germany) with dual ECG and respiratory gating (SA Instruments, Stony Brook, NY).

**Statistical Analysis**

The paired/unpaired Student t test and simple linear regression (Origin version 6.0; MicroCal LLC, Northampton, MA) and the paired interobserver comparisons with Cohen kappa statistics (SPSS version 15.0) were used to assess differences in the experimental data sets, and the measurement of the target-to-background ratios and ΔCNR within rats with different inflammation grades were assessed by 1-way ANOVA testing. Differences were considered significant at P < 0.05. All data are presented as mean ± SD.

**Results**

**Infiltrated Inflammatory Cells Actively Take Up MNPs in Inflamed Hearts**

The highly biocompatible and bifunctional (superparamagnetic and fluorescent) iron oxide MNPs were used in this study for the detection of both fluorescent and magnetic properties in vitro and in vivo. As derived from iron mea-
measurements of the blood samples, the blood half-life of the MNPs was 2.8±0.5 hours (online-only Data Supplement Figure 1). We sought to determine the feasibility of MNP-CMR to detect myocardial cellular inflammation. Experimental autoimmune myocarditis 21D rats showed markedly enlarged hearts with dilated ventricles and thickened myocardium. Whereas control rats did not contain any inflammatory foci (Figure 1A), the EAM rats showed extensive inflammatory foci of varying sizes scattered throughout the myocardium (Figure 1D), matching with the macroscopic discolored areas. Interstitial inflammatory cell infiltration and edema in association with degenerated myocytes and myocardial necrosis were observed in the inflammatory lesions, as reported previously.16,20 Macrophages densely infiltrated the myocardium of EAM rats, and their distribution in IHC precisely correlated with inflamed areas in H&E sections (Figure 1D and 1E): percentage inflammation area for EAM versus control rats, 48.7±8.0% versus 0% (Figure 1I, P<0.001), and percentage macrophage area for EAM versus control rats, 31.3±0.2% versus 1.0±0.3% (Figure 1K, P<0.001). Fluorescence microscopy demonstrated that strong fluorescence signals arising from the accumulated MNPs in EAM hearts were scattered throughout the inflamed myocardium and matched well with the distribution of inflamed foci and macrophages in histology though little fluorescence was observed in the control myocardium (Figure 1D and 1F). Target-to-background ratio of fluorescence signals in the heart sections of EAM rats was 130% higher than in control rats (2.3±0.2 versus 1.0±0.3; P<0.001; Figure 1L). In confocal laser scanning microscopy, we could confirm that the signal originated from interstitial MNP accumulation mainly in macrophages and not from the vasculature. Merged images showed most of the red fluorescent signals indicative of MNPs colocalized with macrophage signals (Figure 1G). In addition, TEM demonstrated that ingested intracellular MNPs were placed within the lysosomes of macrophages (Figure 1H and 1I).

Feasibility and Performance of MNP-CMR in the Assessment of Myocardial Inflammation

On the basis of ex vivo histopathological evidence of MNPs’ accumulation in inflammatory cellular infiltrates, we used CMR to investigate its feasibility in a preclinical model of myocarditis as the first step of clinical translation. Thickened ventricular walls and pericardial effusion, pleural effusion, or both in some instances were noted in 21D EAM rats. Pre–magneto-fluorescent nanoparticle CMR showed slightly higher T2*-weighted signal intensity in the myocardium of EAM rats (Figure 2A and 2E), leading to a 9% CNR increase in the left ventricular (LV) myocardium of EAM rats compared with control rats (1.08±0.10 versus 0.99±0.05; P=0.048), suggesting the contribution of tissue edema to increased signals.21 Magneto-fluorescent nanoparticle injection immediately induced a relatively homogeneous and marked negative contrast throughout the ventricular walls in both groups that was due to the presence of MNPs in the vasculature (data not shown). At 30 minutes after the injection, both the control and 21D EAM rats showed a similar CNR decrease (ΔCNR) of about 75% in the myocardium (data not shown). Although the negative contrast markedly recovered within 24 hours in the control group (Figure 2F–2H; ΔCNR=4%; pre-CNR=0.99±0.05 versus post-CNR=0.95±0.06; P=0.347), much of the negative contrast persisted until 24 hours in the 21D EAM rats (Figure 2B–2D; ΔCNR=56%; pre-CNR=1.08±0.10 versus post-CNR=0.48±0.20; P<0.001). These in vivo MNP-CMR
results were strikingly in accordance with the ex vivo histological observation of robust MNP accumulation within the inflamed myocardium and probably within the infiltrated inflammatory cells.

