Molecular Imaging of Innate Immune Cell Function in Transplant Rejection

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Background—Clinical detection of transplant rejection by repeated endomyocardial biopsy requires catheterization and entails risks. Recently developed molecular and cellular imaging techniques that visualize macrophage host responses could provide a noninvasive alternative. Yet, which macrophage functions may provide useful markers for detecting parenchymal rejection remains uncertain.

Methods and Results—We transplanted isografts from B6 mice and allografts from Balb/c mice heterotopically into B6 recipients. In this allograft across major histocompatibility barriers, the transplanted heart undergoes predictable progressive rejection, leading to graft failure after 1 week. During rejection, crucial macrophage functions, including phagocytosis and release of proteases, render these abundant innate immune cells attractive imaging targets. Two or 6 days after transplantation, we injected either a fluorescent protease sensor or a magnetofluorescent phagocytosis marker. Histological and flow cytometric analyses established that macrophages function as the major cellular signal source. In vivo, we obtained a 3-dimensional functional map of macrophages showing higher phagocytic uptake of magnetofluorescent nanoparticles during rejection using magnetic resonance imaging and higher protease activity in allografts than in isografts using tomographic fluorescence. We further assessed the sensitivity of imaging to detect the degree of rejection. In vivo imaging of macrophage response correlated closely with gradually increasing allograft rejection and attenuated rejection in recipients with a genetically impaired immune response resulting from a deficiency in recombinase-1 (RAG-1^−/−).

Conclusions—Molecular imaging reporters of either phagocytosis or protease activity can detect cardiac allograft rejection noninvasively, promise to enhance the search for novel tolerance-inducing strategies, and have translational potential. (Circulation. 2009;119:1925-1932.)

Key Words: imaging ■ inflammation ■ leukocytes ■ rejection ■ transplantation

A cute parenchymal rejection causes most graft failure in the first year after heart transplantation, and rejection episodes predispose to the development of chronic allograft vasculopathy.1,2 Thus, close surveillance of transplanted organs remains mandatory. The current clinical standard of repetitive invasive endomyocardial biopsies is prone to sampling error, entails a risk of complication, and causes discomfort and anxiety for the patients.3 Therefore, developing noninvasive yet quantitative diagnostic tools to monitor parenchymal allograft rejection would fulfill a compelling need.

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T cells orchestrate allograft rejection; however, macrophages represent an abundant innate immune cell type in these allografts. Various roles of macrophages emerge in ischemia/reperfusion injury and in the alloimmune reaction.4 Whereas in ischemia/reperfusion injury macrophages function as major effector cells in the inflammatory response during the reperfusion phase, in the allogeneic response their functions include facilitating adaptive immunity as antigen-presenting cells, contributing to cell and tissue damage as inflammatory effector cells, and promoting healing and repair once the graft recovers from acute insults.5 The large number of macrophages and the key role of their effector functions during rejection, which include phagocytosis and release of proteases, render them attractive molecular imaging targets.

Recently developed probes report on different biological functions, including phagocytosis and protease activity.6,7 Yet, it remains uncertain which probes best detect and quantify the macrophage response during parenchymal rejec-
Flow Cytometry
After euthanasia, we processed samples of the donor heart for flow cytometric analysis as described in the online Data Supplement. For each sample, 100,000 events were collected. Total number of cell types was determined by multiplying the total cell number per 1 mg tissue (obtained with trypan blue) by the percentage of a given cell type within the living gate (obtained with flow cytometry). Data were acquired on an LSRII device (BD Biosciences, San Jose, Calif). To detect ProSense-680 or CLIO-VT780, samples were excited with the red laser (635 nm) and detected with 680/70 and 695/70 filter configuration.

