Dual Channel Optical Tomographic Imaging of Leukocyte Recruitment and Protease Activity in the Healing Myocardial Infarct

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Abstract—Inflammatory responses after myocardial infarction profoundly impact tissue repair. Yet, efficient tools to serially and noninvasively assess cellular and molecular functions in postinfarct inflammation are lacking. Here we use multichannel fluorescent molecular tomography (FMT) for spatiotemporal resolution of phagocytic and proteolytic activities mediated by macrophages and neutrophils in murine infarcts. We performed FMT imaging to compare the course of efficient and impaired healing in wild-type and FXIII−/− mice, respectively. Mice subjected to coronary ligation received simultaneous injections with Prosense-680, an activatable fluorescence sensor reporting on cathepsin activity, and CLIO-VT750, a magneto-fluorescent nanoparticle for imaging of phagocyte recruitment. On FMT, Prosense-680 infarct signal was 19-fold higher than background \( P < 0.05 \). Protease activity was higher in the infarcted lateral wall than in the remote, uninjured septum on ex vivo fluorescence reflectance imaging (contrast to noise ratio 118±24). CLIO-VT750 FMT signal coregistered with contrast enhancement in the hypokinetic infarct on MRI. Microscopic fluorescence signal colocalized with immunoreactive staining for cathepsin, macrophages and neutrophils. Flow cytometry of digested infarcts revealed monocytes/macrophages and neutrophils as the source of the fluorescence signal. Phagocytic activity peaked on day 6, and proteolytic activity peaked on day 4 after myocardial infarction. FMT detected impaired recruitment of phagocytes and protease activity in FXIII−/− mice \( P < 0.05 \). FMT is a promising noninvasive molecular imaging approach to characterize infarct healing. Spectrally resolved imaging agents allow for simultaneous assessment of key processes of in vivo cellular functions. Specifically, we show that in vivo FMT detects impaired healing in FXIII−/− mice. (Circ Res. 2007;100:1218-1225.)

Key Words: molecular imaging ■ myocardial infarction ■ inflammation ■ cathepsin ■ Factor XIII

Myocardial infarction (MI) triggers a local and systemic inflammatory response characterized by recruitment of neutrophils and macrophages into the injured myocardium. These phagocytic cells clear away necrotic debris and orchestrate infarct healing and consequent left ventricular remodeling.1–7 Inefficient infarct healing leads to infarct expansion and heart failure, an event with high prevalence and mortality. Because infarct healing likely depends on timely and efficient leukocyte recruitment, understanding dynamics of this recruitment may improve prognosis.1,4,5,8 Leukocytes that invade infarct regions actively control the myocardial extracellular matrix by secreting an array of proteinases (eg, matrix metalloproteinases, MMPs)9 and lysosomal proteases (eg, cathepsins).10–12 For instance, MMP-9 activity increases in infarcts after MI13 and participates in myocardial wound healing.2 Breakdown of extracellular matrix also requires cathepsin, a prominent protease of the cysteine family that is upregulated in rats and dogs after MI10–12 and in patients with dilated cardiomyopathy.14 Efficient cardiac repair after MI involves temporally regulated protease activity for sequential inflammation, new tissue formation and tissue remodeling.3 Yet, these findings are based on in vitro and ex vivo experimental approaches, as tools for directly investigating these events in vivo are missing.

Fluorescence-mediated molecular tomography (FMT) imaging has facilitated in vivo molecular imaging in cancer research.15–17 Successful application of this technique after MI promises to give new insights into cellular and molecular events governing the healing process and could facilitate development of more efficient therapies. Here we aimed to establish multichannel cardiac FMT as a noninvasive tool to investigate cardiac healing after coronary ligation in mice. Specifically, we used dual channel imaging to assess quantitatively and spatiotemporally two key features of inflamma-
tory leukocytes in lesions: (1) recruitment of professional phagocytes based on uptake of CLIO-VT750 nanoparticles; and (2) lysosomal cathepsin B protease activity based on lys-lys cleavage of Prosense-680. CLIO-VT750 is a magneto-fluorescent iron oxide nanoparticle with fluorochromes emitting light at 780 nm and is efficiently ingested by inflammatory phagocytes.18,19 Prosense-680 is a protease-activatable fluorescence sensor based on a polymeric scaffold that allows imaging of Cathepsin B activity (and to a lesser extent cathepsins K, L, and S).20 The fully assembled Prosense-680 scaffold consists of near infrared fluorochromes, specific lys-lys peptide substrates and partially methoxypolyalated graft copolymers. Proteolytic cleavage of the scaffold releases the fluorochromes and results in extensive fluorescence generation (dequenching) at 700 nm.