We next sought to perform a comparison of MNP-CMR with LGE-CMR to assess myocardial inflammation in EAM rats. Late gadolinium enhancement CMR and MNP-CMR were sequentially performed in the same EAM rats. On T1W-CMR 20 minutes after Gd-DTPA injection, weakly enhanced areas in the inferior and posterolateral LV wall (Figure 3B, white arrowheads) were detected with a nonsignificant CNR change of about 8% (Figure 3C; from baseline 1.18±0.05 to postinjection 1.27±0.07, P=0.062). However, on the subsequent T2W-CMR 24 hours after MNP injection in the same rats, additional inflammatory areas (Figure 3E, yellow arrows) were detected with a significant CNR change of 46% (Figure 3F; from baseline 1.08±0.06 to postinjection 0.58±0.15, P<0.001). As shown in a representative case, MNP-CMR detected new inflammatory areas in the myocardium of EAM rats that were not evident in LGE-CMR. Histologically, negative contrast areas in MNP-CMR showed agreement with inflammatory foci in H&E (Figure 3G), macrophage areas in IHC (Figure 3H), and MNP-derived fluorescence signals in FM (Figure 3I).
In Vivo Cellular Distribution of MNPs in Inflamed Hearts

We next determined, using flow cytometry, how different inflammatory cell populations contributed to the overall accumulation of the MNPs in inflamed EAM hearts. In 21D EAM rat hearts, there were at least 4 types of leukocytes, which were identified as macrophages (CD11b/CD45RA/CD3/Granulocytes), B-cells (CD11b/CD45RA/CD3/Granulocytes), T-cells (CD11b/CD45RA/CD3/Granulocytes), and granulocytes (CD11b/CD45RA/CD3/Granulocytes). Macrophages were the most frequently detected inflammatory cells (72.3%), followed by granulocytes (14.6%), T-cells (12.0%), and B-cells (1.1%) in the inflamed myocardium (Figure 4A and 4B). Of the total sorted inflammatory cells, the number of cells that were double-positive for cell markers and MNPs was 2.8 \times 10^6 cells (34.1%). Coregistration of MNPs and each cell type revealed that macrophages were the primary source of altered negative contrast effect in inflamed myocardium of measured MNP-CMR images (78.4%; Figure 4C and 4D). T-cells also took up MNPs and contributed to the myocardial T2* effect of MNPs to some extent. However, considering a cellular contribution of MNP source and accumulation of MNPs in macrophages 4 times more than in T-cells,22,23 Macrophages accounted for at least 93% of myocardial MNP accumulation in inflamed hearts. Thus we found that negative contrast effect in MNP-CMR could identify the extent and activity of infiltrated inflammatory cells, mainly macrophages, during the myocardial inflammatory process. Microscopic examination of fluorescence-activated cell-sorting subsets after Giemsa staining confirmed their typical morphological features (Figure 4E).

Effectiveness of MNP-CMR in Discriminating Inflammation Grades

We classified all rats into control, G1, G2, and G3 groups using the reported inflammation grading system (see the online-only Data Supplement) by measuring grades of inflammation from H&E images. This grading system discriminated well with the extent and intensity of negative-contrast area in MNP-CMR images (column post-MNP of A) match well with inflammation grade grouped from H&E-stained images and with the extent of macrophage signals in IHC-stained images. Contrast-to-noise ratios in G2 and G3 groups are significantly lower than in G1 and control (B): 0.47 ± 0.21 (G2) and 0.47 ± 0.13 (G3) versus 0.93 ± 0.06 (G1) and 0.95 ± 0.06 (control) (G2 and G3 versus G1 and control; \( P < 0.001 \)). The Cohen’s kappa value for interobserver reliability was 0.83. Scale bars, 5 mm. MNP indicates magneto-fluorescent nanoparticle; H&E, hematoxylin and eosin; IHC, immunohistochemistry; and CNR, contrast-to-noise ratio.
There were no significant differences in within the myocardium (Figure 6B; R\textsuperscript{2}=0.833, P<0.001) and IHC-positive area of myocardium (R\textsuperscript{2}=0.863, P<0.001). There was no significant difference in ΔCNRs within G2 and G3 or within G1 and control (56% for G2 and G3; 3%–4% for G1 and control). ΔCNR indicates change in contrast-to-noise ratio; MΦ, macrophages.

**Figure 6.** Quantitative correlation of MNP-CMR and histopathological findings. The change in CNR between pre- and post-MNP-CMR images (ΔCNR) correlated with the percentage inflammation area in H&E images (A) and the percentage macrophage area (% MΦ area) in IHC images (B) in the control (closed triangles), G1 (open triangles), G2 (closed diamonds), and G3 (open diamonds) groups. Change in CNR correlated in a linear fashion with the inflamed area (R\textsuperscript{2}=0.833, P<0.001) and IHC-positive area of myocardium (R\textsuperscript{2}=0.863, P<0.001). There was no significant difference in ΔCNRs within G2 and G3 or within G1 and control (56% for G2 and G3; 3%–4% for G1 and control). ΔCNR indicates change in contrast-to-noise ratio; MΦ, macrophages.