 Nanoparticles and Imaging
The following 2 imaging agents were coinjected into the tail vein 24 hours before in vivo fluorescence molecular tomography (FMT) and magnetic resonance imaging (MRI): ProSense-680 (excitation wavelength, 680±10 nm; emission, 700±10 nm) for imaging of the protease activity, 5 nmol in 150 μL PBS, and CLIO-VT750 nanoparticles (excitation wavelength, 750±10 nm; emission, 780±10 nm) for imaging of the phagocytic activity, 15 mg Fe per 1 kg body weight. For flow cytometry, we injected ProSense-680 or CLIO-VT780 nanoparticles. FMT was performed on a dual-channel imaging system (FMT 2500, VisEn Medical, Woburn, Mass) as previously described.5,10 MRI studies were performed with a 7-T horizontal-bore scanner (Bruker Pharmascan, Billerica, Mass) after injection of the phagocytosis sensor and following a protocol described in the online Data Supplement. The contrast-to-noise ratio (CNR) between the myocardium and the skeletal muscle was calculated as follows: $\text{CNR} = (\text{myocardial signal} - \text{skeletal muscle signal})/\text{SD of the noise}$.

After euthanasia, native hearts and donor heart were excised, and sections were visualized with a custom-built fluorescence reflectance imaging system (FRI) system (BonsAI, Siemens, San Francisco, Calif) as detailed in the online Data Supplement.

 Statistical Analysis
Data are reported as mean±SEM. When comparing 2 groups, we used Student’s t test; for multiple comparisons, we used ANOVA with subsequent Bonferroni correction. Differences, indicated by an asterisk, were considered statistically significant at P<0.05. We performed statistical analysis with GraphPad Prism 4.0c for Macintosh (GraphPad Software, Inc, San Diego, Calif).

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

 Results
 Macrophages and Cathepsin-Expressing Cells Accumulate Abundantly in Rejecting Cardiac Allografts
As in human heart transplantation, allografting in mice requires periods of warm and cold ischemia, conditions that can provoke nonimmunologic parenchymal injury. Study of isografts permits isolation of the consequences of ischemia/reperfusion from immunologically mediated tissue damage.12 Our prior studies have described in detail the cellular and inflammatory sequences of events in hearts allografted under these conditions.1,13 Here, we performed immunohistochemistry not only to define the sequence of inflammatory cell accumulation in heart grafts but also to quantify macrophages and cathepsin-expressing cells in these allografts. Analysis of the sections stained with anti-mac3, anti–NIMP-R14, anti–CD4, anti–CD8, anti–cathepsin B, and anti–cathepsin S of isografts and allografts at postoperative day (POD) 3 and 7 indicated the number of positive cells (Figure 2). This technique detected only a few neutrophils in the heart grafts.
at POD 3 and 7, with no difference among groups. Macrophages, abundant innate immune cells and important amplifiers of T-cell–driven response in graft rejection, accumulated predominantly in the allografts over time. As expected, CD4 and CD8 T lymphocytes accumulated almost exclusively in the allografts, but later and to a lesser extent than did macrophages. Inflammatory cells in the graft expressed both cathepsins B and S, and the allografts had more protease-positive cells than did the isografts.

Fluorescent Signals From Both Probes Colocalize With Immunoreactive Macrophages and Cathepsin B

After coinjection of the fluorescent sensor reporting on protease activity and a magnetofluorescent phagocytosis sensor, we assessed the fluorescent signal by microscopy at different wavelengths on the same section of heart grafts. In the 680-nm channel, the ProSense-derived fluorescence signal colocalized with positive staining for cathepsins B and S as well as macrophages (Figure 3A, 3B, 3C, and 3E). In the channel for the CLIO-VT750 emission, the signal also colocalized with macrophages (Figure 3C and 3E). Neutrophils (sparse in the myocardium and accumulating mainly in the left ventricular thrombus) and nonhematopoietic cells did not colocalize with the fluorescent activity (data not shown). Acquiring images in the FITC channel facilitated the conclusion that autofluorescence contributes negligibly to the fluorescent signal in the channels specific for each probe (Figure 3F).