After simultaneous injection of the two spectrally resolved molecular imaging agents into the same mouse we used FMT to follow the time course of leukocyte recruitment and proteolytic activity in infarcted hearts with efficient wound healing (C57BL6 wild-type). We further investigated mice with impaired wound healing (FXIII–/– mice) to assess the capacity of the tomographic approach to resolve altered phenotypes.

Materials and Methods

Mouse Model and Treatment Groups
A total of 51 C57BL6 mice (Jackson Labs, Bar Harbor, Maine), 9 FXIII–/– mice (Harlan, Germany, a gift of Dr G. Dickneite, ZLB Behring, Marburg) and 9 CBA mice (Jackson Labs) were used in this study (FMT dose ranging, validation, ischemia/reperfusion injury, and concentration of fluorochromes. After initial dose finding experiments, the following imaging agents were cojected into the tail vein 24 hours before imaging: Prosense-680 (excitation wavelength 680±10 nm, emission 700±10 nm) for imaging of cathepsin B activity, 5nmol in 150 µL PBS, and CLIO-VT750 (excitation wavelength 750±10 nm, emission 780±10 nm) for imaging of leukocyte recruitment, 15 mg of Fe/kg bodyweight. The magnetic iron oxide nanoparticles have an overall size (volume weighted) in aqueous solution of 38 nm, an R1 of 21 mmol/L sec 1, an R2 of 62 mmol/L sec -1 (37°C, 0.5 T).

In a set of validation experiments, mice were also cojected with Prosense-680 and 5nmol Angiosense-750 (excitation wavelength 750±10 nm, emission 780±10 nm, VisEn Medical) to determine the region of interest in the FMT images by using the blood pool signal from the heart. Before imaging, mice were shaved and depilated to remove all hair within the imaging region of interest, because dark hair can absorb light and interferes with optical imaging. Mice were anesthetized by inhalation of isoflurane and placed in the imaging chamber. Planar reflectance images were taken, the chamber was filled with index matching fluid (VisEn Medical) warmed to 37°C and intrinsic and fluorescent scans were obtained in two channels (Prosense-680: excitation 680 nm, emission 700 nm; CLIO-VT750 or Angiosense-750: 750/780 nm). We acquired 30 frontal slices of 0.5 mm thickness in z-direction, with an in-plane resolution of 1x1 mm. Total imaging time for acquisition in 2 channels was typically ~5 minutes. Following image acquisition, data sets were analyzed using a normalized Born forward equation22,23 to calculate fluorochrome concentration expressed in nM fluorescence per voxel. A 3D dataset was reconstructed and a volume of interest was defined in the heart region.

MRI
CLIO-VT750 is a magneto-fluorescent iron oxide nanoparticle which enhances image contrast on T2* weighted MRI. Therefore, we performed MRI to validate the source of fluorescence signal observed in FMT imaging. Bright-blood cine images were obtained with ECG and respiratory gating (SA Instruments, Stony Brook, NY) using a gradient echo FLASH-sequence on a 7 Tesla horizontal bore scanner (Phasmarcan, Bruker, Billerica, Mass). Imaging parameters were as follows: echo time (TE), 2.7 and 5 ms; 16 frames per RR interval (TR 7.0 to 10.0 ms); in-plane resolution 250×250 µm; slice thickness 1 mm; NEX 8.

Flow Cytometry
Following euthanasia, hearts were excised and infarct tissue was harvested and minced with fine scissors. Thereafter, the tissue was digested with a cocktail of collagenase I, collagenase XI, DNase I and hyaluronidase (Sigma-Aldrich, St. Louis, Mo) and shaken at 37°C for 1 hour.24 Cells were then passed through nylon mesh and centrifuged (15 minutes, 500g, 4°C). The resulting single cell suspensions were washed with HBSS supplemented with 0.2% (wt/vol) BSA and 1% (wt/vol) FCS. For visualization of macrophages, monocytes, and neutrophils, cells were incubated with a cocktail of monoclonal antibodies against T cells (CD90-PE), B cells (B220-PE), NK cells (DX5-PE and NK1.1-PE), granulocytes (Ly-6G-PE), myeloid cells (CD11b-APC), macrophages/dendritic cells (CD11c-biotin-Strep-PerCP, all from BD Biosciences, San Jose, Calif).7 Data were acquired on an LSRII (BD Biosciences) with 670/LP and 695/40 filter configuration to detect Prosense-680 and 755/LP and 780/60 to detect CLIO-VT750.

