Transplantation remains an important option for patients with advanced stages of heart failure. Detecting parenchymal rejection and monitoring immunosuppressive therapy present an ongoing clinical challenge. The current approach to surveillance for rejection involves performing serial multiple endomyocardial biopsies obtained by invasive transvenous access. Typically, the right ventricle is biopsied in 3 to 4 locations.\(^1\) The procedure, which is performed biweekly in the first months after transplantation, causes complications in \(\leq 3\%\) of the cases, including valvular insufficiency, arrhythmia, infection, and bleeding.\(^2\) Sampling errors can also confound biopsy surveillance because the biotome often misses areas of myocardial inflammation. Moreover, inflamed myocardium beyond the endocardial surface cannot be detected with this procedure. There is thus a need for a better, less invasive method for monitoring rejection in transplant recipients.

**Clinical Perspective on p 573**

Nanoparticle PET-CT Detects Rejection and Immunomodulation in Cardiac Allografts

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**Background**—Macrophages predominate among the inflammatory cells in rejecting allografts. These innate immune cells, in addition to allospecific T cells, can damage cardiomyocytes directly.

**Methods and Results**—We explored whether sensitive positron emission tomography–computed tomography (PET-CT) imaging of macrophage-avid nanoparticles detects rejection of heart allografts in mice. In addition, we used the imaging method to follow the immunomodulatory impact of angiotensin-converting enzyme inhibitor therapy on myeloid cells in allografts. Dextran nanoparticles were derivatized with the PET isotope copper-64 and imaged 7 days after transplantation. C57BL/6 recipients of BALB/c allografts displayed robust positron emission tomography signal (standard uptake value allograft, 2.8±0.3; isograft control, 1.7±0.2; \(P<0.05\)). Autoradiography and scintillation counting confirmed the in vivo findings. We then imaged the effects of angiotensin-converting enzyme inhibitor (5 mg/kg enalapril). Angiotensin-converting enzyme inhibitor significantly decreased nanoparticle signal (\(P<0.05\)). Histology and flow cytometry showed a reduced number of myeloid cells in the graft, blood, and lymph nodes and diminished antigen presentation (\(P<0.05\) versus untreated allografts). Angiotensin-converting enzyme inhibitor also significantly prolonged allograft survival (12 versus 7 days; \(P<0.0001\)).

**Conclusions**—Nanoparticle macrophage PET-CT detects heart transplant rejection and predicts organ survival by reporting on myeloid cells. (Circ Cardiovasc Imaging. 2013;6:568-573.)

**Key Words:** heart transplantation ■ imaging ■ macrophage ■ positron emission tomography/computed tomography

Prior research has established lymphocytes as central orchestrators of tolerance and rejection. Macrophages (MΦ) and their circulating monocyte precursors, parts of the innate immune system, traditionally received attention as mediators of myocardial damage during rejection. An increasing body of work in models of inflammatory disease suggests that myeloid cells play very active roles in immunity.\(^3\) Human biopsy studies revealed that MΦ comprise \(\leq 60\%\) of the inflammatory cell population in allografts.\(^4\) Initiating inflammatory events recruit monocytes into the graft where they differentiate into MΦ, which then amplify inflammation by release of soluble factors such as tumor necrosis factor-\(\alpha\), interleukins, myeloperoxidase, inducible nitric oxide synthase, and reactive oxygen species. Monocytes and MΦ are involved in cell killing, scavenging of debris, and phagocytosis of nonself material and remodel the extracellular matrix through proteolysis, fibrosis, and angiogenesis.\(^5\) Moreover, monocytes and MΦ can prime allospecific T cells by alloantigen presentation.\(^6\)
Many of these functions may have important diagnostic and therapeutic implications in transplant rejection. Indeed, a clinical trial concluded that kidney allograft dysfunction correlates more closely with MΦ than T-cell content in biopsies. On the basis of these considerations, nanoparticle imaging of MΦ emerged as a modality to follow rejection, mostly relying on MRI detection of their iron oxide cores. Because positron emission tomography (PET) offers the most sensitivity and quantifiability among imaging modalities currently available in the clinic, we here explored PET/computed tomography (CT) imaging of monocyte/MΦ infiltration in cardiac mouse allografts. To this end, we derivatized phagocytosable nanoparticles with the long-lived PET isotope copper-64, hypothesizing that the PET signal would reflect organ rejection and effects of MΦ-modulating therapy.