Macrophages: The Major Cellular Source for the Fluorescent Signal

Determining how different inflammatory cell populations contributed to the overall fluorescent signal entailed flow cytometry with single-cell suspensions of digested donor hearts. First, using specific antibodies, we separated and visualized the cell populations of monocytes/macrophages, neutrophils, and CD11b-negative cells (Figure 4A). Second,
after gating on specific cell populations, staining intensities were determined after either protease sensor or phagocytosis sensor injection (Figure 4A). At POD 7, allografts from animals injected with the phagocytosis sensor had markedly higher staining intensity and more monocytes/macrophages than did isografts, with protease sensor injection producing a similar result (Figure 4A). Third, the representation of the relative contribution of each cell population to the overall fluorescent signal illustrated that although the monocyte/macrophage population was the major cellular contributor in most groups, neutrophils and nonmyeloid cells or CD11b-negative cells also contributed to the signal (Figure 4B and 4C). The area of the pie charts indicates the total fluorescence signal in a given group and revealed a higher signal in allografts than in isografts at POD 7 (Figure 4C).

**Figure 4.** Flow cytometry shows that monocytes/macrophages (Mo/Mφ) contribute predominantly to the imaging signal. A, Dot plots show living cells from allografts (gray) and isografts (black) stained with CD11b and CD90/B220/CD49b/NK1.1/Ly-6G. Cells were identified as monocytes/macrophages (gate i), neutrophils (gate ii), and CD11b-negative cells (including lymphocytes) (gate iii). For each cell population, representative histograms depict the fluorescence intensities of the phagocytosis sensor (CLIO 680) and the protease sensor (ProSense 680) in both allografts (gray) and isografts (black). The number of dots and the height of the histogram peaks illustrate the difference in cell number per 1 mg tissue retrieved from allografts and isografts on POD 7. A negative control for each cell population (animal not injected with sensor), shown as a dashed vertical line, demarcates the border between autofluorescence and positive signal. Mean fluorescent intensities (±SD) are shown. B, Bar graphs display the cell number per 1 mg tissue, calculated as total cell numbers per 1 mg tissue multiplied by percent of gated population. Statistical analysis for each cell type was performed with 1-way ANOVA with Tukey’s multiple comparison test (*P<0.001). C, Relative contribution to the overall fluorescence for each cell population. Pie chart area corresponds to mean total imaging signal (calculated as cell number per 1 mg that is positive for the sensor). The relative contribution of each cell population is shown as percentage of overall signal and pictorially represented by the magnitude of each slice. Monocyte/macrophages dominated as a cellular contributor to the fluorescence signal.

**Allografts Exhibit Higher Overall Protease Activity**

After defining cellular and biological characteristics of the protease sensor, we explored its ability to detect the expected difference in inflammatory activity between isografts (B6 into B6) and allografts (Balb/c into B6) in vivo. We assessed the protease activity after protease sensor injection using FMT at POD 3 and 7. At POD 7, allografts showed higher fluorescence than isografts, and the signal emanating from the allografts increased from POD 3 to 7 (Figure 5A through 5C). We used 2 control groups: sham-operated mice, which showed only background signal (Figure 5C), and native hearts in the same mouse showing only minimal fluorescence (data not shown). Fluorescence measured by FMT correlated with the number of macrophages enumerated by histological examination ($R^2=0.42$, $P=0.0014$; Figure 5D) and cellular staining for cathepsins B and
Figures 5. In vivo FMT demonstrates higher protease activity in the allograft (allo) than in the isograft (iso) of mice injected with protease sensor. A, B, FMT after injection of protease sensor at POD 7, with the signal representing the quantity of fluorescence. A, The FMT signal in the isograft indicated continuing ischemia/reperfusion injury. B, In the allograft, the FMT signal exceeded that in the isograft, reflecting the inflammatory response resulting from the alloimmune reaction. C, The quantity of fluorescence detected in the volume of interest is given in picomoles. The fluorescence was 7- to 10-fold higher in heart grafts than in sham-operated mice (n=3), and graft rejection resulted in a significant difference between the isograft and allograft at POD 7. Additionally, fluorescence increased in allografts from POD 3 to 7. D, Fluorescence in picomoles detected by FMT at POD 3 and 7 in isografts and allografts correlates with numbers of immunoreactive macrophages (r²=0.42, P=0.014). E and F, Validation of in vivo imaging with FRI after injection of protease sensor at POD 7, with the signal representing counts per pixels. E, The signal in the isograft was significantly higher than in the native heart (inset shows native heart of same animal), indicating an ongoing ischemia/reperfusion injury. F, The signal in the allograft was higher than in not only the native heart (inset shows native heart of same animal) but also the isograft (E). G, The TBR was calculated from the counts per pixels of heart grafts normalized to the counts of native hearts. A significant difference of the TBR between isograft and allograft at POD 7 and an increase in TBR in allografts from POD 3 to 7 confirmed the in vivo findings (A through C). Additionally, allografts had higher TBR than isografts at POD 3. HPF indicates high-power field.