In Vivo Fluorescent Molecular Tomographic Imaging
Fluorescent molecular tomography was performed on a dual channel imaging system (VisEn Medical, Woburn, Mass), which delivers true 3D spatial information about fluorochrome distribution and concentration.13 Spatial encoding is achieved by varying the spatial location of the excitation laser, whereby fluorochromes are excited by 80 different point sources distributed over the area of interest behind the imaging chamber, and the resulting fluorescence is recorded by a CCD camera facing the mouse frontally. Light is directed through the mouse at different projections and signal is detected from multiple points of the body surface. A post processing algorithm then uses light propagation properties and the information of these 80 raw images to tomographically reconstruct the location and concentration of fluorochromes. After initial dose finding experiments, the following imaging agents were cojected into the tail vein 24 hours before imaging: Prosense-680 (excitation wavelength 680±10 nm, emission 700±10 nm) for imaging of cathepsin B activity, 5nmol in 150 µL PBS, and CLIO-VT750 (excitation wavelength 750±10 nm, emission 780±10 nm) for imaging of leukocyte recruitment, 15 mg of Fe/kg bodyweight. The magnetic iron oxide nanoparticles have an overall size (volume weighted) in aqueous solution of 38 nm, an R1 of 21 mmol/L sec 1, an R2 of 62 mmol/L sec -1 (37°C, 0.5 T).

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infrared fluorescence (NIRF) images were obtained with respective exposure times of 75 ms and 60 seconds. The infarct contrast to noise ratio (CNR) was calculated as: CNR=(infarct signal-background signal)/(standard deviation of the noise). TCT staining was performed to visualize the infarct and reliably locate the source of fluorescence on short axis rings.

**Histopathological Analysis**

Directly after euthanasia, hearts were excised and rinsed in PBS and embedded in OCT (Sakura Finetek, Torrance, Calif). Serial 6 μm thick sections were used for immunohistochemical staining of neutrophils (NIMP-R14, Abcam, Cambridge, Mass), macrophages (MAC-3, BD Pharmingen, San Diego, Calif), and cathepsin B (sc-6493, Santa Cruz biotechnology, Santa Cruz, Calif) using appropriate secondary antibodies.

**Statistics**

Results are expressed as mean±SD. Statistical comparisons among 2 groups were evaluated by Student’s t-test, and corrected by ANOVA for multiple comparisons. P<0.05 was considered to indicate statistical significance.

**Results**

**CLIO-VT750 Allows for Dual Modality FMT and MR Imaging of Phagocytes After MI**

Myocardial infarction triggers substantial phagocyte recruitment into the injured tissue, and these phagocytes ingest iron oxide nanoparticles. FMT imaging of mice with MI that received CLIO-VT750 magneto-fluorescent nanoparticles showed robust fluorescence in the heart region 4 days post infarction (Figure 1A). To validate the source of the FMT signal, we subjected the same mice to T2* weighted MR imaging. Figure 1B shows decreased signal within the hypokinetic, apical infarct region, indicating uptake of CLIO-VT750 into the scar. Furthermore, the FMT signal colocalized with the infarct on autopsy (Figure 1C).