**Head-to-Head Performance Comparison Between MNP-CMR and Conventional CMR**

The performance of MNP-CMR in the detection of focal and less-severe myocardial inflammation was assessed by a head-to-head comparison of MNP-CMR with T2W-, EGE- and LGE-CMR in additional EAM rats (n=10). Conventional (T2W, EGE, and LGE) CMR was sequentially performed preceding MNP-CMR in the same EAM rats, followed by IHC staining. Representative serially obtained T2W-, EGE-, and LGE-CMR images (Figure 7A) in 3 EAM rats are shown with corresponding IHC-stained images (Figure 7B). In the EAM#1 and EAM#2 (first and second rows in Figure 7A, group 1; 5 rats out of 10 EAM rats have similar myocardial inflammation pattern), large inflammation areas (green arrows) with small or focal inflammation regions (yellow arrows of EAM#1 and red arrow of EAM#2) were detected by MNP-CMR whereas only focal inflammation areas, less-severe inflammation areas, or a combination of the 2 were detected in EAM#3 (third row in Figure 7A, group 2; 5 rats out of 10 EAM rats have similar myocardial inflammation pattern) by MNP-CMR (column Post-MNP in Figure 7A). Relatively large areas of myocardial inflammation (green arrows) in EAM rats of group 1 were well detected by conventional T2W-, EGE-, LGE-CMR whereas small-sized inflammation regions (orange arrows) consistently failed to be detected. The superiority of MNP-CMR to conventional CMR was clearly shown for focal inflammation (red arrows), in which only MNP-CMR could detect this minute degree of inflammation. Contrast-to-noise ratio change was greater in MNP-CMR than in any other conventional CMR sequence (Figure 7C–7E). Compared with control rats, only EAM rats imaged by MNP-CMR showed a significant CNR change of ≈32% (Figure 7E, P=0.018). In EAM rats of group 2 with their myocardial negative contrast area (MNP area in the online-only Data Supplement Figure II) <25%, the myocardial inflammation could not be detected by T2W-, EGE-, or LGE-CMR (online-only Data Supplement Figure II A–II C). There was no significant difference in ejection fractions measured from cine MR images between control and EAM rats (online-only Data Supplement Figure II D).

**MNP-CMR Monitoring of the Evolution of Myocardial Inflammation**

Serial MNP-CMR was conducted in the same rats to monitor transition from mild to severe inflammation stages in order to assess dynamic evolution of myocardial inflammation. Magneto-fluorescent nanoparticles were intravenously injected twice on 15D and 20D after immunization, followed by post-MNP-CMR 24 hours after MNP injection on 16D and 21D in the same rats (n=3). Serial pre- and post-MNP-CMR in 2 representative slices of the same EAM rat are shown in Figure 8. On the 16D post-MNP-CMR (Figure 8B and 8E), several negative contrast inflammation spots were detected (arrowheads) with a significant CNR change of about 41% (Figure 8G; pre-MNP 0.95±0.02 to post-MNP 0.56±0.24; P=0.049). At 21D after MNP-CMR following the second MNP injection on 20D, inflammatory regions were additionally detected (arrows) and the CNR change tended to be higher (0.31±0.15; P=0.002 versus pre-MNP) compared with the first post-MNP-CMR. There were differences in the contrast pattern and area between EAM rats as inflammation developed. Magneto-fluorescent nanoparticle CMR was further conducted in order to serially track macrophages labeled with MNPs once in vivo in order to understand if the MNP-labeled-macrophages move or expand into nearby myocardium. After pre-MNP-CMR in EAM rats (n=3) on 18D, MNPs were intravenously injected, then MNP-CMR was serially performed at 24, 72, and 120 hours after MNP injection in the same rats. On MNP-CMR images obtained serially 3 times at 24, 72, and 120 hours after MNP injection, the negative contrast areas (red arrows in the online-only Data Supplement Figure III) in the first MNP-CMR remained almost in the same inflammation regions without further expansion into newly inflamed sites (blue arrows of online-only Data Supplement Figure III) for 120 hours after MNP injection.
To date, reliable tools to noninvasively diagnose cellular inflammation in myocarditis are lacking. Here we demonstrate that the application of MNPs with superparamagnetic and fluorescent properties combined with targeted CMR permit noninvasive and robust imaging of myocardial inflammatory cellular infiltrates, enabling spatial mapping, quantification, and assessment of inflammation in EAM. Magneto-fluorescent nanoparticle CMR more accurately detected scattered foci of inflammation within the myocardium of EAM rats and provided higher-contrast images and better conspicuity of small and less-severe myocardial inflammation (red arrows; note that the diameter of macrophage cluster in the IHC image (red arrow of EAM#2) was ~200 μm). The CNR changes between pre- and post-MR images were more significant in MNP-CMR (D, P<0.001) compared with EGE- and LGE-CMR (C, P=0.002 and P=0.014 in EGE and LGE, respectively), and the CNR change between control and EAM rats was significant only in MNP-CMR (~32% from 0.98±0.04 to 0.65±0.26; P=0.018) (E). The Cohen’s kappa values for interobserver reliability of CNRs in T2W-, EGE-, LGE-, and MNP-CMR were 0.67, 0.70, 0.64, and 0.83, respectively. Scale bars, 5 mm. *P<0.05, **P<0.01, ***P<0.001. T2W indicates T2-weighted; Gd, gadolinium; EGE, early gadolinium enhancement; LGE, late gadolinium enhancement; MNP, magneto-fluorescent nanoparticle; EAM, experimental autoimmune myocarditis; IHC, immunohistochemistry; MΦ, macrophages; and CNR, contrast-to-noise ratio.