Imaging Signal Intensity in Allografts Reports on Magnitude of Allograft Rejection

To demonstrate that the signal intensity (picomole fluorescence for FMT and CNR for MRI) arising from in vivo imaging approaches reflects the severity of the immune response, we compared protease activity and phagocytosis in allografts of B6 wild-type recipients at POD 3 and 7 with grafts implanted into genetically immunodeficient RAG1−/− mice, which lack mature T and B cells. In vivo assessment by FMT showed not only increasing fluorescence in B6 wild-type recipients from POD 3 to POD 7 but also a lower imaging signal in RAG1−/− recipients than in B6 wild-type recipients at POD 7 (Figure 7A). Similarly, evaluating the phagocytic activity with in vivo MRI showed that CNR of allografts in B6 wild-type recipients intensified with time, whereas grafts in RAG1−/− recipients had a lower CNR than those in B6 wild-type recipients at POD 7 (Figure 7B). Ex vivo FRI corroborated these findings after protease sensor (Figure 7C) and phagocytosis sensor injection (Figure 7D). Likewise, for both probes, flow cytometry (Figure 7E) and immunohistochemical study (Figure 7F through 7H) validated these imaging results. Finally, to further link the in vivo imaging signal to the magnitude of rejection, we show correlations between phagocytosis as assessed by MRI and number of macrophages obtained from histological enumeration (Figure 7I; r²=0.85, P=0.001) and between protease activity as assessed by FMT and cellular cathepsin B staining (Figure 7J; r²=0.33, P=0.006).