**Methods**

**Transplantation**

Female C57BL/6j (B6 WT, H-2b), BALB/c (H-2b) mice were purchased from the Jackson Laboratory. Animals were enrolled at 6 to 10 weeks of age (body weight, 20–25 g). The studies were approved by the Subcommittee on Animal Research Care at Massachusetts General Hospital (13th St, Charlestown, MA). In the treatment cohort, enalapril was administered at a dose of 5 mg/kg daily by gastric gavage starting 2 days before surgery and continuing until grafts were rejected. Ketamine (50 mg/mL; Bioniche Pharma) and xylazine (100 mg/mL; Vedco) were used as anesthetics. Vascularized intra-abdominal heterotopic heart transplantation was performed using microsurgical techniques as described previously. Hearts were transplanted into the peritoneal cavity of recipients by establishment of an end-to-side anastomosis of the donor aorta to the recipient aorta and an end-to-side anastomosis of the donor pulmonary trunk to the recipient inferior vena cava. The total surgery time was ≈45 minutes. If myocardial contractions were not palpated through the abdomen immediately postoperatively, the procedure was considered a failure and ≈1 in 10 recipients were excluded from the study. We investigated total allografted grafts (donor mouse, BALB/c, recipient C57/B6, H-2d into H-2b) and compared those with syngeneic isograft controls (donor C57/BL6, recipient C57/BL6). The use of isograft controls permitted differentiation of alloimmune-mediated injury of parenchymal rejection from background signals arising from ischemic injury (because of harvesting and anastomosis), pericardial inflammation (because of surgery), and the occasional formation of intracavitary thrombi within the vascularized but nonpumping grafts. Cardiac allograft survival was subsequently assessed through daily palpation. Rejection was defined as the complete cessation of cardiac contractions and was confirmed by direct visualization.

**Nanoparticle Preparation**

Copper-64-cross-linked iron oxide nanoparticles labeled with VivoTag 680 (64Cu-CLIO-VT680) were prepared as described previously, resulting in a particle diameter of 20 nm. Briefly, CLIO-47 was incubated with diethylamine pentaacetic acid (DTPA; Sigma, St. Louis, MO) for 2 hours at room temperature and then purified with a PD-10 column. DTPA-CLIO-VT680 (100 µg) was labeled with 64CuCl2 (252 ± 44 MBq; n = 3) in ammonium acetate buffer (400 mmol/L, pH 6.5) at 95°C for 1 hour. Filtration (molecular weight cutoff, 50 kDa) of the reaction mixture provided the labeled nanoparticle (34 ± 18 MBq) in 72.0% decay-corrected radiochemical yield. 64Cu-CLIO-VT680 was prepared for injection by resuspension in phosphate-buffered saline.

**PET-CT Imaging**

Mice were imaged with PET-CT using an Inveon small animal scanner (Siemens) 7 days after transplantation (n = 8–9 transplant recipients per group). This time point was chosen on the basis of previous flow cytometric studies. Imaging was done 24 hours after injection of 64Cu-CLIO-VT680. Mice were anesthetized with isoflurane (1.5%; O2 2 L/min) for the duration of imaging. After acquisition of 600 million counts, a high-resolution Fourier algorithm was used to rebin sinograms, and a filtered back-projection algorithm was then used to reconstruct 3-dimensional PET images. Image voxel size was 0.806 × 0.806 × 0.866 mm, for a total of 128 × 128 × 159 voxels. Calibration of the PET signal with a cylindrical phantom containing 64Cu isotopes was performed before scanning. Data are expressed as standard uptake values, which normalizes activity for body weight and injected activity. Mean injected activity was 2.1 ± 0.15 mCi in 60 ± 10 µL saline, and the mean weight of mice was 22.8 ± 0.8 g. CT images were reconstructed from 360 cone-beam x-ray projections with a power of 80 kV and 500 µA. The isotropic voxel size of the CT images was 110 µm. During CT acquisition, iodine contrast was infused into the tail vein at a rate of 35 µL/min to enhance intravascular contrast. Projections were acquired at end expiration, which was identified using a BioVet gating system (M2M Imaging, Cleveland, OH). The CT acquisition time was ≈10 minutes. Reconstruction of data sets, PET-CT fusion, and image analysis were done using Inveon Research Workplace (Siemens). Three-dimensional visualizations were produced using the DICOM (digital imaging and communications in medicine) viewer OsiriX (The OsiriX Foundation, Geneva, Switzerland).