**Figure 7.** Head-to-head performance comparison between MNP-CMR and conventional (T2W-, EGE-, and LGE-) CMR. Three representative T2W-, EGE-, LGE-, and MNP-CMR (Post-MNP) images (A) sequentially obtained in the same EAM rats (n=10) are shown with corresponding IHC-stained images (IHC) (B). In the EAM#1 and EAM#2 (first and second rows in A, case 1; 5 rats out of 10 EAM rats), large inflammation areas (green arrows) with small or focal inflammation regions (yellow arrows of EAM#1 and red arrow of EAM#2) were detected by MNP-CMR, whereas only focal inflammation areas, less severe inflammation areas, or a combination of the 2 were detected in EAM#3 (third row in A, case 2; 5 rats out of 10 EAM rats) by MNP-CMR (column Post-MNP in A). In EAM rats of Case 1, large inflammation areas (green arrows) were detected by conventional and MNP-CMR (except for T2WI in EAM#2) whereas small-sized inflammation regions could be detected only by MNP-CMR. In EAM rats of group 2, only MNP-CMR could detect focal and less severe inflammation (red arrows; note that the diameter of macrophage cluster in the IHC image (red arrow of EAM#2) was ~200 μm). The CNR changes between pre- and post-MR images were more significant in MNP-CMR (D, P<0.001) compared with EGE- and LGE-CMR (C, P=0.002 and P=0.014 in EGE and LGE, respectively), and the CNR change between control and EAM rats was significant only in MNP-CMR (~32% from 0.98±0.04 to 0.65±0.26; P=0.018) (E). The Cohen’s kappa values for interobserver reliability of CNRs in T2W-, EGE-, LGE-, and MNP-CMR were 0.67, 0.70, 0.64, and 0.83, respectively. Scale bars, 5 mm. *P<0.05, **P<0.01, ***P<0.001. T2W indicates T2-weighted; Gd, gadolinium; EGE, early gadolinium enhancement; LGE, late gadolinium enhancement; MNP, magneto-fluorescent nanoparticle; EAM, experimental autoimmune myocarditis; IHC, immunohistochemistry; MΦ, macrophages; and CNR, contrast-to-noise ratio.

**Discussion**

To date, reliable tools to noninvasively diagnose cellular inflammation in myocarditis are lacking. Here we demonstrate that the application of MNPs with superparamagnetic and fluorescent properties combined with targeted CMR permit noninvasive and robust imaging of myocardial inflammatory cellular infiltrates, enabling spatial mapping, quantification, and assessment of inflammation in EAM. Magneto-fluorescent nanoparticle CMR more accurately detected scattered foci of inflammation within the myocardium of EAM rats and provided higher-contrast images and better conspicuity of small and less-severe myocardial inflammation than conventional T2W-, EGE-, and LGE-CMR. Multiple inflammatory cellular subsets contributed to MNP accumulation within the myocardium, with macrophages as the dominant cell type. Notably, we found that changes in CNR caused by MNP accumulation were strongly linked to alterations in percentage inflammation area and percentage macrophage area, suggesting that MNP-CMR could be an effective monitoring tool to image the evolution of inflammation and response to anti-inflammatory therapy in myocarditis. In addition, this imaging strategy showed regional distribution of myocardial inflammatory cellular activity in EAM rats.

Accurate and efficient tools for the diagnosis of myocarditis via serial noninvasive imaging of myocardial inflammation along with image-guided biopsies for histopathological analysis remain unmet clinical needs. First, early and prompt diagnosis may change the therapeutic strategy adopted for myocarditis. With current diagnostic tools, it is difficult to recognize the onset of myocarditis and diagnose the initial phase. Even for cases in which myocarditis was suspected, most cases were confirmed only by postmortem or after congestive heart failure.7 Second, a novel tool to monitor the response of patients with myocarditis after therapeutic intervention to reduce myocardial inflammation or prevent progression to dilated cardiomyopathy is an important clinical need. In particular, early recognition of individual responses after the administration of newly introduced drugs could provide reliable approaches for preventing unnecessary or harmful administration. In this study, we showed that MNP-CMR could be a highly efficient tool for visualizing myocardial inflammation in the early course of the disease, with a potential for quantifying the response to specific therapies for myocarditis. Furthermore, this MRI method along with histopathological analysis of MRI-guided biopsies will assist in...
the diagnosis of the focal manifestations of myocarditis. Cardiac MRI is now a routine diagnostic test for suspected myocarditis in patients.\textsuperscript{1,2} Since Gagliardi’s first report on the use of MRI for the diagnosis of human myocarditis,\textsuperscript{24} many researchers have reported interesting results on the diagnostic utility of MRI for human myocarditis. Because active myocarditis is associated with myocyte injury including edema and cellular swelling, the assessment of relaxation times provides a sensitive measurement for the detection of myocarditis. Abdel-Aty et al reported that the global noncontrast $T_2$ signal-to-noise ratio was significantly higher in patients with acute myocarditis than in control subjects\textsuperscript{11}; the calculated sensitivity, specificity, and diagnostic accuracy of $T_2$ at a cutoff value of 1.9 were 84%, 74%, and 79%, respectively. On the basis of our findings from preclinical animal models, it can be proposed that a combination of precontrast $T_2^*$ CNR and long-circulating MNP-induced contrast could result in enhanced diagnostic accuracy for patients with myocarditis. The established safety and clinical use of commercial $T_2^*$ agents, together with the finding of a previous MNP-based study on humans with prostate cancer,\textsuperscript{25} further increase the possibility for applying this method in humans.