**Dual Channel FMT Shows Increased Protease Activity in Hearts With MI**

Four days after MI, injection of the activatable protease sensor Prosense-680 resulted in strong focal fluorescence in the cardiac region on frontal FMT slices of the anterior thorax. To determine the source of protease activity, we coinjected the blood pool agent Angiosense-750 and performed dual channel FMT. The blood-pool signal from the cardiac region was fused with the 680 channel. In the merged channels, we observed colocalization of both signals, establishing that Prosense-680 was activated in the heart. The activatable near-infrared protease sensor was tested in a total of 8 mice 4 days after coronary ligation, and a mean fluorescence of 653±95 nM was detected (Figure 2A). We compared this fluorescence signal to 2 control groups, and very little signal was observed in 5 sham operated mice without infarction, that also received 5 nmol Prosense-680 (signal at 12% of MI Prosense-680 group, P<0.01, Figure 2B). Furthermore, 4 mice with MI were injected with saline without infarction, that also received 5 nmol Prosense-680 (signal at 12% of MI Prosense-680 group, P<0.01 Figure 2C). These in vivo FMT findings were corroborated by ex vivo fluorescence reflectance images from excised myocardial rings (Figure 2E and 2G). Figure 2E shows the typical nonuniform, patchy activation of the protease sensor over the infarct, and the signal was very low in the noninfarcted remote myocardium.

**In Vivo Observations by FMT Are Corroborated by Histology and Flow Cytometry**

Capitalizing on the fluorescent properties of the agents, we next aimed to determine the cellular and molecular source of signal observed in macroscopic in vivo imaging. Activated Prosense-680 in fluorescence microscopy colocalized with immunoreactive cathepsin B in adjacent sections (Figure 3A and 3D). Fluorescence signal from CLIO-VT750 colocalized with specific immunoreactive stains for macrophages and neutrophils (Figure 3B, 3E, and 3F). Autofluorescence contributed minimally to the signal observed by fluorescence microscopy. (Figure 3C).

To further validate the source of the fluorescence signal, we performed flow cytometry of cellular suspensions yielded from digested murine infarcts. Monocytes/macrophages as identified by their phenotype CD11b<sup>b</sup> (CD90/B220 DX5/NK1.1/Ly-6G)<sup>b</sup> were the major contributor to signal in both channels, however, neutrophils, CD11b<sup>b</sup> (CD90/B220 DX5/NK1.1/Ly-6G)<sup>b</sup>, also contributed to some of the intracellular signal (Figure 4, mean fluorescence intensity Prosense-680 channel: macrophages 6482±1298, neutrophils 2576±829; CLIO-VT750 channel: macrophages 1258±465, neutrophils 440±60). All other cell types (CD11b<sup>b</sup>) did not contribute to signal (Figure 4). Also, cells from an uninjected control mouse with MI exhibited negligible fluorescence intensity (Figure 4).
Time Course of Phagocytic and Proteolytic Activity After MI

To assess phagocytic ingestion of iron oxide nanoparticles and cathepsin activity as a function of time after coronary ligation we performed FMT imaging in several animals at various time points (n=3 to 6 at each time point). The highest CLIO uptake was observed on day 6 (Figure 5A), and the highest proteolytic activity occurred on day 4 after MI (Figure 5B).

Ischemia/Reperfusion Injury

Compared with permanent ligation, 30 minutes of ischemia followed by reperfusion resulted in comparable phagocytic and proteolytic activity on day 2 post surgery (Figure 5C and 5D).

In Vivo FMT Detects Impaired Inflammatory Response in FXIII Deficiency

FXIII−/− mice rupture their infarcts starting on day 3 after MI because of poor infarct healing. We therefore performed dual channel FMT to compare recruitment of phagocytes and protease activity in 6 FXIII−/− mice and 6 CBA wild-type mice on day 2 after MI. In FXIII−/− mice, a significantly decreased fluorescence signal was detected in both channels (Figure 6). Ex vivo validation by immunohistochemistry also revealed less cathepsin B, macrophages and neutrophils in FXIII−/− than in wild-type mice (Figure 7). Quantitative flow cytometry analysis of cell suspensions from explanted hearts showed a 56% decreased neutrophil recruitment (Figure 8A) and 54% decreased macrophage recruitment (Figure 8B). These results confirm impaired recruitment of phagocytes into injured hearts of FXIII-deficient mice as identified by FMT. Furthermore, neutrophils (but not macrophages) in FXIII−/− mice showed a somewhat reduced phagocytic activity (Figure 8C) and cathepsin activity (Figure 8D), that may also have contributed to lower FMT signals.

Discussion

Dual channel optical fluorescence tomography resolves in vivo protease activity and phagocyte recruitment in the heart. This novel approach promises to be valuable for investigation of myocardial injury and repair. We report that 1) neutrophils and macrophages but not nonmyeloid cells ingest CLIO-VT750 magneto-fluorescent nanoparticles and therefore are

Figure 2. Prosense-680 is specifically activated in the infarct. A, FMT of mouse with myocardial infarction after injection of Prosense-680. In this maximum intensity projection, focal signal can be observed in the heart region. Panels A through C are windowed identically. B, FMT of mouse without myocardial infarction (sham surgery involves thoracotomy only), 24 hour after injection of Prosense-680. Very little fluorescent signal is detected in the heart region, because no cathepsin activity occurs in the uninjured heart, to activate Prosense-680. C, FMT of mouse with myocardial infarction after injection of saline. This experiment demonstrates that the autofluorescence background signal is negligible. D, Quantification of Prosense-680 activation 4 days after MI. The signal is 19-fold higher than in saline injected MI, and 8-fold higher than in sham operated controls. E, Fluorescence reflectance image of excised heart 4 days after MI. The fusion of fluorescence image with the white light image shows Prosense activation mainly in the thin infarct scar (top). Almost no signal is observed in the remote myocardium in the septum and right ventricle (bottom). The infarct can be readily identified as the unstained, pale area in the TTC stain (f). G, The contrast to noise ratio of excised hearts calculated from fluorescence reflectance images shows significantly higher CNR in the infarcted mice after Prosense-680 injection. *P<0.01

Figure 3. Histological distribution of fluorescence signal. A through C, Fluorescence microscopy was performed on the same section in different wavelengths. A, The protease sensor Prosense-680 is activated in the infarct. B, The overall CLIO-VT750 signal distribution resembles the activation of the protease sensor Prosense-680. C, Acquisition in the FITC channel demonstrates little contribution of autofluorescence to the signal in A and B. D, IHC for Cathepsin B presence colocalizes with Prosense-680 activation (A). E and F, Immunohistochemistry for macrophages and neutrophils colocalizes with Cathepsin B presence and fluorescence in A and B. Both cell types ingest CLIO-VT750 and produce proteases which cleave Prosense-680. Magnification ×200.
selectively detected by FMT. 2) Recruited neutrophils and macrophages exclusively contribute to cysteine protease activity as identified by lys-lys cleavage of the protease-activatable sensor Prosense-680. Cystine protease activity was found to be increased 8-fold in the infarcted areas. 3) The sensitivity of the FMT approach allows one to assess the time course of phagocytic and protease activity after MI, to compare the impact of ischemia/reperfusion injury to permanent coronary ligation, and to noninvasively detect impaired recruitment of neutrophils and macrophages and decreased local protease activity in FXIII−/− mice, a model of deficient infarct healing.

Coregistration of FMT with MRI was facilitated by a hybrid magneto-optical nanoparticle and verified the infarct as the origin of the fluorescent signal (Figure 1). In addition, we validated the heart as the FMT signal source by showing that the Prosense-680 activation colocalized with the cardiac blood-pool signal of Angiosense-750. Fluorescence microscopy, immunohistochemistry and flow cytometry corroborated the results at microscopic and cellular levels. Flow cytometry established not only macrophages, but also neutrophils as the cellular source of signal in both channels. Although macrophages dominantly ingested CLIO-VT750 nanoparticles as described before, we also found considerable uptake of nanoparticles by neutrophils. Likewise, macrophages and neutrophils ingest and activate Prosense-680, indicating intracellular cystein protease activity. Of note, neutrophils have recently been identified as a source of MMP-9. For certain applications that aim to interrogate one cell type only, future development of agents based on nanoparticle library screens may provide for phagocytic cell type specificity.

Figure 4. Flow cytometry reveals macrophages and neutrophils activate Prosense-680 and internalize CLIO-VT750. Flow cytometry was performed on digested infarcts. The panel on the left shows the placement of gates within all living cells: (i) neutrophil gate, (ii) macrophages/monocyte gate, and (iii) all other cell types. Panels on the right show the histograms with respective intracellular signal in the Prosense and CLIO channel. Shaded areas were acquired in a mouse that was injected with Prosense-680 and CLIO-VT750, the non-shaded data were acquired in an uninjected control mouse and depict autofluorescence background. The major signal contribution to both channels derives from the monocyte/macrophage cell type, however, neutrophils also contribute to the signal. All other cell types do not contribute to the fluorescence signal.