**Measurement of Ex Vivo Tissue Activity**

All mice were placed in a well counter (CRC-127R, Capintec, Ramsey, NJ) after injection of 64Cu-CLIO-VT680 and again after imaging was completed before dissection, to record total corporeal activity. After euthanization, grafts were excised, and radioactivity was measured using a gamma counter (1480 Wizard 3”, PerkinElmer, Boston, MA). Finally, tissues were exposed on phosphor imaging plates that were then read using a Typhoon 8600 scanner (GE).

**Histological Study**

Cardiac allografts were harvested 7 days after transplantation and embedded in optimal cutting temperature compound (Sakura Finetek). Fresh-frozen 6-µm-thick serial sections were examined by hematoxylin-eosin and immunohistochemical staining for both CD11b+ myeloid cells (anti-CD11b primary antibody, clone M1/70, BD Biosciences) and MAC-3+ MΦ (anti-Mac3 antibody, clone M3/84 BD Biosciences). Immunoperoxidase staining was performed using a biotinylated secondary antibody and ABC kit (Vector Laboratories Inc.), and the reaction was visualized using a 3-amino-9-ethylcarbazole substrate (AEC, DAKO California). Slides were scanned using a Nanozoomer 2.0RS (Hamamatsu, Japan), and the percentage positive area was measured with IPlab (version 3.9.3; Scanalytics) in 4 to 5 high-power fields per slide.

**Flow Cytometry**

Transplanted hearts, para-aural lymph nodes, spleen, and blood were collected and processed immediately (n = 5 per group). Single-cell suspensions were produced in flow cytometry (FACS) buffer. Approximately 3 million cells were diluted in 200 µL FACS buffer for staining with antibodies against leukocyte lineage markers (B220, CD49b, CD90, Ly6G, NK1.1, and Ter119), CD11b, CD11c, F4/80, Ly6C, and major histocompatibility complex class II (BD Pharmingen and eBioscience). To detect antigen presentation by flow cytometry, we stained the harvested cell suspensions with Y-Ae antibody (1:30; eBioscience), which recognizes BALB/c Ea52-68 peptide presented in context with B6 IAb (major histocompatibility complex class II). The stained cells were then analyzed using a LSRII flow cytometer (BD Biosciences). The data were analyzed using flowjo software (Tree Star, Inc.).

**Statistics**

Results are expressed as mean±SEM. Statistical comparisons between 2 groups were evaluated using the Student t test and corrected using ANOVA for multiple comparisons. Kaplan-Meier survival graphs were constructed, and a log-rank comparison of the groups.
was used to calculate $P$ values. A value of $P<0.05$ was considered statistically significant. We used Prism 6 (Graphpad) for statistical analysis.

**Results**

We found that the in vivo nanoparticle PET signal in the allografts was higher than that in isograft controls (standard uptake value, 2.8±0.3 versus 1.7±0.2; $P<0.05$; Figure 1A and 1B). The target-to-background ratio, using the native heart for normalization to background radioactivity, was also increased in allograft recipients (2.1±0.2 versus 1.4±0.1; $P<0.01$; Figure 1C), reflecting lower uptake of nanoparticles by the native, noninflamed heart. To validate these in vivo findings, we next harvested the hearts for ex vivo analysis. Scintillation counting of the organ and autoradiography both showed increased graft activity ($P<0.05$; Figure 2). In agreement with previous data,9,12 allografts also exhibited increased levels of $\text{CD11b}^+ \text{myeloid cells and MAC-3}^+ \text{MΦ}$ (Figure 3A), cells that avidly ingest dextran nanoparticles.9,14–16

Next, we tested this imaging approach in a trial examining the immunomodulatory actions of angiotensin-converting enzyme inhibitor (ACEi; 5 mg/kg enalapril per gavage). On the basis of our previous findings that angiotensin II triggers release of inflammatory monocytes into the blood,17,18 we hypothesized that enalapril treatment would reduce inflammation in mice with allograft rejection by dampening myeloid cell flux into the heart. Indeed, both flow cytometry and immunoreactive histology showed that administration of the ACEi diminished the presence of $\text{CD11b}^+$ myeloid cells and MAC-3$^+$ MΦ in allografts on day 7 after transplantation (Figure 3A and 3B). ACEi treatment also reduced the number of myeloid cells in the blood and draining lymph nodes of allograft recipients (Figure 4). We then examined whether ACEi reduces antigen presentation, a process that initiates and maintains the adaptive immune response to foreign tissue, including transplanted organs. To this end, we used flow cytometric analysis after staining cell suspensions with a Yaε antibody. This antibody detects a peptide from the I-Ea chain bound to I-Ab, reporting on major histocompatibility complex class II presentation of BALB/c antigen mice that had served as heart donors. In graft-draining lymph nodes and the spleen, the number of Yaε$^+$ cells was significantly lower in recipients that were treated with enalapril ($P<0.05$; Figure 5).