Vascular leakage and the uptake of long-circulating MNPs by macrophages may possibly be the key mechanism for the continued observation of negative contrast in the myocardium. As a contrast agent, we used MNPs having a relatively long blood half-life (\approx 2.8 hours) in rats, which facilitates uptake of MNPs by circulating monocytes or macrophages at the inflammatory site of the myocardium. The findings in Figure 1 suggest that MNPs eluted from the fenestrated myocardial capillaries were phagocytosed by infiltrated inflammatory cells including macrophages and still existed in the inflamed myocardium 24 hours after injection, whereas the leaky MNPs disappeared almost completely from normal myocardium. Confocal laser scanning microscopy revealed the accumulated MNPs in macrophages, which induced negative contrast. In addition, the intimate correlation between $\Delta$CNR and percentage inflammation/macroage area suggests that alterations in $T_2^*$ signals increase in close relationship with the number of MNP-engulfed macrophages. Interestingly, no MNP deposit was visible in necrotic areas lacking macrophages (in 40D EAM hearts; data not shown). The correlation between CMR contrast and histological inflammatory change from mild to severe inflammation further validated the MNP accumulation in infiltrated macrophages. Thus, this imaging strategy can be useful for the noninvasive assay of myocardial macrophage activity. Though macrophages are the dominating cell type, MNPs can also be phagocytosed by tissue lymphocytes and neutrophils,\textsuperscript{26} as in our study (\approx 7% contribution to MNP uptake within the myocardium), suggesting that MNPs may be useful for lymphocytic or bacterial myocarditis, but this remains to be tested. The efficacy of visualizing inflammation in EAM rats between MNP-CMR and LGE-CMR, a current standard diagnostic tool in clinics, was directly compared in the same animals. Magneto-fluorescent nanoparticle CMR provided better image resolution with more-pronounced changes in CNR compared with LGE-CMR and identified inflammatory lesions with higher specificity. In humans, contrast enhancement after gadolinium administration matched well with predominantly macrophage-rich inflammation associated with myocyte damage.\textsuperscript{27} However, because the mechanism of gadolinium enhancement is via diffusion of the contrast agent into areas of damaged myocytes and inflammation, and because myocarditic areas often have living myocytes between the islands of necrosis,\textsuperscript{27,28} contrast enhancement after gadolinium injection may be weak and not specific for macrophage infiltration in contrast with MNP-induced enhancement as in our study.

This study has several limitations. First, the preclinical model that we employed in this study resembles more closely human giant-cell myocarditis than viral myocarditis. Thus the clinical translatablety of MNP-MRI needs further studies in a model of viral myocarditis. However, macrophages have been reported to be the major cellular infiltrates in human myocarditis\textsuperscript{28} and in coxsackievirus B3–induced mouse myocarditis.
carditis,29,30 as shown in this model of EAM. Second, the application of MNPs as a contrast agent has a limitation in terms of quantification. Signal loss in MRI depends not only on the amount of iron but also on how densely packed these MNPs are, including saturation behavior in negative contrast, as well as field strength and MRI pulse sequence (eg, T2 versus T2*). For this, we first verified the quantitative correlation between signal loss and MNP-labeled cell concentrations in vitro and expanded the results to the in vivo setting in order to assess the pattern and density of macrophage infiltrates in CMR of EAM rats after MNP administration. Third, CNR quantification was very susceptible to the selected regions of interest of CMR, and the area could extend outside the borders of LV myocardium, especially if there were dense monocyte/macrophages with enough MNPs near the border area. Thus we tried to encircle the ROI within the LV by avoiding border regions of the myocardium. Alternative approaches to quantifying super-paramagnetic iron oxide deposition based on positive contrast sequences31 may improve quantification approaches. Fourth, although MNP-CMR performed best in the setting of EAM rats with at least moderate inflammatory response, it failed to detect mild (G1) inflammation (Figure 6) both visually and quantitatively, partly because we adopted the global measurement of CNR in the entire LV myocardium. However, MNP-CMR were excellent at detecting focal inflammation spots (red arrows in Figure 7).

In conclusion, the application of long-circulating MNPs with superparamagnetic properties and appropriate in vivo CMR permitted noninvasive and robust imaging of myocardial inflammatory cellular infiltrates, enabling the spatial mapping, quantification, and assessment of inflammation severity, and also monitoring the dynamic evolution of myocardial inflammation in a preclinical model of EAM. Magneto-fluorescent nanoparticle CMR registered more accurately scattered and focally involved foci of inflammation within the myocardium of EAM rats and provided more discernible images than conventional CMR. However, MNP-CMR performed best in EAM rats with at least moderate inflammatory response.