Discussion

The clinical diagnosis of cardiac allograft rejection currently requires invasive biopsy and histological examination based on morphological rather than functional criteria. Macro-
phagocytosis, increasingly recognized as key inflammatory amplifiers in T-cell–driven organ rejection, make up a large part in grafts of immunodeficient hosts. A, The fluorescence (pmol) detected by in vivo FMT increased in allografts of B6 wild-type (WT) recipients from POD 3 to 7 and decreased in RAG1−/− recipients compared with B6 wild-type recipients at POD 7. The number of monocyte/macrophages per 1 mg tissue was calculated as total cell numbers per 1 mg tissue multiplied by percent of gated monocyte/macrophage population. B, The CNR resulting from the negative signal enhancement detected by MRI intensified in grafts of B6 wild-type recipients from POD 3 to 7 and diminished in RAG1−/− recipients compared with B6 wild-type recipients at POD 7. C and D, MRI correlated with the number of macrophages as assessed by histology. E, Flow cytometry corroborated imaging findings revealing a gradually increasing number of macrophages (Mφ) in allografts and a substantially reduced number of macrophages in RAG1−/− allograft recipients compared with B6 wild-type recipients at POD 7. The number of monocyte/macrophages per 1 mg tissue was calculated as total cell numbers per 1 mg tissue multiplied by percent of gated monocyte/macrophage population. F through H, The number of cells per high-magnification field that were positive for a macrophage marker (F), cathepsin B (G), and cathepsin S (H) increased in allografts with time and were lower in RAG1−/− recipients than in B6 wild-type recipients at POD 7. I and J, Detection of phagocytosis by FMT and MRI detect increasing protease and phagocytic activity in allografts and attenuated rejection in allografts of immunodeficient hosts. A, ECG with electrodes on the front and back paws shows QRS complexes and comparable heart rates of both native and transplanted hearts. However, this combined trace would not allow triggering MRI acquisition to the graft. B, ECG with electrodes attached to hind limbs reports activity of transplanted heart only. C, Long-axis view of the transplanted heart in an intra-abdominal location, with the dotted line indicating the position of the short-axis view in D. E through G, T2*-weighted MRI after phagocytosis sensor injection resulted in negative signal enhancement in the grafted heart, with the signal represented as CNR. E and F, At POD 7, the contrast was stronger in the allograft (F) than in the isograft (E). G, Quantitatively, the CNR was significantly higher in allografts than in isografts at POD 7, and the CNR increased in allografts from POD 3 to POD 7. H through J, FRI confirmed the in vivo imaging findings that the fluorescence intensity of the heart graft normalized to native heart in the same animal increased in B6 wild-type allograft recipients compared with B6 wild-type recipients at POD 7, illustrating the ability of the FMT to detect the severity of the rejection. B, The CNR resulting from the negative signal enhancement detected by MRI intensified in grafts of B6 wild-type recipients from POD 3 to 7 and diminished in RAG1−/− recipients compared with B6 wild-type recipients at POD 7. C and D, FRI confirmed the in vivo imaging findings that the fluorescence intensity of the heart graft normalized to native heart in the same animal increased in B6 wild-type allograft recipients with time and diminished in the RAG1−/− allograft recipients at POD 7 after protease sensor (C) and phagocytosis marker (D) injection. E, Flow cytometry corroborated imaging findings revealing a gradually increasing number of macrophages (Mφ) in allografts and a substantially reduced number of macrophages in RAG1−/− allograft recipients compared with B6 wild-type recipients at POD 7. The number of monocyte/macrophages per 1 mg tissue was calculated as total cell numbers per 1 mg tissue multiplied by percent of gated monocyte/macrophage population. F through H, The number of cells per high-magnification field that were positive for a macrophage marker (F), cathepsin B (G), and cathepsin S (H) increased in allografts with time and were lower in RAG1−/− recipients than in B6 wild-type recipients at POD 7. I and J, Detection of phagocytosis by MRI correlated with the number of macrophages as assessed by histology ($r^2=0.85, P<0.001$), and in vivo protease activity correlated with cellular staining of cathepsin B ($r=0.33, P=0.006$). Correlations included allografts in B6 wild-type recipients on POD 3 and 7 and RAG1−/− recipients on POD 7. HPF indicates high-power field.
tion of parenchymal rejection, and these imaging approaches that interrogate macrophage functions during rejection could permit investigative evaluation of novel therapeutic strategies and the longitudinal assessment of individualized immunosuppressive regimens.

In the classic sequence of inflammatory cell accumulation in solid organ allografts, neutrophils arrive early and disappear first, followed by macrophages, present throughout the life of the graft, in the company of the CD4 and CD8 T lymphocytes. Although the presence of different cathepsins in animal and clinical studies in transplantation research has been reported, their roles remain largely unknown. In our study, immunostaining defined not only the differences in macrophage accumulation and cathepsin staining between isografts and allografts but also the relative abundance of macrophages in allografts, highlighting their appeal as target cells.

To explore the cellular and molecular distribution of the protease sensor and the phagocytosis sensor in the grafted hearts, we took advantage of their fluorescent moieties. The fluorescent signal of both probes colocalized microscopically with cathepsins B and S as well as Mac-3 staining, suggesting that macrophages predominantly activate the protease sensor and internalize the nanoparticle. Stronger fluorescence emanated from the protease sensor than from the phagocytosis sensor, possibly because 1 molecule of protease can cleave multiple molecules of the protease sensor, resulting in signal amplification. To profile the cellular signal distribution, we performed flow cytometry, which established macrophages as the major cellular contributor to the imaging signal for both probes. As previously reported, the contribution of neutrophils decreased over time with waning ischemia-related injury. CD11b-negative or nonmyeloid cell populations contributed negligibly to the signal (between 5% and 7% in allografts at POD7). This heterogeneous cell population includes not only lymphocytes but most likely also stromal vascular cells, and low uptake of this phagocytosis reporter by non-“professional” phagocytes can occur. Finally, the fluorescent features of both probes revealed that although the protease sensor seems to target macrophages more specifically than the phagocytosis sensor (81% versus 62%, respectively), the difference in signal between allografts and isografts, although significant for both, is more pronounced with the phagocytosis sensor. Taken together, fluorescence microscopy and flow cytometry illustrate how fluorescent nanoparticles facilitate better understanding of the exact cellular imaging signal source.