Figure 5. Time course and comparison to ischemia/reperfusion injury. A, Time course of CLIO-uptake after coronary ligation as assessed by FMT. The signal in the 750 channel peaks on day 6 after coronary ligation. Three to 6 mice were studied per time point. B, Time course of cathepsin activity as assessed by activation of the protease sensor Prosense-680 by FMT. The signal peaks on day 4 after coronary ligation. Three to 6 mice were studied per time point. C and D, Comparison of permanent coronary ligation to 30 minutes of ischemia followed by reperfusion. Both, phagocytic (C) and protease activity (D) were not significantly different between MI and ischemia/reperfusion on day 2 after surgery.
enon: 1) Prosense-680 is also activated by other proteases than cathepsin B, and 2) the fluorescence image takes advantage of signal amplification, because one molecule of protease can cleave multiple Prosense-680 molecules.

During the myocardial response to ischemia, a profound macrophage infiltration follows the invasion of neutrophils. Monocytes, triggered by chemokines like MCP-1 (corresponding receptor on monocytes: CCR-2) and expression of adhesion molecules, infiltrate the injured zone and differentiate into macrophages.3 This phagocyte recruitment is assessed noninvasively by CLIO-VT750 uptake with FMT. The peak uptake was detected on day 6 after MI, which is in accordance with previous histological assessments.3,28–30

Macrophages degrade the preexisting extracellular matrix, thus creating room for a newly forming collagen scaffold. They secrete proteases such as cathepsins and metalloproteinases to degrade the extracellular matrix and also to facilitate their migration through the inflamed tissue. These enzymes have been shown to have a major role in the healing process,2 and it appears that although macrophage activity, including the secretion of proteinases, can be detrimental,1,2 this activity is nevertheless necessary, at least at a basic level, to promote healing. In our study, the maximal cathepsin activity occurred on day 4. Previously, MMP-2 and 9, proteases also involved in infarct healing, were reported to peak on day 7 after MI.13

We used FMT to investigate FXIII−/− mice, an established model of impaired wound healing.6,31 These mice rupture their infarcts because of poor healing on day 3 to 5 after coronary ligation.6 Using in vivo FMT, we found significantly diminished phagocyte recruitment in FXIII−/− mice. Flow cytometric analysis of digested infarcts and immunohistochemistry staining validated these data. Future experiments will establish the mechanisms involved in impaired inflammatory responses. FXIII−/− mice may have decreased MCP-1 levels or altered monocytic responses to chemokine signals. Interestingly, intracellular FXIII promotes monocyte recruitment to inflamed lesions by crosslinking agonist-induced AT1-receptors in a mouse model of atherosclerosis.32 We also detected reduced neutrophil recruitment in infarcts of FXIII−/− mice (Figure 8A). Previously, a lower number of granulocytes has been reported using histology.6 The capacity of FMT to detect reduced cell recruitment and protease activity in FXIII−/− mice underline its potential for cellular and molecular imaging during myocardial healing.

Previous studies3,28–30 suggest that optimal infarct healing is characterized by a delicate, multifactorial balance. We believe that noninvasive molecular imaging tools such as multichannel FMT are needed to monitor biological activities regulating the healing process.33 Clinically, it is desirable to achieve optimized healing to form a sufficient scar and to prevent heart failure. From a perspective of personalized

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**Figure 6.** Dual channel FMT detects impaired protease activity and leukocyte recruitment in FXIII−/− mice. A and B, D and E. Maximum intensity projection of FMT signal in wild-type and FXIII−/− mice (both genotypes windowed identically). C and F. In FXIII−/− mice, fluorescence signal was significantly diminished in both channels, consistent with decreased protease activity and leukocyte recruitment. *P < 0.05

**Figure 7.** Immunohistochemistry confirms FMT results of decreased neutrophil recruitment and protease activity in FXIII−/− mice. Upper panel (A through C), Infarct area of wild-type mice, 2 days after coronary ligation, lower panel (D through F) depicts staining in FXIII−/−. In FXIII deficient mice, staining for cathepsin B, neutrophils and macrophages is decreased when compared with CBA wild-type mice. Magnification ×200.
medicine, molecular imaging tools may enable physicians to control the healing process in patients individually.

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**Disclosures**

Dr Weissleder is a shareholder of VisEn Medical in Woburn, Mass.

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