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Disclosures
None.

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Myocarditis is defined as inflammation of myocardial tissue with characteristic inflammatory cellular infiltration into the myocardium. To date, reliable tools to noninvasively diagnose cellular inflammation in myocarditis are lacking. Here we demonstrate that the application of long-circulating magno-fluorescent nanoparticles combined with cardiac magnetic resonance imaging permit noninvasive and robust imaging of myocardial inflammatory cellular infiltrates, enabling spatial mapping, quantification, and assessment of inflammation in experimental autoimmune myocarditis. Compared with clinically conventional cardiac magnetic resonance, magno-fluorescent nanoparticle cardiac magnetic resonance more accurately detected scattered foci of inflammation and provided better conspicuity of small and less severe myocardial inflammation. Early and prompt diagnosis may lead to a paradigm shift in the therapeutic strategy of myocarditis. In addition, this magno-fluorescent nanoparticle cardiac magnetic resonance approach could be an effective clinical application in monitoring the evolution of inflammation and response to anti-inflammatory therapy in myocarditis.
Noninvasive Assessment of Myocardial Inflammation by Cardiovascular Magnetic Resonance in a Rat Model of Experimental Autoimmune Myocarditis
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**TITLE:** Noninvasive Assessment of Myocardial Inflammation by Cardiovascular Magnetic Resonance in a Rat Model of Experimental Autoimmune Myocarditis

**AUTHORS:** Hyeyoung Moon, MS; Hyo Eun Park, MS; Jongeun Kang, MS; Hyunseung Lee, MS; Chaejoon Cheong, PhD; Yong Taik Lim, PhD; Sang-Hyun Ihm, MD, PhD; Ki-Bae Seung, MD, PhD; Farouc A. Jaffer, MD, PhD; Jagat Narula, MD, PhD; Kiyuk Chang, MD, PhD; and Kwan Soo Hong, PhD

**Supplemental Methods and Results**

**Iron oxide nanoparticles:** PEGylated superparamagnetic iron oxide (SPIO) particles were synthesized in our laboratory as reported previously.\(^1\,^2\) Each particle consisted of a SPIO core of about 12-nm diameter, a rhodamine isothiocyanate (RITC)-incorporated silica coating of approximately 20-nm thickness, and polyethylene glycol (PEG) (molecular weight of 460-590; Gelest Inc., Tullytown, PA, USA) conjugated onto the silica coating. The final mean particle size was ~85 nm, measured by dynamic light scattering (DLS), and the T\(_2\) relaxivity (\(r_2\)) at 4.7 T was about 130 s\(^{-1}\)mM\(^{-1}\). These home-made highly biocompatible and bifunctional iron oxide magnetic nanoparticles (MNPs) enable the detection of both fluorescent and magnetic properties *in vitro* and *in vivo*. To determine their blood half-life, the MNPs were intravenously injected (10 mg Fe/kg) into the rats (n = 4), and blood samples (~300 μl each) were collected from pre-cannulated jugular veins from 10 min to 6 h after MNP injection. The amount of iron in the serially obtained blood samples was measured by an inductively coupled plasma atomic emission spectrophotometry (ICP-AES) system (Optima 4300DV, Perkin Elmer, Waltham, MA, USA), and then fitted to a mono-exponential decay curve. The measured blood half-life of the MNPs was about 2.8 ± 0.5 h (Supplement Figure S1). This value means that these MNPs remain in circulation for enough time to be phagocytosed by tissue monocytes/macrophages at the site of inflammation.
**Induction of the EAM rat model:** EAM was induced in 7-week-old male wild-type Lewis rats as described previously.\(^3\)\(^5\) Briefly, 1 mg (0.1 ml) of porcine heart myosin (Sigma, St Louis, MO, USA) with an equal volume of complete Freund’s adjuvant (Sigma) was subcutaneously injected into the rear footpads of the rats on days 0 and 6. On day 1, the rats were intraperitoneally injected with 500-ng *Bordetella pertussis* toxin (Fluka, Switzerland).

**Transmission electron microscopy (TEM):** After *in vivo* MRI, the rat hearts were perfused with PBS containing 20 U/ml heparin, and extracted hearts were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) and transversely cut into 20-μm-thick sections with cryomicrotome (CM1850, Leica Microsystems, Nussloch, Germany). Sectioned tissues were fixed with 2.5% glutaraldehyde buffer in grid glass bottom dish coated with poly-L-lysine at 4°C for 2 h. The tissues were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer on ice for 1 h, dehydrated in an ethanol series, and embedded in Epon 812. Ultra-thin sections with 70 nm were cut on an ultra-microtome (Ultracut-UCT, Leica, Germany) with a diamond knife (Diatome, Switzerland), and stained with uranyl acetate for 20 min followed by lead citrate for 10 min. TEM images were obtained by an electron microscope (Tecnai G2 Spirit TWIN, FEI, USA) at 120-kV accelerating voltage.