After evaluating the biological behavior of both probes during parenchymal rejection of heart grafts, we used fluorescence tomography. This technique allows a volumetric reconstruction of the source of fluorescent light in the intact, living animal. In our study, FMT enabled the in vivo detection of protease activity, deploying a 3-dimensional map of macrophage function. Compared with the prevailing standard of histological appearance, the protease activity detected by FMT correlates well with immunoreactive macrophage staining. High background signal caused by the biliary excretion of the phagocytosis sensor and lower average target signal intensities explain in part the difficulties of detecting significant differences in FMT signals between isografts and allografts heterotopically implanted in the abdominal cavity. We overcame this problem with MRI of phagocytosis sensor uptake, which provides superior spatial resolution and soft tissue contrast.

Magnetic nanoparticles detect rejection by MRI, serving as blood pool agents for perfusion imaging or in delayed MRI. Comparable to previous studies, MRI resolved higher phagocytosis sensor uptake in allografts. No significant signal difference between allograft and isograft occurred 3 days after transplantation, when the inflammation resulting from the ischemia/reperfusion injury in both isograft and allograft predominates the effect of the gradually increasing alloimmune response in the allograft.

To evaluate the ability of the imaging approach to report on the severity of the immunologically mediated injury, we used cardiac transplantation into RAG1−/− mice recipients. These mice lack mature T and B lymphocytes, and totally mismatched allografts do not undergo rejection. Noninvasive macrophage-targeted imaging with either probe detected not only a gradual signal increase in allografts but also lower macrophage accumulation in hearts grafted into RAG1−/− recipients compared with B6 recipients. In addition, the signal measured by both FMT and MRI correlated with the prevailing standard of histological appearance. Beyond demonstrating the ability of noninvasive imaging to report on the magnitude of the allogeneic immune response, these results illustrate the potential to study loss- or gain-of-gene functions by imaging in mice rather than in transplanted rat hearts as in previous reports.

Iron oxide nanoparticles with coatings comparable to the preparation used in this study are already in clinical use (ferumoxides) or trials (ferumoxtran, ferumoxytol) for liver and macrophage-targeted imaging in cancer and atherosclerosis. Therefore, the MRI findings presented here should be readily translatable. Clinical FMT imaging is limited by depth and, given the physical limits, is unlikely to cover the entire human heart. However, recent developments in catheter design allowed probing protease activity in vivo with intravascular access. This approach might serve to guide biopsies in patients and thus reduce the sampling error currently associated with fluoroscopically guided procedures.

Conclusions

The present study demonstrates the potential of quantifying myocardial macrophage content and function in vivo. The imaging tools described here could facilitate the search for new immunosuppressive and tolerogenic therapies, and its application might improve clinical graft surveillance.

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CLINICAL PERSPECTIVE
Since the advent of effective immunosuppressive regimens, cardiac transplantation has become a routine treatment of end-stage heart failure. Acute rejection episodes occur in most heart transplant recipients during the first year after surgery, and their severity and frequency seem to predict the long-term survival of the graft. Thus, rapid and precise detection of graft rejection is vital. Repetitive invasive endomyocardial biopsies remain the most commonly performed diagnostic test but are invasive, increase morbidity, and have a considerable sampling error. The development of a noninvasive yet quantitative diagnostic tool to identify graft rejection would significantly improve the care of heart transplant recipients. Assessing macrophage-host responses by the use of recently developed molecular and cellular imaging techniques could provide a noninvasive alternative. As a result of the uncertainty about which macrophage functions provide the most useful markers for detecting parenchymal rejection, we compared 2 imaging probes reporting on key macrophage functions, protease activity and phagocytosis. In vivo imaging with both probes provided a functional 3-dimensional map of macrophage accumulation, with the signal intensity correlated to the severity of graft rejection. The imaging approaches described here could advance the investigation of novel therapeutic strategies and the longitudinal assessment of individualized immunosuppressive regimens, and their application may well facilitate the monitoring of transplant recipients.