![Figure 1](image1.png) **Figure 1.** In vivo nanoparticle positron emission tomography–computed tomography (PET-CT) 7 days after transplantation. **A**, Heterotopic grafts in the recipient's abdomen. The allograft (Allo) showed higher PET signal compared with the isograft (Iso). **B**, Allografts showed significantly higher standard uptake value (SUV) and (C) target:background ratios (TBR) than Iso controls. Data are mean±SEM (*$P<0.05$; **$P<0.01$).

![Figure 2](image2.png) **Figure 2.** Ex vivo validation of rejection imaging by autoradiography of native and transplanted hearts. **A**, Representative autoradiographic images showed higher signals in allografts (Allo). **B**, Scintillation counting of explanted grafts yields higher percentage injected dose per organ (%IDO) in allografts. **C**, Target:background ratio (TBR) of grafted hearts. The native heart served as background. Data are mean±SEM (*$P<0.05$; **$P<0.01$). Iso indicates isograft.

![Figure 3](image3.png) **Figure 3.** Angiotensin-converting enzyme inhibitor (ACEi) treatment decreased the accumulation of $\text{CD11b}^+$ cells. **A**, ACEi administration significantly reduced $\text{CD11b}^+$ monocyte/macrophage numbers in grafted hearts compared with untreated recipients. **B**, Hematoxylineosin (H&E) and immunohistological stains using $\text{CD11b}$ and MAC 3 showed that ACEi treatment decreased inflammation in allografts (Allo). Scale bar indicates 50 μm. Data are mean±SEM (*$P<0.05$). HPF indicates high-power fields; and Iso, isograft controls.
We subsequently studied the anti-inflammatory actions of ACEi in vivo using MΦ-targeted nanoparticle PET-CT. Enalapril treatment was found to significantly reduce the mean PET standard uptake value and target-to-background ratio (Figure 6A–6C). Interestingly, the mean allograft survival time increased from 7 days in the control group to 12 days in recipients treated with the ACEi (n=7 per group; P<0.0001; Figure 6D).

Discussion

On receiving a heart allograft, patients are treated with lifelong immunosuppressive therapy to suppress rejection of the organ, which is recognized as foreign by the immune system of the host. These agents, however, have profound side effects and are thus given at the lowest doses possible. In cases when inflammation of the organ occurs, immunosuppressive therapy must be intensified, which consequently necessitates close monitoring of the patient and the graft. Unfortunately, serial heart biopsy, which is the method recommended by current guidelines,1 is associated with several shortcomings, including complications19,20 and discomfort to the patient.

There is thus an urgent need for alternative monitoring strategies such as leukocyte imaging8,9,12,21 or, possibly more cost-effective in routine surveillance, by following circulating biomarkers.22 This study used sensitive MΦ-targeted nanoparticle PET imaging to detect transplant rejection in mice. Copper-64-derivatized dextran nanoparticles were able to sense monocytes/MΦ in rejected heart grafts and could thus be used to monitor therapeutic immunomodulation in mice with total allo-mismatched hearts.

PET imaging has significant advantages in terms of sensitivity. 18F-fluorodeoxyglucose (18F-FDG), a clinically predominant PET agent, reports on glucose uptake by cells and can identify sites where MΦ have accumulated and been activated.
to increase glucose transport. High glucose uptake by cardiac myocytes, however, renders \(^{18}\)F-FDG less suitable for imaging inflammation in the heart, particularly when the target is interstitial inflammation as it is in transplant rejection. In contrast, although nanoparticles undergo avid uptake by myeloid cells, they undergo negligible uptake by other cardiac cells. We therefore used dextran nanoparticles, which closely resemble nanoparticles in current clinical use,\(^23\) to image cardiac graft rejection. The particles used in this study have a blood half-life of several hours. We thus chose a PET isotope with relatively slow radioactive decay (copper-64, \(t_{1/2} = 12.7\) hours) to match the pharmacokinetics of these particles. In doing so, imaging could be delayed sufficiently to allow nanoparticles not taken up by \(\Phi\) to exit the bloodstream. Future studies will now explore whether smaller nanoparticles (possibly approaching the 5-nm-diameter renal clearance cutoff) accelerate particle excretion so that shorter injection-imaging sequences become feasible; this would, in turn, enable use of the more widely available radionuclide fluoride-18 (\(t_{1/2} = 110\) minutes).

Heterotopic transplantation into the abdominal cavity of recipient mice has limitations; namely, it results in a beating but nonworking heart, and it has contact with the peritoneum. Therefore, translating results from this model to heart transplantation requires caution. In previous preclinical studies, it was demonstrated that MRI could be used to detect heart transplant rejection in rodents.\(^8,9,24\) In a recent clinical trial, it was likewise demonstrated that nanoparticle MRI could detect monocyes/\(\Phi\) in the heart of patients with acute myocardial infarction.\(^25\) This suggests that a similar approach may also work for imaging heart transplant rejection. However, the number of myeloid cells per gram of myocardium (or imaging voxel) is particularly high in acute myocardial infarction and likely exceeds leukocyte levels in early-stage organ rejection by orders of magnitude. The higher sensitivity of PET compared with MRI should facilitate imaging of allograft rejection, particularly in its early stages. Moreover, PET is more readily quantifiable and allows serial data to be compared. This would be particularly useful in clinical trials that use PET biomarkers as imaging end points.

We used PET detection of monocyes/\(\Phi\) in heart grafts to study the effects of administration of an ACEi on organ rejection. In mice treated with enalapril, the PET signal fell significantly, and allografts survived twice as long compared with untreated allograft controls. Although ACEi treatment did not abolish rejection (all hearts ceased beating by day 14), our data suggest this therapy could serve as an adjunct to standard immunosuppressive regime. The rationale for investigating ACE inhibition was based on our previous finding that angiotensin II triggers release of proinflammatory monocyes from a splenic reservoir into the bloodstream.\(^17,18\) Indeed, treatment with enalapril reduced the number of circulating monocyes in allograft recipients in the present study, and consequently, fewer of these cells accumulated in the graft as assessed by imaging, histology, and flow cytometry. Previous clinical reports described prolonged graft survival in patients taking an ACEi or angiotensin II receptor blocker in kidney transplants\(^26\) and heart allografts,\(^27\) whereas others did not.\(^28\)

A prospective trial of ACEi administration in patients after heart transplantation could provide clarity in this regard. In conclusion, our study shows that \(\Phi\) targeted nanoparticle PET imaging may offer a quantitative and noninvasive alternative to the myocardial biopsies performed after heart transplantation. The method was able to detect both myeloid cells in allografts and their diminution after therapeutic intervention. By optimizing the nanoparticles further, for instance through enhancing their pharmacokinetics, but still maintaining their phagocyte targeting, it is possible that their clinical translation will be accelerated. Ultimately, our goal is to develop a detection method that will eliminate the need for myocardial biopsies in allograft recipients.

Sources of Funding

This work was funded by grants from the National Heart, Lung, and Blood Institute (R01HL095629, R01HL114477, contract No. HHSN2682010000044C). Dr Ueno is a recipient of the American Heart Association Scientist Development Grant (11SDG5150000). Dr Dutta is a recipient of the American Society of Transplantation Basic Science Fellowship Grant (AST-129). Dr Majmudar was supported by an NIH T32 training grant (T32-HL094301).

Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Although heart transplantation saves lives of patients with end-stage heart failure, the recipient’s immune system detects the new heart as foreign and will thus reject it. Therefore, transplantation patients require close monitoring and efficient immunosuppression. The current standard of monitoring, heart biopsies, is inconvenient, prone to missing inflammatory tissue, and may result in complications. Because macrophages are among the most numerous cells invading a graft that is being rejected, they may serve as an imaging target. Here, we used a macrophage-avid nanoparticle derivatized with a positron emission tomography isotope to detect rejection in mice after allotransplantation and found that this imaging approach was a sensitive noninvasive method to follow rejection. It further detected immunomodulatory effects of angiotensin-converting enzyme inhibitor therapy. Thus, nanoparticle positron emission tomography promises to provide a useful 3-dimensional sensing technique in allograft rejection and a noninvasive alternative to biopsies.
Nanoparticle PET-CT Detects Rejection and Immunomodulation in Cardiac Allografts
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*Circ Cardiovasc Imaging*. 2013;6:568-573; originally published online June 14, 2013; doi: 10.1161/CIRCIMAGING.113.000481
*Circulation: Cardiovascular Imaging* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-9651. Online ISSN: 1942-0080

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