**In vivo cardiac MRI protocol:** MRI was performed by using a 4.7 T MRI system (BioSpec 47/40; Bruker, Ettlingen, Germany) with dual electrocardiographic (ECG) and respiratory gating (SA Instruments, Stony Brook, NY, USA). To acquire *in vivo* MR images, the animals were anesthetized with inhalational isoflurane (5% induction, 1.5% maintenance) in a O\(_2\):N\(_2\)O (3:7) mixture, and body temperature was maintained at 37 ± 1°C using warm water ducts into the animal bed. A quadrature birdcage RF resonator (Bruker) with an inner diameter of 72 mm was used for signal transmission and reception. For MR image acquisition, ECG signals were obtained from needle electrodes secured to the fore and hind limbs, and the R-wave was thereby used to generate a trigger pulse for image acquisition. T2*W-CMR was obtained with a gradient-echo FLASH sequence in the axial direction. The imaging parameters were as follows: FOV = 60 × 50 mm\(^2\), matrix size = 256 × 256, slice thickness = 1 mm, number of slice = 10, TR = 130 ms, TE = 6 ms, flip angle = 30°, number
of averages = 8, total scan time = 4 min 27 s. Also, T1W-CMR was obtained with a black-blood FLASH cine sequence in the center, upper and lower side of the heart in the axial direction. The imaging parameters were as follows: FOV = 60 × 60 mm², matrix size = 256 × 256, slice thickness = 1.5 mm, number of slices = 1, TR = 8 ms, TE = 2.8 ms, flip angle = 30°, number of averages = 6, total scan time = 3 min 16 s. In the serial MRI study of T2W and T1W with T2*W, spin-echo T2W and fat-saturated spin-echo T1W sequences were used with the following parameters: number of averages = 4, FOV = 80 × 60 mm², matrix = 192 × 192, slice thickness = 1.15 mm, number of slices = 10, TE/TR = 40/1,000 ms (T2W) or 5/150 ms (T1W).

**Image analysis:** Regions-of-interest (ROIs) for the measurements of contrast-to-noise ratio (CNR) and contrast area were manually traced by two blinded observers within left ventricular (LV) wall. Intercostal muscle area was used as reference in the three slices. To compare the signal contrasts in the inflamed myocardium, we measured the CNR of the myocardium in the acquired MR images of the EAM and control rats. The following equation was used to compare the change in CNR (ΔCNR) due to the MNPs in the myocardium before and after the MNP injection: ΔCNR = CNR_{pre} - CNR_{post}.

**FACS analyses of inflammatory cellular infiltrates within EAM hearts:** 24 hours after intravenous administration of the MNPs (10 mg Fe/kg), EAM rats were anesthetized and the hearts were perfused with PBS containing 20 U/ml heparin. Pulled-out rat hearts were chopped and micro-dissected using blades. Harvested hearts were digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1, and 450 U/ml collagenase type I (all enzymes were obtained from Sigma-Aldrich) in PBS containing 20 mM HEPES at 37°C for 1 h in a shaking incubator. A cell suspension was passed through a 70-μm strainer. Red blood cells (RBCs) were lysed after incubation with RBC lysis buffer (Qiagen, MD, USA) for 5 min and washed twice with PBS containing 20 mM HEPES. The remaining cells were incubated with a cocktail of antibodies including PE-CD45RA, PE-CD3, PE-CD11b/c, PE-Granulocytes (BD Biosciences, NJ, USA) for 30 min at 4°C. After washing, cells were detected and sorted by flow cytometry & cell sorter (Moflo, Beckman Coulter, USA). The sorted cells were stained with Giemsa, and were viewed with a fluorescence attached microscope (Olympus AX70 TR-62A02, Tokyo, Japan), and images were digitally captured with a
**Histopathological examination:** After *in vivo* MRI, the rat hearts were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) and transversely cut into 5-μm-thick sections. Quantitative distribution of MNP accumulation within the myocardium was compared between the EAM and Control rats by using a fluorescence stereoscopic microscope (Olympus SZ61TR, Tokyo, Japan) with the following parameters: excitation, 500 ± 550 nm; emission, 575 ± 650 nm; exposure time, 0.2 s. To identify cellular localization of MNPs in inflamed myocardium, a CLSM system (LSM 510 Meta; Carl Zeiss, Germany) was employed. After staining with the mouse anti-rat macrophage monoclonal antibody (clone ED-1; Chemicon International, Temecula, CA, USA) followed by Alexa Fluor488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), we investigated MNP and macrophage colocalization by color-coding the final CLSM images with red for RITC (MNP), green for Alexa Fluor488 (macrophages), and blue for DAPI (nuclei). H&E staining was used for observing the general morphology and determining the inflammation degree such as G1, G2, and G3. The inflammation grades were determined according to the Okura’s microscopic scoring method as follows: Control (normal), G1 (a few small lesions in a section, the extent of which did not exceed 0.25 mm²), G2 (multiple small lesions or a few moderate lesions, the extent of which did not exceed 6.25 mm²), and G3 (multiple moderate lesions or large lesions). For the IHC, we used the avidin biotin peroxidase method with the same antibodies. Briefly, sections treated with 0.3% hydrogen peroxide were incubated for 60 min with primary antibody, followed by biotinylated secondary antibody peroxidase conjugate (Invitrogen, CA, USA). The reaction was visualized with a substrate chromogen mixture and counterstained with hematoxylin. Adjacent sections treated with nonimmune IgG acted as the controls for antibody specificity. Stained tissue sections were viewed with a fluorescence attached microscope (Olympus AX70 TR-62A02; Tokyo, Japan), and images were digitally captured with a DP-70 digital camera (Olympus; Tokyo, Japan).

**Measurement of ejection fraction (EF):** For the measurement of ejection fraction of the heart, 2D cine images, with 7 slices covering almost left ventricle of the heart, obtained in diastolic and systolic phases were
used. A steady-state free-precession (SSFP) cine pulse sequence was used with the following parameters: TE/TR = 2.2/8.0 ms, number of frames = 30, number of averages = 4, FOV = 60 × 50 mm², matrix = 256 × 256, slice thickness = 1.5 mm. Inner area of left ventricular (LV) wall was measured from all 7 slices, and multiplied by 1.5 mm in order to calculate the LV volumes in diastolic (V_{dia}) and systolic (V_{sys}) phases. Ejection fraction (EF) was measured as $EF\,(\%) = 100 \times (V_{dia} - V_{sys})/V_{sys}$. The measured EFs were between 68% and 83%, and there was no significant difference depending on inflammation degree in the EAM (n = 10) and control (n = 5) rats used (Supplement Figure S2D).

**MR image-tracking of MNP-labeled macrophages in vivo.** MNP-CMR was conducted in order to serially track macrophages labeled with MNPs in vivo. After pre-MNP CMR in EAM rats (n = 3) on 18D, MNPs (10 mg Fe/kg) were intravenously injected, then post-MNP CMR images were serially obtained at 24, 72, and 120 hrs after MNP injection in the same rats. Immediately after performing MNP-CMR, the hearts were extracted and used for H&E and IHC for macrophages (MΦ) in order to measure tissue inflammation. In the first post-MNP CMR images at 24 hrs after MNP injection, several negative contrast regions were well detected (red arrows of Supplement Figure S4), resulting from macrophages, in vivo labeled with MNPs, in inflammation sites on 18D-19D. The negative contrast areas remained almost with the same regions without further expansion to newly inflamed sites (blue arrows of Supplement Figure S4) for 120 hrs after MNP injection.
### Supplemental Table S1. Summary of experiments.

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EAM (n = 75) & Control (n = 15)

MNP indicates magnetic nanoparticle; CMR, cardiac magnetic resonance; H&E, hematoxylin and eosin; IHC, immunohistochemistry; FM, fluorescence microscopy; CLSM, confocal laser scanning microscopy; TEM, transmission electron microscopy; LGE, late gadolinium enhancement; T2W, T2-weighted; EGE, early gadolinium enhancement; EAM, experimental autoimmune myocarditis.

*The rats (n = 4) were used additionally to get CNR values in Fig. 2.
Supplemental Figure S1. Measurement of blood half-life of the magnetic nanoparticles (MNPs) in rats. Time dependent iron content measured in blood samples from 4 mice after intravenous injection of the MNPs. The data were fitted with an exponential function (straight line), and then blood half-life was $t_{1/2} = 2.8 \pm 0.5$ h. This relatively long blood circulation provides a potential for the MNPs to be phagocytosed in infiltrated macrophages with improved possibility. Note that y-axis is in log-scale.
**Supplemental Figure S2.** Comparison of contrast area between MNP-CMR (MNP area) and conventional CMR (EGE, EGE area; LGE, LGE area; T2W, T2W area) together with ejection fraction (EF). In EAM rats (circle data points; group 2 of Figure 7) with MNP area lower than 25%, the myocardial inflammation could not be detected by T2W-, EGE-, and LGE-CMR (A-C). There was no significant difference of ejection fractions measured from cine MR images between control (80 ± 4%, closed triangles in D) and EAM (76 ± 4%) rats ($P = 0.1$). The Cohen’s kappa values for inter-observer reliability of T2W, EGE, LGE, and MNP area were 0.82, 0.82, 0.82, and 0.88, respectively.
Supplemental Figure S3. MR image-tracking of MNP-labeled macrophages in vivo. After pre-MNP CMR in EAM rats (n = 3) on 18D, MNPs (10 mg Fe/kg) were intravenously injected, and MNP-CMR images were serially obtained at 24 (19D), 72 (21D), and 120 hrs (23D) after MNP injection in the same rats, then the hearts were extracted and used for H&E and IHC for macrophages (MΦ) in order to confirm inflammation regions. In the first MNP-CMR images at 24 hrs after MNP injection on 19D, several negative contrast regions (red arrows) were well assigned as inflammation sites, resulting from macrophages in vivo labeled with MNPs on 18D-19D. Then, the negative contrast areas remained almost in the same inflammation regions without further expansion to newly inflamed sites (blue arrows). Note that inflammation was further expanded in other heart regions (blue arrows in H&E and IHC images) during CMR imaging. Scale bars, 5 mm.

Supplemental References:


