Imaging Granzyme B Activity Assesses Immune-Mediated Myocarditis

Masanori Konishi,* S. Sibel Erdem,* Ralph Weissleder, Andrew H. Lichtman, Jason R. McCarthy, Peter Libby

Rationale: The development of molecular imaging approaches that assess specific immunopathologic mechanisms can advance the study of myocarditis.

Objective: This study validates a novel molecular imaging tool that enables the in vivo visualization of granzyme B activity, a major effector of cytotoxic CD8+ T lymphocytes.

Methods and Results: We synthesized and optimized a fluorogenic substrate capable of reporting on granzyme B activity and examined its specificity ex vivo in mice hearts with experimental cytotoxic CD8+ T lymphocyte-mediated myocarditis using fluorescence reflectance imaging, validated by histological examination. In vivo experiments localized granzyme B activity in hearts with acute myocarditis monitored by fluorescent molecular tomography in conjunction with coregistered computed tomographic imaging. A model anti-inflammatory intervention (dexamethasone administration) in vivo reduced granzyme B activity (vehicle versus dexamethasone: 504±263 versus 194±77 fluorescence intensities in hearts; P<0.002).

Conclusions: Molecular imaging of granzyme B activity can visualize T cell–mediated myocardial injury and monitor the response to an anti-inflammatory intervention.

(Circ Res. 2015;117:502-512. DOI: 10.1161/CIRCRESAHA.115.306364.)

Key Words: dexamethasone ■ granzyme ■ immunology ■ molecular imaging ■ myocarditis

The diagnosis and treatment of immune-mediated myocarditis and the rejection of cardiac allografts remain clinical challenges. Myocardioctyolysis mediated by CD8+ T cells contributes to viral and autoimmune myocarditis, and to acute allograft rejection. The current clinical standard of repetitive invasive endomyocardial biopsies can entail discomfort for patients, sampling error, and risks of serious complications, including perforation and pericardial tamponade.1 Existing imaging techniques for myocarditis detection include echocardiography,2 nuclear imaging with gallium-67 or indium-111–labeled antimyosin antibodies,3,4 and magnetic resonance imaging. The ability to visualize specific molecular targets5-6 could provide quantitative imaging tools to assess the cellular and molecular functions of myocarditis. Recently developed probes can assess different biological functions, such as phagocytosis and protease activity in atherosclerotic lesions and infarcted hearts, using both fluorescence reflectance imaging (FRI) and fluorescent molecular tomography in conjunction with coregistered computed tomographic (FMT-CT) imaging.7-9 This study uses a newly developed molecular probe that detects the cytotoxic T cell effector molecule granzyme B to assess acute myocarditis mediated by antigen-specific CD8+ T cells. The transgenic mouse strain CMy-mOva expresses ovalbumin (Ova) in cardiac myocytes.10 Adoptive transfer of T cell receptor transgenic Ova peptide (SIINFEKL)-specific CD8+ T cells to CMy-mOva transgenic (CMy-Tg) mice induces progressive myocarditis of varying severity, depending on the number of T cells transferred.11 This type of experimental myocarditis involves a fundamental mechanism implicated in many kinds of human myocarditis.

In This Issue, see p 483

Granzyme B released from CD8+ T cells induces apoptotic death of target cells by caspase-dependent mechanisms.12 Sustained expression of granzyme B in myocarditis indicates ongoing immunologic myocardial cell damage.13 Cellular and humoral immunity probably trigger the long-term sequelae of many forms of myocarditis, therefore, suggesting immunosuppression...
Nonstandard Abbreviations and Acronyms

CMy-Tg mice CMy-mOva transgenic mice
EAM experimental autoimmune myocarditis
FMT-CT fluorescent molecular tomography in conjunction with coregistered computed tomographic imaging
FRI fluorescence reflectance imaging
WT wild-type

as a treatment. Yet, the systemic administration of many immunosuppressive agents has yielded mixed results. The lack of tools that provide reliable monitoring of effector mechanisms limits the development and evaluation of novel therapies.

Methods

Granzyme B-Sensitive Nanoprobe Synthesis

General

All chemicals and solvents were purchased from Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO) and used as received without purification, with the exception of mPEG-succinimidyl succinate (5000 MW) that was purchased from Laysan Bio (Arab, AL). Peptides were received on resin from the MGH Peptide/Protein Core Facility, and were synthesized using Fmoc chemistries on resin amide resin. CyAl5.5B was synthesized as described previously.15 The polylysine graft copolymer was synthesized as described previously. The loading of methoxy polyethylene glycol was quantified by nuclear magnetic resonance spectroscopy as 32%. UV-vis spectra were recorded on a Varian Cary 50 UV-vis spectrophotometer (Palo Alto, CA). Fluorescence data were collected with a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA). Liquid chromatography–mass spectrometry data were collected with a Waters 2695 high-performance liquid chromatography system (Milford, MA) equipped with a 2996 diode array detector, a Micromass ZQ4000 ESI-MS module, and an Agilent Pursuit XRS 100b×2.0 mm column at a flow rate of 0.3 mL/min. Gradients were run with buffer A (H2O/0.1% trifluoroacetic acid) and buffer B (90% acetonitrile/10% H2O/0.1% trifluoroacetic acid). For analytic high-performance liquid chromatography, a C-18 reverse phase column (Agilent Pursuit XRS 10 mm) was used at a flow rate of 2695 mL/min. Trifluoroacetic acid (TFA) was used as the mobile phase.

Dye Modification of Peptide Resin

The succinimidyl ester of CyAl5.5B (2 equiv, 54 mg) and triethylamine (4 equiv, 17 μL) was added to the granzyme B-sensitive peptide (GIEFDSGGC) on resin (96 mg resin, 3×10−5 mol) in 1.5 mL N,N-dimethylformamide. The reaction was allowed to proceed for 16 hours, at which point the resin was washed 3× with dimethylformamide and 3× with methanol. The peptide was subsequently cleaved from the resin by reaction with 3 mL of a mixture of trifluoroacetic acid/triisopropylsilane/water (95/2.5/2.5) for 2 hours, followed by filtration to remove the resin. The product was precipitated from the solution via the addition of 12 mL methyl tert-butyl ether. The precipitate was recovered by centrifugation and washed twice more with methyl tert-butyl ether. The product was purified by high-performance liquid chromatography using a gradient of 50% buffer A to 0% buffer A during 32 minutes, observing at 640 nm (retention time=8.3 minutes). All fractions containing the pure product were combined and lyophilized to give a blue powder.

+ESI-MS (30 V, CH3CN0.1% trifluoroacetic acid) m/z=1663.8 (M+).

Granzyme B-Sensitive Probe Synthesis

To 16-mg polylysine graft copolymer in 3-mL phosphate-buffered saline (PBS, 1×) was added excess N-succinimidyl iodoacetate (27 mg, 9.5×10−3 mol) in 3-mL dimethyl sulfoxide. The reaction was allowed to proceed for 4 hours, at which time it was diluted to 60 mL with PBS and concentrated using centrifugal filtration (Amicon Ultra-15, 100 kDa cut off). The solution was washed a further 3× with PBS and then diluted to 15 mL, also with PBS. The dye-labeled peptide (40 mg, 2.3×10−3 mol) in 3-mL dimethylformamide/dimethyl sulfoxide/PBS (1/1/1) was added to this solution. This brought the total volume to 20 mL by the addition of 5 mL of PBS, and the reaction was allowed to proceed for 16 hours. On completion, the product was purified by dialysis against distilled water (Spectra/Por 3, 3500 kDa cut off). After dialysis, the solution was lyophilized to give the final product.

Experimental Cytotoxic CD8+ T Lymphocyte–Mediated Myocarditis and Study Protocols

OT-1 cytotoxic T lymphocytes were suspended in PBS and injected intraperitoneally into CMy-mOva transgenic (CMy-Tg) mice and wild-type (WT) C57BL/6 mice (10- to 12-week-old male). In a survival study, CMy-Tg and WT mice received 2.0, 3.5, or 5.0×106 CD8+ OT-1 T cells. Their survival was monitored for 28 days. To evaluate a model anti-inflammatory intervention, half of the CMy-Tg or WT mice received intraperitoneal injections of either dexamethasone (No. D2915, Sigma-Aldrich) or PBS (each group: n=8). Dexamethasone was dissolved in sterile PBS immediately before use and injected at a volume of 100 μL and a concentration of 0.75 mg/kg once a day for 4 days. The control mice received an equivalent volume of PBS. In the granzyme B expression study, CMy-Tg mice and WT mice received 3.5×106 CD8+ T cells, and the samples were isolated from euthanized mice after 0, 3, 5, or 7 days (each group: n=6). In an imaging study, CMy-Tg mice and WT mice received 2.0, 3.5, or 5.0×106 CD8+ T cells intraperitoneally. They also received intraperitoneal injections of either dexamethasone or PBS once a day for 4 days (each group received 2.0×106 CD8+ T cells in the CMy-Tg groups: n=12, in the WT groups: n=4; each group received 3.5×106 CD8+ T cells in the CMy-Tg groups: n=12, in the WT groups: n=4; each group received 5.0×106 CD8+ T cells in the CMy-Tg groups: n=8, in the WT groups: n=4). Four days after T cell injection, mice received the granzyme B-sensitive probe intravenously. One day (24 hours) after the probe administration, anesthetized mice underwent in vivo FMT-CT imaging. The probe was dissolved in sterile PBS immediately before use (final concentration 1 μmol/L CyAl5.5B). Each mouse received 50 μL of the solution. The samples isolated from euthanized mice underwent assessment by microscopic ex vivo FRI and other methods. In a study designed to evaluate the optimum time for analysis, CMy-Tg mice and WT mice received 3.5×106 CD8+ T cells or PBS intraperitoneally (CMy-Tg group: n=5; WT group: n=5). Four days after T cell injection, mice received the granzyme B-sensitive probe intravenously. Six, 24, and 48 hours after the probe administration, the mice were euthanized in vivo and FMT-CT imaging was performed. In an imaging study with ProSense 680 (No. NEV10003, PerkinElmer, Waltham, MA), CMy-Tg mice and WT mice received 3.5×106 CD8+ T cells, and received intraperitoneal injections of PBS (CMy-Tg group: n=12; WT group: n=12). Four days after T cell injection, the mice received 2 μmol/150 μL of ProSense 680 intravenously in accordance with the manufacturer’s instructions. One day after the administration, samples isolated from euthanized mice underwent assessment by microscopic ex vivo FRI and other methods.

Macroscopic Ex Vivo FRI

For macroscopic ex vivo imaging, excited hearts and sections were visualized with a fluorescence microscope at ×4 magnification using OV-110 (Olympus, Center Valley, PA) and Image Station 4000MPPro (Kodak, Rochester, NY) after euthanasia. Near infrared fluorescence images were obtained in the 680-nm channels (excitation filter: 630 nm; emission filter: 700 nm) with progressive exposure times: for 1 minute (tissues), 10 minutes (heart sections from cytotoxic CD8+ T lymphocyte–mediated myocarditis), or 30 minutes (heart sections from experimental autoimmune myocarditis (EAM)). White images were obtained without filtration for 0.05 s. Images were analyzed using OsirIX (freeware, Geneva, Switzerland). Signal intensities
counts per pixel were measured by tracing a manual region of interest in the left ventricular myocardium, yielding average signal intensity.

Macroscopic In Vivo FMT
For in vivo imaging, mice were anesthetized (isoflurane 1.5%; O2 2 L/min). After registration, FMT-CT was performed on a dual channel imaging system (FMT 2500, VisEn Medical, Woburn, MA), which reported three-dimensional (3D) spatial information about fluorophore distribution and concentration. Total imaging time for FMT acquisition was typically 5 to 8 minutes. Data were postprocessed using a normalized Born forward equation to calculate 3D fluorophore concentration distribution. CT angiography was immediately followed by FMT to guide selection of the heart and aortic root as the region of interest. The imaging cartridge lightly immobilized the anesthetized mouse between optically translucent windows and, thereby prevented motion during transfer to the CT (Inveon PET-CT, Siemens, Erlangen, Germany). The CT x-ray source was operated at 80 kVp and 500 μA with an exposure time of 370 to 400 ms to acquire 360 projections. The effective 3D CT resolution was 80-μm isotropic. The CT reconstruction protocol was performed by bilinear interpolation, using a Shepp–Logan filter, and scaled pixels to Hounsfield units. Data were imported into OsiriX to coregister FMT and CT images. Fiducials on the imaging cartridge were visualized and tagged in FMT and CT images with point markers to define their XYZ coordinates. Using these coordinates, data were resampled, rotated and translated to match the image matrices, and finally displayed in 1 hybrid image.

Results
Development of a Fluorogenic Probe for Granzyme B
The introduction of the serine protease granzyme B into a target cell affects apoptosis via cleavage of apical (caspases 8 and 10) and executioner (caspases 3 and 7) caspases, as well as through cleavage of BH3-interacting domain death agonist. This process engendered the hypothesis that a quenched substrate with cleavage site-mimicking caspase 3 generates a fluorogenic probe that can detect granzyme B that activated cytotoxic T lymphocyte use as an effector. The detection of mouse granzyme B in experimental myocarditis requires consideration of species specificity because murine granzyme B does not readily cleave the human sequence for caspase 3. This study modified the peptide sequence GIEFDSGGC on the N terminus with Cy5.5-analogous fluorescent dye CyAl5.5B (Figure 1A). Its ease of synthesis and optimal photophysical properties render this dye ideal for the generation of a fluorogenic probe, whereas its hydrophobicity enhances the required intermolecular quenching. Conjugation of the fluorophore-labeled peptide to a polylysine graft copolymer followed. Initial functionalization of the free polylysine graft copolymer amines with succinimidyl iodoacetate, followed by reaction of the cysteine-terminated peptide, yielded the fluorogenic granzyme B nanoprobe after dialysis to remove the unreacted peptide. Ultimately, dynamic light scattering analysis of the product revealed a particle with an average diameter of 122 nm (Figure 1B).

Purified murine granzyme B enabled assessment of the fluorogenic properties of this nanoprobe (Figure 1C). The addition of granzyme B to an assay buffer containing the probe triggered a 3.7-fold increase in fluorescence intensity during the course of the experiment because of the cleavage of the peptide substrate and release of the fluorophores from the polymer backbone.

Figure 1. Synthesis and characterization of the granzyme B-sensitive fluorogenic nanoprobe. A, Modification of the poly-lysine graft copolymer and fluorescence activation: (i) succinimidyl iodoacetate; (ii) CyAl5.5B-modified GIEFDSGGC peptide; (iii) granzyme B. B, Nanoprobe size characterization via dynamic light scattering reveals an average hydrodynamic diameter of 122 nm. C, Enzymatic assay of nanoprobe activation. The probe was incubated with purified murine granzyme B (0.01 mg/mL), chymotrypsin (0.01 mg/mL), granzyme A (0.01 mg/mL), trypsin (0.01 mg/mL), or bovine serum albumin ([BSA] 0.1% in water) and the fluorescence increase was recorded over time. Fold increase vs the initial sample fluorescence (each group: n=3). The P value is compared with the granzyme B group. ***P<0.0001.
Studies to assess enzyme specificity used granzyme A, which may also be present in the biological milieu within a heart undergoing myocarditis,13 and trypsin and chymotrypsin. When incubated with granzyme A, even at a 12-fold excess of enzymatic activity, the probe did not display appreciable increase in fluorescence. Similarly, no increase was observed during incubation with the serine protease trypsin (300-fold excess). Yet, when incubated with the serine endopeptidase chymotrypsin (82-fold excess activity), with an affinity for large hydrophobic amino acids including the phenylalanine contained within the cleavage sequence, the probe demonstrated activation comparable in intensity to granzyme B.

Investigation of the use of the nanoprobe in cell culture followed. Western blot demonstrated that coculture of cardiomyocytes isolated from CMy-Tg mice with OT-1 cytotoxic T-lymphocyte cells yielded a significant increase in granzyme B and cleaved caspase 3 (Online Figure IA). Incubation of T cells with WT cardiomyocytes did not produce an increase in either of these proteases. Media from the CMy-Tg cocultures also contained granzyme B (Online Figure IB). A microplate assay using the cocultures served to investigate probe uptake and activation. Wells containing both transgenic cardiomyocytes and transgenic T cells demonstrated increases in fluorescence, whereas other wells exhibited negligible signal (Online Figure IC). Immunofluorescence microscopy permitted further investigation of the cocultures incubated with the nanoprobe. CMy-Tg cardiomyocytes incubated with the OT-1 T cells demonstrated colocalization between granzyme B and the fluorogenic nanoagent (Online Figure ID).

The Number of OT-1 CD8+ T Cells Adoptively Transferred Directly Correlates With the Lethality of Myocarditis

Although all WT mice survived, all CMy-Tg mice injected with 5.0×10^6 or 3.5×10^6 OT-1 cytotoxic T-lymphocyte cells died within 8 to 12 days. Half the CMy-Tg mice injected with 2.0×10^6 of OT-1 CD8+ T cells survived for 28 days (Figure 2A). Necropsy revealed ascites, lobulated enlarged livers that associate with hepatic congestion, and foamy lungs consistent with pulmonary edema in all mice that succumbed to myocarditis. Five and 7 days after the transfer of 3.5×10^6 OT-1 CD8+ T cells, CMy-Tg mouse hearts had higher concentrations of granzyme B and cleaved caspase 3 than WT mouse hearts (Figure 2B). CMy-Tg mouse serum also had higher concentrations of granzyme B and cardiac troponin-I than serum of WT mice (Figure 2C).

Dexamethasone Mitigates the Severity of Myocarditis

Dexamethasone (0.75 mg/kg IP) for 4 days post transfer of OT-1 cytotoxic T lymphocytes increased survival of CMy-Tg mice. Similar to WT mice, all dexamethasone-treated CMy-Tg mice injected with 2.0×10^6 OT-1 cells survived for 28 days (Figure 3A). The injection of 3.5×10^6 or 5×10^6 OT-1 cells into dexamethasone-treated CMy-Tg mice yielded a survival...
rate between 0.6 and 0.8 (Figure 3A). Untreated mice experienced a much lower survival rate (Figure 2A). CMy-Tg mouse hearts had higher protein levels of granzyme B and active caspase-3 than WT mouse hearts 5 days post transfer of 3.5×10⁶ OT-1 CD8⁺ T cells. Dexamethasone reduced these concentrations (Figure 3B). CMy-Tg mice had higher serum concentrations of granzyme B, interferon-γ, and cardiac troponin-I than WT mice 5 days post transfer of 3.5×10⁶ OT-1 CD8⁺ T cells. Dexamethasone treatment limited these increases (Figure 3C).

The hearts of CMy-Tg mice contained abundant inflammatory cells after the transfer of 3.5×10⁶ OT-1 CD8⁺ T cells (Figure 4A). CMy-Tg groups harbored considerably more CD8⁺ T cells, CD68⁺ macrophages, and NIMP-R14⁺ neutrophils evaluated by immunohistochemical examination than those from the WT groups (Figure 4B). All groups, however, revealed a similar number of scattered CD4⁺ T cells. Apoptotic cardiac myocytes detected as the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling–positive nuclei increased significantly after T cell transfer in CMy-Tg mice but not in WT mice. A reduction in inflammatory and apoptotic cells in hearts indicated that dexamethasone mitigated myocarditis (Figure 4C).

The hearts of CMy-Tg mice had higher concentrations of mRNAs that encode cytokines, mediators of apoptosis, and adhesion molecules, such as interferon-γ, tumor necrosis factor-α, interleukin-2, interleukin-6, caspase 3, caspase 8, BH3-interacting domain death agonist, Fas, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 than WT mice after the transfer of OT-1 CD8⁺ T cells. Dexamethasone limited the expression of these mediators in the hearts (Online Figure II).

In vitro, granzyme B protein concentrations in OT-1 CD8⁺ T cells (Online Figure IIIA) and their culture medium (Online Figure IIIB) increased above baseline after 48 hours of anti-CD3 stimulation. Dexamethasone did not mute this rise. Yet, granzyme B and cleaved caspase-3 protein levels in CMy-Tg cardiomyocytes cocultured with OT-1 CD8⁺ T cells (Online Figure IIIC) and granzyme B protein concentrations in their culture medium (Online Figure IIID) increased above baseline after 24 hours. Dexamethasone suppressed these levels significantly depending on the duration of incubation. Dexamethasone also decreased the probe signals that colocalized with granzyme B activity in the CMy-Tg cardiomyocytes cocultured with OT-1 CD8⁺ T cells, as assessed by confocal microscopy (Online Figure IIIE).
The Granzyme B-Sensitive Nanoprobe Reported on Myocarditis and the Effects of Dexamethasone

Twenty-four hours after the probe injection, CMy-Tg mice revealed higher signals from the probe in heart tissues and sections than WT mice in the ex vivo FRI (Figure 5A) and in vivo FMT (Figure 5B). The CT fusion images permitted the anatomic localization of the fluorescent signals within hearts. Ex vivo FRI imaging of ProSense 680 did not demonstrate a significant difference between CMy-Tg and WT mouse hearts (Online Figure IV A and IVB). The granzyme B-sensitive nanoprobe also reported on the CD8+ T cell-mediated myocardial injury of CMy-Tg mice injected with 2.0×10^6 or 5.0×10^6 OT-1 cytotoxic T lymphocytes in the in vivo FMT (Online Figure V A and VB). The fluorescent intensities correlated linearly with myocarditis grade (R^2=0.591; P<0.001; Online Figure VC). The background signals minimized 24 hours after the probe injection in both CMy-Tg and WT mice in the in vivo FMT (Online VIA and VIB). Plasma stability of the probe kept a baseline level ≤24 hours (Online Figure VIC). Immunofluorescence microscopy of heart sections permitted the colocalization of probe signals with CD8 and granzyme B expression (Figure 6). Dexamethasone reduced granzyme B probe activity, as detected in ex vivo FRI, in vivo FMT, and ex vivo fluorescence microscopy (Figure 5A, 5B, 6; Online Figure V A and VB).

The Granzyme B-Sensitive Nanoprobe Reported on Cardiac Myosin–Induced EAM

Further experiments addressed the ability of the probe to visualize a more chronic autoimmune myocarditis induced by immunization with cardiac myosin. Twenty-one days after initial immunization, Balb/cByJ mice had severe myocarditis shown by hematoxylin and eosin staining (Figure 7A), and contained more myocardial neutrophils, macrophages, and CD4+ T cells as assessed by immunohistochemical examination than WT mice (Figure 7B). In this form of...
chronic myocarditis, the heart sections had only scattered CD8+ cells, and lower amounts of granzyme B and cleaved caspase-3 protein when compared with those measured in the acute CD8+ T cell–induced myocarditis (Figure 7C).

Twenty-four hours after the probe injection, Balb/cByJ mice revealed higher signals from the probe in heart tissues and sections than WT mice in the ex vivo FRI (Figure 7D). In keeping with the histological and biochemical results, the molecular imaging signal in the chronic immune myocarditis was less pronounced than in the acute OT-1 CD8+ T cell–induced disease.

Specificity of the Granzyme B-Sensitive Nanoprobe In Vitro and In Vivo

In vitro, granzyme B protein concentrations in Balb/c WT CD8+ T cells increased above baseline after 48 hours of anti-CD3 stimulation, whereas Balb/c granzyme B–deficient CD8+ T cells did not show this rise (Online Figure VIIA). The fluorescent signal from the probe incubated with the lysates of WT CD8+ T cells stimulated with anti–CD-3 increased above baseline after 48 hours, yet decreased significantly, and in a concentration-dependent manner, when incubated with granzyme B inhibitors (Online Figure VIIIB). When incubated with the lysates of granzyme B–deficient CD8+ T cells, the nanoprobe did not demonstrate a comparable increase. In vivo, nonspecific probe activation was observed in the stomach of both WT- and granzyme B–deficient mice at 24 hours after the probe injection (Online Figure VIIIC).

Discussion

Endomyocardial biopsies assist in the clinical evaluation of acute myocarditis and the rejection of cardiac allografts. Yet this procedure entails the potential for complications and sampling errors. Current noninvasive imaging approaches to evaluating myocardial inflammation, including ultrasound and nuclear and magnetic resonance imaging, lack molecular specificity. This study developed, optimized, and validated a fluorogenic molecular imaging agent that visualizes granzyme B activity, a target directly involved in CD8+ T cell–mediated myocardioctyolysis. The inclusion of a cleavage sequence

Figure 5. Investigation of the use of the nanoprobe in CD8+ T cell–mediated myocarditis. A, Ex vivo fluorescence reflectance imaging (FRI) of the heart tissues and sections, and immunofluorescent staining (IF) of heart sections in CMy-mOva transgenic (CMy-Tg) and wild-type (WT) mice 5 days after the injection of 3.5×10^6 CD8+ T cells (each group of CMy-Tg mouse: n=12; each group of WT mice: n=4). The mice received PBS (vehicle) or dexamethasone at days 1, 2, 3, and 4 intraperitoneally and the probe at day 4 intravenously after the CD8+ T cell injection. The left panels show the white images and the right panels show the color images. The figure shows the mean signal intensities. In IF, the blue signals indicate nuclei stained with 4',6-diamidino-2-phenylindole and the red signals indicate the probe location. Closed bars indicate the vehicle control group of each mouse, and open bars indicate the dexamethasone group of each mouse. B, In vivo fluorescent molecular tomography in conjunction with coregistered computed tomographic (FMT-CT) images for CMy-Tg and WT mice 5 days after the injection of 3.5×10^6 CD8+ T cell (each group of CMy-Tg mouse: n=12; each group of WT mouse: n=4). The P value refers to comparison with the vehicle control of each group. *P<0.05, †P<0.001, §P<0.0001. IF indicates immunofluorescent staining; and RGB, red-green-blue color.
derived from caspase 3, one of the intracellular targets of the enzyme, enhances the signal produced by this imaging probe. Without activation by granzyme B, this probe displays minimal fluorescence.

The purified enzyme and medium harvested from cocultures of Ova-specific CD8+ cytotoxic T-lymphocyte cells and ovalbuin-expressing cardiomyocytes isolated from CMy-Tg mice validated the imaging agent in vitro. The incubation of probes with purified granzyme B yielded an almost 4-fold increase in fluorescence signal in solution. The incubation of probes with lysates of CD8+ T cells affirmed its selectivity for granzyme B. Cocultures of transgenic mouse cells allowed for further validation of the probe. The cardiomyocytes from the CMy-Tg mice present ovalbumin peptides in conjunction with major histocompatibility complex class-1 molecules on the cell surface, which activates the receptor on the surface of the CD8+ T lymphocytes. This process triggers the release of granzyme B, with incubation time directly correlating with the extent of release. Incubation of cocultures with the fluorogenic probe caused a concentration-dependent increase in fluorescence signal. These data corroborated the results obtained with the purified enzyme.

The amount of CD8+ T cells transferred into CMy-Tg mice related directly with myocarditis severity and lethality. The injection of fewer CD8+ T cells into CMy-Tg mice triggered the development of transient heart inflammation and recovery without apparent sequelae. The transfer of a larger number of cells, however, proved lethal. Our previous investigation demonstrated maximal cardiac damage at 96 to 144 hours after transfer.10 This study used conditions that yielded increased granzyme B expression and produced cardiac damage while minimizing mortality 5 days after transfer. In vivo FMT imaging of the time course of probe uptake, enzymatic cleavage, and clearance informed the choice of 24 hours postinjection to study the nanoprobe, a time that allows localization and activation while minimizing background signals. The nanoprobe remains stable in serum for over 24 hours without displaying activation. The injection of myocarditic mice with the probe yielded a significant fluorescence signal localized to the heart via noninvasive FMT-CT imaging, whereas control WT mice exhibited no signal. In mice with mild myocarditis, the weak fluorescence signal only covered a small region of the heart. Administration of the probe to WT or granzyme B–deficient mice yielded a

![Figure 6. Demonstration of probe specificity in CD8+ T cell–mediated myocarditis.](image)

Immunofluorescent staining in heart sections in CMy-mOva transgenic (CMy-Tg) and wild-type (WT) mice 5 days after the injection of 3.5×10^6 CD8+T cells (each group of CMy-Tg mouse: n=12; each group of WT mouse: n=4). The mice received PBS (vehicle) or dexamethasone at days 1, 2, 3, and 4 intraperitoneally and the probe at day 4 intravenously after CD8+ T cell injection. Original magnification in the upper panels is ×100. Bars indicate 200 μm. The blue signals indicate nuclei stained with 4′,6-diamidino-2-phenylindole. The green signals indicate the location of granzyme B, CD8, or NIMP-R14. The red signals indicate the location of the probe. The lower graph shows the probe colocalization with granzyme B, CD8, or NIMP-R14 positive area quantified using ImageJ software. Closed bars indicate the vehicle control group of each mouse, and open bars indicate the dexamethasone group of each mouse. *P<0.05, ‡P<0.005, §P<0.0001.
fluorescence signal in the abdomen, regardless of the presence of myocardial disease or the enzyme of interest, indicating other metabolic clearance pathways for the agent. Further investigation localized fluorescence to the stomach. The enzyme survey we conducted pointed to chymotrypsin as responsible for probe activation in the gastrointestinal tract. As demonstrated in the in vitro findings, chymotrypsin breaks down the probe, and the enzyme is present in the intestine. Acid hydrolysis may also activate the probe in the stomach. Chymotrypsin should not give rise to a signal in the cardiovascular system, rendering this activity unlikely to confound the use of the probe described here for investigation of myocarditis. FRI and fluorescence microscopy ex vivo corroborated the in vivo findings. Immunofluorescent staining of heart sections further demonstrated the colocalization of the probe signal with both granzyme B and CD8+ T cells. Although previous studies show the expression of granzyme B by neutrophils,19 the colocalization of the probe signal reported on fewer neutrophils than CD8+ T cells in this study. This study also compared the capabilities of the granzyme B probe to the previously studied protease sensor, ProSense 680, which readily visualized macrophage host responses in the setting of acute rejection of mouse heart allografts, a close parallel the CD8+ T cell–mediated myocardial injury in this study.20 The granzyme B-sensitive probe demonstrated a substantially higher signal in this model than ProSense 680. Further study should evaluate the use of this probe in acute rejection of cardiac allografts and in viral myocarditis.

This study used dexamethasone as a model anti-inflammatory agent to determine if the fluorogenic granzyme B nanoprobe can serve as a molecular monitor of therapeutic efficacy. Previous studies revealed that treatment with immunosuppressive agents may improve the prognosis of giant cell myocarditis and that early administration of dexamethasone might have utility for the treatment of fulminant viral myocarditis.
myocarditis. Dexamethasone also acts on leukocytes and endothelial cells to attenuate the leukocyte–endothelial cell interactions and reduces the generation and release of proinflammatory cytokines and mediators. In vitro, incubation of cocultures with dexamethasone limited CD8+ T cell–cardiomyocyte interactions and reduced granzyme B expression in the targeted cardiomyocytes, depending on the duration of exposure. These experiments demonstrated that dexamethasone does not modulate granzyme B expression in CD8+ T cells after anti-CD3 stimulation. The treatment of myocarditic mice with dexamethasone and the nanoprobe yielded a significant reduction in fluorescence signal within the heart in vivo when compared with control mice receiving saline. Mice receiving this agent also displayed decreased expression of inflammatory cytokines, adhesion molecules, and apoptotic pathways within the heart tissue.

Experimental cardiac myosin–induced EAM results in a more chronic form of the myocarditis. When mice bearing this disease were treated with the fluorogenic nanoprobe, longer exposure times were required to acquire an appropriate image via ex vivo FRI, indicating reduced probe activation. CD4+ T cells that do not release granzyme B mainly mediate EAM. The signal observed in hearts with EAM originates from the CD8+ T cells are 6-fold less abundant than CD4+ T cells, further affirming the specificity of this probe.

In conclusion, these studies generated and validated a novel fluorogenic probe for the detection of granzyme B activity in vivo in mouse myocarditis. The fluorescent probe should prove useful to evaluate pathogenic mechanisms and evaluate experimental therapies in mice. With respect to clinical translation, the depth dependence of fluorescence imaging currently limits the study of large animal or human hearts. Yet, the validation of granzyme B as a novel molecular imaging target presented here justifies future efforts to develop more readily translatable magnetic resonance or radionuclide methods. This study establishes the principle that a molecular target of myocarditis can enable noninvasive imaging of this process in vivo. These results point the way toward the future development of further novel tools that can investigate the mechanisms of immune-mediated cardiac processes, including acute cardiac transplant rejection, and evaluate the effects of therapeutic interventions.

Acknowledgments
We thank Chelsea Swallom for her editorial contributions.

Sources of Funding
This study was supported, in part, by National Institutes of Health National Heart, Lung, and Blood Institute contract HHSN268201000044C and R01HL121363-01. Grant-in-Aid for Scientific Research from the National Institutes of Health; Translational Program of Excellence in Nanotechnology, Japan Society for the Promotion of Science; Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation, and The Uehara Memorial Foundation; Research Fellowship.

Disclosures
None.

References
What Is Known?

- The diagnosis and treatment of immune-mediated myocarditis and the rejection of cardiac allografts remain clinical challenges.
- Endomyocardial biopsies have many drawbacks in assessing cardiac inflammation.
- The killer T-lymphocyte enzyme granzyme B participates causally in CD8+ T cell–mediated myocardiocytolysis.

What New Information Does This Article Contribute?

- This study developed, optimized, and validated a fluorogenic molecular imaging agent that visualizes granzyme B activity in mice with experimental immune-mediated myocarditis.
- The results establish that targeting granzyme B activity can enable noninvasive imaging of immune-mediated myocarditis in vivo and monitor a therapeutic intervention.

Endomyocardial biopsies assist in the clinical evaluation of acute myocarditis and the rejection of cardiac allografts. Yet this procedure risks of complications and sampling errors. Current noninvasive imaging approaches to imaging myocardial inflammation generally lack molecular specificity. This study developed, optimized, and validated a fluorogenic molecular imaging agent that visualizes granzyme B activity, a target directly involved in CD8+ T cell–mediated myocardiocytolysis. This probe emits low fluorescence in its uncleaved, quenched form, but fluorescence brightly when cleaved by granzyme B. The probe produced a signal in hearts of mice with experimental immune-mediated myocarditis that associated with the severity of the lesions. The treatment of mice exhibiting myocarditis with a conventional anti-inflammatory agent, dexamethasone, significantly reduced the cardiac granzyme B signal. This study establishes the principle that a molecular target related to a particular pathophysiologic pathway involved in immune-mediated myocarditis could enable noninvasive imaging of this process in vivo. The findings establish the feasibility of developing molecularly targeted imaging agents to investigate the mechanisms of immune-mediated cardiac diseases, and evaluate the effects of therapies.
Imaging Granzyme B Activity Assesses Immune-Mediated Myocarditis
Masanori Konishi, S. Sibel Erdem, Ralph Weissleder, Andrew H. Lichtman, Jason R. McCarthy and Peter Libby

_Circ Res._ 2015;117:502-512; originally published online July 21, 2015;
doi: 10.1161/CIRCRESAHA.115.306364
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/117/6/502

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/07/21/CIRCRESAHA.115.306364.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
1. Detailed Methods

1.1. Generation of mice

Wild-type C57BL/6 (WT) mice were purchased from the Jackson Laboratory. CMy-mOva transgenic (CMy-Tg) mice,\(^1\) which express membrane-bound ovalbumin exclusively on cardiomyocytes, were maintained on a C57BL/6 Thy1.2 (SD90.2) background. The TCR transgenic OT-1 mouse strain, in which most T cells are CD8\(^+\) and specific for Ova peptide 257-264 (SIINFEKL) bound to the class I MHC molecule H-2K\(^b\), were maintained on a C57BL/6 Thy1.1 (CD90.1) background, allowing for the distinction of the transferred CD8\(^+\) T cells from recipients with endogenous CD90.2 CD8\(^+\) T cells. The granzyme B-deficient mice were provided by Dr. Judy Lieberman (Boston Children’s Hospital, Harvard Medical School) and maintained on a Balb/c background.\(^2\) Mice consumed a low-fluorescence diet (AIN-76A Purified diet, Harlan Teklad, Madison, WI) for one week before imaging studies.

1.2. Ethical aspects

Animal care and procedures have been reviewed and approved by the Harvard Medical School Standing Committee on Animals and performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

1.3. Cardiac myosin-induced experimental autoimmune myocarditis (EAM)

Balb/cByJ male mice between 8 and 20 weeks of age (Jackson Laboratory) were immunized with a peptide derived from the murine α-myosin H chain, Myhc-α 614–634 (Ac-RSLKLMATLFSYASADR-OH; AnaSpec, Fremont, CA), as described.\(^3\) The peptide was diluted in PBS, 1 mg/mL, and emulsified 1:1 with Freund’s complete adjuvant (Sigma-Aldrich). The 100 μg of the peptide in 200 mL of the emulsion was injected subcutaneously on day 0 and day 7. The control mice were injected with PBS. Samples were harvested from mice at day 21. In the imaging study, EAM and control mice received an intravenous injection of the granzyme B probe at day 20 (the EAM group: \(n = 16\), the control group: \(n = 4\)). One day after the administration, samples isolated from euthanized mice were assessed by microscopic \textit{ex vivo} FRI and by other experimental methods. The probe was dissolved in sterile PBS immediately before use at a concentration of 1 μmol/L CyAl5.5B. Each mouse received 50 μL of the solution.

1.4. Enzymatic activation assays for murine granzyme B

In all fluorogenic assays with purified enzymes, the granzyme B nanoprobes was diluted to 10 μg/mL (optical density at 673 nm ~ 0.4) in the assay buffer (0.5 mmol/L Tris HCl, 150 mmol/L NaCl, 0.01 % Triton X-100, pH = 8.0). Concomitantly granzyme A (R&D Systems, 2905-SE, 9.0 μmol/min chromogenic substrate/mg), granzyme B (Sigma-Aldrich, SRP3202, 0.75 μM/min chromogenic substrate/mg enzyme), chymotryptsin (Sigma-Aldrich, C4129, 62 μmol/min substrate/mg), or trypsin from bovine pancreas (Sigma Aldrich, T1426, 12885 BAEE units/mg)
protein, 224 µmol/L/min chromogenic substrate/mg enzyme) were diluted to 10 µg/mL in distilled water with 0.1 % bovine serum albumin (BSA). To 250 µL of the probe, 30 µL of either the respective enzyme, water, or water with 0.1 % BSA as a control was added, and the initial fluorescence of each sample was measured (λ<sub>ex</sub> = 670 nm, λ<sub>em</sub> = 693 nm). The enzymatic reaction was allowed to proceed for 8 hours, at which point the fluorescence of each sample was measured and the fold increase for each sample was calculated. In an assay for the lysate of cytotoxic CD8<sup>+</sup> T cells, a total of 4 × 10<sup>6</sup>PBS-washed viable cells was re-suspended in 300 µL of hypotonic lysis buffer solution (0.5 mmol/L Tris HCl, 20 mmol/L NaCl, 0.01 % Triton X-100, pH = 8.0) for 10 min and sonicated on ice. Supernatant was collected following a 10-min microfuge spin (10,000 x g). The granzyme B probe was diluted to 20 µg/mL (optical density at 673 nm ~ 0.4) in the assay buffer (0.5 mmol/L Tris HCl, 150 mmol/L NaCl, 0.01 % Triton X-100, pH = 8.0). To 50 µL of the probe, 50 µL of lysate, or hypotonic lysis buffer as a control was added, and the initial fluorescence of each sample was measured (λ<sub>ex</sub> = 670 nm, λ<sub>em</sub> = 693 nm). The enzymatic reaction was allowed to proceed for 8 hours, at which point the fluorescence of each sample was measured and the fold increase for each sample was calculated. In a study designed to evaluate the plasma stability, 50 µL of the mouse plasma was added to 50 µL of the probe. The enzymatic reaction was allowed to proceed for 0, 4, 8, 12, 16, 20, 24, and 28 hours, at which point the fluorescence of each sample was measured and the fold increase for each sample was calculated.

1.5. CD8<sup>+</sup> T cell preparations

All cell cultures were placed in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 2 mmol/L Na-pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mmol/L HEPES (Invitrogen).<sup>4</sup> Spleens were harvested from OT-1 TCR transgenic mice (8 – 10 weeks, male) and single-cell suspensions were prepared by expressing spleens through a stainless-steel mesh and passing the cells through a 70-µm nylon strainer (BD Biosciences, Palo Alto, CA). The resulting suspensions were treated with Tris-ammonium chloride (TAC) buffer to lyse red blood cells, and CD8<sup>+</sup> cells were purified using a MACS CD8a (Ly-2) MicroBeads kit (Miltenyi Biotec, Auburn, CA). This protocol typically yielded >95% CD44<sup>low</sup> CD8<sup>+</sup> CD4<sup>-</sup> cells, as assessed by flow cytometry. The T cells were cultured on plate-bound anti-CD3 (#100302, Biolegend, San Diego, CA), applied at 2 µg/mL, and the medium was supplemented with 2 µg/mL anti-CD28 (BD Pharmingen, Palo Alto, CA), 50 U/mL recombinant mouse IL-2 (R&D Systems, Cambridge, MA), and 10 ng/mL recombinant mouse IL-12 (R&D Systems). The cultures were incubated at 37°C, 5 % CO<sub>2</sub>. After 3 days of stimulation, all cultures were diluted 1: 1 with fresh medium containing 40 U/mL IL-2 and differentiated OT-1 cytotoxic T lymphocytes were harvested for use at day 5.

1.6. Mouse cardiomyocyte isolation and treatment

Neonatal cardiomyocytes from 1- to 3-day-old CMY-Tg or WT mice were isolated using a commercially available kit (Thermo Scientific, Rockford, IL) and cultured in vitro, as described previously.<sup>5</sup> The cardiomyocytes were added onto 24-well or 96-well plates (Corning, Tewksbury, MA) and incubated in Eagle’s minimum essential medium (Sigma-Aldrich) supplemented with 10 % heat-inactivated FBS for 5 days at 37°C. Cardiomyocytes were
isolated from the CMy-Tg or WT mice, seeded in 24-well or 96-well plates (seeding density was $2.5 \times 10^5$ cells/cm$^2$), and divided into 6 subgroups: the control group, in which only PBS was added; the co-cultured with OT-1 CD8$^+$ T cells group, in which 10 times the amount of OT-1 CD8$^+$ T cells were co-cultured together; the control nanoprobe (1 nmol/L) group, in which only the probe was added (the final concentration was 1 nmol/L); the control nanoprobe (10 nmol/L) group, in which only the probe was added (the final concentration was 10 nmol/L); the nanoprobe (1 nmol/L) co-cultured with OT-1 CD8$^+$ T cells and the probe group, in which 10 times the amount of OT-1 CD8$^+$ T cells and the probe were added and incubated together; the nanoprobe (10 nmol/L) co-cultured with OT-1 CD8$^+$ T cells and probe group, in which 10 times the amount of OT-1 CD8$^+$ T cells and the probe were added and co-cultured together (each group: $n = 4$). After 0, 6, 12, or 24 hours post the addition of PBS or OT-1 CD8$^+$ T cells and the probe, the cardiomyocytes were washed 3 times using PBS. For the immunofluorescent staining, the 15 mm round coverslips with high molecular weight Poly-D-lysine coating (neuVitro, Braunschweig, Germany) were placed at the bottom of 24-well plates.

1.7. Microplate assay

After the cardiomyocytes in the 96-well plates were washed, 100 µL of PBS were added into each well. The signals were measured by Imaging Station 4000MMPro (Kodak, Rochester, NY). Near-infrared fluorescence (NIRF) images were obtained in the 680 nm channels (excitation filter; 630 nm, emission filter; 700 nm) with progressive exposure time for 1 minute. White images were obtained without using any filter for 0.05 seconds.

1.8. Assessment of the effects of dexamethasone on CD8$^+$ T cells

CD8$^+$ T cells isolated from OT-1-Tg mice ($5.0 \times 10^6$) were cultured in 24-well plates with PBS or the anti-CD3 ($\#100302$, Biolegend, San Diego, CA) solution (50 ng/mL) for 48 hours and divided into 6 groups: the control group, in which only PBS was added; and the dexamethasone groups, in which dexamethasone was added (the final concentration was 10 µmol/L) for 1, 6, 12, 24, or 48 hours after the anti-CD3 stimulation (each group: $n = 4$).

1.9. Assessment of the effects of dexamethasone on cardiomyocytes co-cultured with CD8$^+$ T cells

Cardiomyocytes were isolated from CMy-Tg or WT mice, seeded in 24-well or 96-well plates (seeding density was $2.5 \times 10^5$ cells/cm$^2$), and divided into 6 subgroups: the vehicle control group, in which only PBS was added after being co-cultured with 10 times the amount of OT-1 CD8$^+$ T cells; and the dexamethasone groups, in which dexamethasone was added (the final concentration was 10 µmol/L) for 0, 6, 12, or 24 hours after being co-cultured with 10 times of OT-1 CD8$^+$ T cells (each group: $n = 4$). After 0, 6, 12, or 24 hours from the addition of PBS or OT-1 CD8$^+$ T cells and the probe, the cardiomyocytes were washed 3 times using PBS. For immunofluorescent staining, 15 mm round coverslips coated with high molecular weight poly-D-lysine were placed at the bottom of 24-well plates.

1.10. Assessment of the specificity of the nanoprobe in cytotoxic CD8$^+$ T cell
A granzyme B-inhibitor (Z-AAD-CMK, #368050, Calbiochem, Cambridge, MA) was diluted in dimethyl sulfoxide (DMSO, #D2650, Sigma-Aldrich). Cytotoxic CD8+ T cells isolated from Balb/c WT or Balb/c granzyme B-deficient mice (4.0 × 10^6) were cultured in 24-well plates with PBS or the anti-CD3 (#100302, Biolegend, San Diego, CA) solution (50 ng/mL) for 48 hours. And, the lysate of each group was divided into 3 subgroups: the control group, to which only DMSO was added; the granzyme B-inhibitor groups (1 µmol/L), to which granzyme B-inhibitor was added (the final concentration was 1 µmol/L); and the granzyme B-inhibitor groups (10 µmol/L), to which granzyme B-inhibitor was added (the final concentration was 10 µmol/L) (each group: n = 4).

1.1. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was extracted from cultured cells or myocardium using TRIzol (Invitrogen) or the RNeasy kit (QIAGEN Inc., Valencia, CA), reverse-transcribed using the ThermoScript RT-PCR system and random primers according to the manufacturer’s instructions (Invitrogen), and amplified by real-time PCR with SYBR Green PCR mix (Applied Biosystems, Foster City, CA) and an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) or Step-One Detection System (Applied Biosystems), according to the manufacturer’s instructions. Levels of specific gene expression in the samples were presented relative to endogenous GAPDH mRNA concentrations in the same sample to normalize for mRNA differences between samples. The sequences of the primers were as shown (Supplemental Table 1). All real-time reactions were carried out on an ABI 5700 Sequence Detection System (Applied Biosystems) and analysis was completed with accompanying software. The RT-PCR was verified by dissociation curve analysis.

1.1.2. Histological grading of myocarditis
After euthanasia, hearts were harvested, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and cut into serial 6 µm sections. Myocarditis was graded by microscopic examination of hematoxylin and eosin (H&E)-stained sections. Grading was performed in a blind fashion by a trained pathologist after microscopically examining the entire area of three sections with a grid ocular, using a 0 – 4 scale, modified as follows: grade 0, no disease; grade 1, up to 5 % of cross-sectional area of the heart section; grade 2, 6 % to 20 %; grade 3, 21 % to 50 %; grade 4, > 50 %.

1.1.3. Immunohistochemistry (IHC)
Staining was performed on frozen sections of heart tissues for immunohistochemical staining of neutrophils (anti-NIMP-R14, #sc-59338, Santa Cruz, Cambridge, MA), macrophages (anti-CD68, #MCA1957, AbD Serotec, Raleigh, NC), CD4+ T cells (anti-CD4, #550278, BD Pharmingen), CD8+ T cells (anti-CD8, #ab-22378, Abcam, Cambridge, MA), granzyme B (#ab-4059, Abcam), and species-appropriate biotinylated secondary antibodies, followed by avidin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Reactions were visualized with 3-amino-9-ethyl carbazole substrate (red reaction product; AEC; Sigma-Aldrich), and sections were counterstained with Mayer’s hematoxylin solution (Sigma-Aldrich). Images were captured with a digital camera.
(Nikon DXM1200-F, Nikon Inc., Melville, NY) using imaging software ACT-1 (Version 2.63). The number of positive cells was counted in a blind fashion by a trained pathologist after microscopically examining the entire area of three sections with a grid ocular and total area was quantified using ImageJ software.

1.14. Immunofluorescent staining (IF)
Staining was performed on cultured cells or frozen sections of heart tissues. The antibodies used for immunohistochemistry included anti-CD8 (anti-CD8, #ab-22378), anti-granzyme B (#ab-4059, Abcam), anti-NIMP-R14 (#sc-59338, Santa Cruz), anti-cardiac troponin-I (#ab-47003, Abcam), and isotype-matched antibodies as controls. For IF, samples were incubated with 4’, 6-diamidino-2-phenylindole (DAPI) (#R37606, Life Technologies, Grand Island, NY), and species-appropriate secondary antibodies conjugated fluorescence including Alexa 488 or Alexa 568 (Life Technologies). Immunofluorescence was examined by microscopy (FV1000, Olympus, Cambridge, MA).

1.15. Assessment of apoptosis
The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed according to the manufacturer’s instructions using a commercially available kit for detecting end-labeled DNA (Roche Diagnostics, Mannheim, Germany).

1.16. Assay of cardiac troponin-I, granzyme B, IFN-γ, and IL-6
Medium and serum concentrations of cardiac troponin-I (Life Diagnostics Inc., West Chester, PA), granzyme B (eBioscience, San Diego, CA), IFN-γ, and IL-6 (Invitrogen) in mice were determined using an ELISA kit in accordance with the manufacturer’s instructions.

1.17. Western blot analysis
Western blotting was performed for cells or tissue lysates using polyclonal antibodies for mouse granzyme B (#14-8822, eBioscience), procaspase-3 (#9662, Cell Signaling Technology, Danvers, MA), cleaved caspase-3 (#9664S), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#2118), and cardiac troponin-I (#ab-47003, Abcam). Membranes were incubated with peroxidase-coupled goat anti-rat or anti-rabbit IgG (Invitrogen). Densitometric analysis was conducted using the ImageJ program.

1.18. Macroscopic ex vivo FRI for abdominal organs
WT Balb/c (Jackson Laboratory) and granzyme B deficient male mice between 10 and 15 weeks of age were received an intravenous injection of PBS or the granzyme B probe (the WT control group: n = 4, the WT group: n = 4, the granzyme B deficient group: n = 4). One day after the administration, the abdominal organs, such as the stomach, intestine, colon, liver, kidney, and spleen were isolated from euthanized mice and were assessed by microscopic ex vivo FRI and other experimental methods. The probe was dissolved in sterile PBS immediately before use at a
concentration of 1 µmol/L CyAl5.5B. Each mouse received 50 µL of the solution. The control mice received PBS (vehicle).

1.19. Statistics

All values are reported as mean ± standard deviation. Quantification yielded the data from the CD8⁺ OT-1 T cells -co-cultured with cardiac myocytes and control groups in vitro, or the myocarditis and control groups ex vivo and in vivo. Data from histology studies, ELISA, Western blot, and RT-PCR was collected and the differences were analyzed with the commercially available SPSS software package (SPSS Japan, Tokyo, Japan). For imaging, 12 mice in each group were used, estimated to yield 85 % power to detect a difference between groups. Data were tested for normality using the D’Agostino-Pearson normality test and for equality of variances using the Bartlett’s test. If normality and equality of variances were not rejected at 0.05 significant levels, the group means were compared using parametric tests, such as t-test (for 2 groups) or ANOVA, followed by Bonferroni correction (for more than 2 groups). Differences were considered significant at $p < 0.05$. For non-normally distributed data and/or data with unequal variances, nonparametric tests were applied, such as Mann-Whitney U (for 2 groups) and Kruskal-Wallis tests, followed by Dunn’s post-test (for more than 2 groups). Data are the mean ± standard deviation (S.D.).

2. Supplemental Figures and Figure Legends

Supplemental Figure I. In vitro investigation of nanoprobe activation.

(A) Western blotting analysis of granzyme B and cleaved caspase-3 in the cardiomyocytes after co-culture with the CD8⁺ T cells for 0, 6, 12, or 24 hours (each group; $n = 4$). The data are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Closed bars indicate the cardiomyocytes isolated from the CMy-Tg mice; open bars indicate the cardiomyocytes isolated from the wildtype (WT) mice. (B) Evaluation of granzyme B levels in medium (each group; $n = 4$). (C) Microplate assay for the signals in the cardiomyocytes co-cultured with CD8⁺ T cells for 24 hours after the 1 or 10 nmol/L of the probe addition (each group; $n = 4$). The upper panels show the white images and the lower panels show the RGB images. The figure shows the mean signal intensities. (D) Immunofluorescent staining (IF) for the CMy-Tg or WT cardiomyocytes co-cultured with CD8⁺ T cells and 1 nmol/L of the probe for 24 hours. The upper panels show analysis of granzyme B and the probe colocalization in the cardiomyocytes. The white signals indicate cardiac troponin-I positive cells. The blue signals indicate nuclei stained with DAPI. The green or red signals indicate the location of granzyme B or the probe. Bars indicate 50 µm. The graph shows the percentages of granzyme B-positive cardiomyocytes (each group; $n = 4$); The $P$-value is compared to each vehicle group. * $P < 0.05$, † $P < 0.001$, ‡ $P < 0.005$, § $P < 0.0001$.

Supplemental Figure II. Messenger RNA (mRNA) expression in CD8⁺ T cell-mediated myocarditis.

The figures show the levels of granzyme B, perforin, interferon-γ, TNF-α, interleukin-2, interleukin-6, caspase-3, caspase-8. Fas, Bid, VCAM-1, and ICAM-1 in hearts of the CMy-Tg and WT mice 5 days post the injection of 3.5 ×
of the CD8\(^+\) T cells (each group of CMy-Tg mouse; \(n = 12\), each group of WT mouse; \(n = 4\)). The mice received PBS (vehicle) or dexamethasone at days 1, 2, 3, and 4 after the CD8\(^+\) T cell injection. The data are normalized to GAPDH. Closed bars indicate the vehicle control group of each mouse; open bars indicate the dexamethasone group of each mouse. The \(P\) –value refers to comparison to the vehicle control of each group. * \(P < 0.05\), † \(P < 0.001\), ‡ \(P < 0.005\), § \(P < 0.0001\). Abbreviation; TNF-\(\alpha\); Tumor necrosis factor-alpha, VCAM-1, vascular cell adhesion molecule-1, ICAM-1, intercellular adhesion molecule-1.

Supplemental Figure III. Investigation of dexamethasone administration in CD8\(^+\) T cell-cardiomyocyte interactions.

(A) Western blot analysis of the granzyme B protein levels in the CD8\(^+\) T cells 48 hours post anti-CD3 activation. The CD8\(^+\) T cells were incubated with dexamethasone (10 \(\mu\)mol/L) for 1, 6, 12, 24, or 48 hours before harvested (each group; \(n = 4\)). (B) Evaluation of granzyme B concentrations in the culture medium (each group; \(n = 4\)). (C) Western blotting analysis of the granzyme B and cleaved caspase-3 protein levels in the CMy-Tg or WT cardiomyocytes co-cultured with CD8\(^+\) T cells for 24 hours (each group; \(n = 4\)). (D) Evaluation of granzyme B levels in medium (each group; \(n = 4\)). (E) Immunofluorescent staining for the CMy-Tg myocytes co-cultured with the CD8\(^+\) T cells and 1 nmol/L of the probe for 24 hours. The cardiomyocytes in the dexamethasone group were incubated in dexamethasone (10 \(\mu\)mol/L) for 12 hours before being harvested. The upper panels show analysis of granzyme B and the probe colocalization with the cardiomyocytes. The white signals indicate cardiac troponin-I positive cells. The blue signals indicate nuclei stained with DAPI. The green or red signals indicate the location of granzyme B or the probe. Bars indicate 50 \(\mu\)m. The lower graph shows the percentages of granzyme B-positive cardiomyocytes (each group; \(n = 4\)). The \(P\) -value refers to comparison with the vehicle group. * \(P < 0.05\), † \(P < 0.001\), ‡ \(P < 0.005\), § \(P < 0.0001\).

Supplemental Figure IV. Investigation of the sensitivity of ProSense 680 in CD8\(^+\) T cell-mediated myocarditis.

(A) Evaluation of hematoxylin and eosin (H&E)-stained heart sections in CMy-Tg and WT mice 5 days post the injection of \(3.5 \times 10^6\) CD8\(^+\) T cells (CMy-Tg mouse; \(n = 12\), WT mouse; \(n = 12\)). Mice received i.p. injections of PBS at days 1, 2, 3, and 4 after the CD8\(^+\) T cell injection. Original magnification in the upper panels is 20\(\times\). The original magnification in the lower panels is 100\(\times\). Bars indicate 200 \(\mu\)m. The figure shows grading of myocarditis grades in each group. Closed bars indicate the CMy-Tg group; open bars indicate the WT group. (B) \(Ex vivo\) FRI for the heart sections in the CMy-Tg and WT mice (CMy-Tg mice; \(n = 12\), WT mice; \(n = 12\)). The mice received 2 nmol / 150 \(\mu\)L of ProSense 680 intravenously at day 4. One day after the administration, the samples isolated from euthanized mice were assessed by microscopic \(Ex vivo\) FRI. The left panels show the white images and the right panels show the RGB images. The figure shows the mean signal intensities. The \(P\) –value refers to comparison to the control. * \(P < 0.05\), † \(P < 0.001\), ‡ \(P < 0.005\), § \(P < 0.0001\).

Supplemental Figure V. Demonstration of the sensitivity of the nanoprobe in CD8\(^+\) T cell-mediated myocarditis.

(A) \(In vivo\) fluorescent molecular tomography in conjunction with co-registered computed tomography imaging
Supplemental Figure VI. Investigation of the optimum time of the nanoprobe in CD8+ T cell-mediated myocarditis.

(A) In vivo FMT images for CMy-Tg and WT mice 6, 24 and 48 hours after the probe administration (both groups received 3.5 × 10^6 CD8+ T cells, CMy-Tg mouse; n = 5, WT mouse; n = 5). The left panels show 2D images and the right panels show the location of heart signals. (B) The mean signal intensities of hearts for CMy-Tg and WT mice 6, 24 and 48 hours after the probe administration. Closed bars indicate the CMy-Tg group; open bars indicate the WT group. The P-value refers to comparison with the WT group of each time. (C) Plasma stability of the nanoprobe. Open bar indicates the baseline fluorescence; closed bars indicate fluorescent increases 4, 8, 12, 16, 20, 24, and 28 hours post the co-incubation of mice plasma and 10 µg/mL of probe (each group; n = 4). * P < 0.05, † P < 0.001, ‡ P < 0.005, § P < 0.0001.

Supplemental Figure VII. Investigation of the specificity of the nanoprobe in cytotoxic CD8+ T cells and abdominal organs

(A) Western blot analysis of the granzyme B protein levels in the CD8+ T cells 48 hours post anti-CD3 activation. The cytotoxic CD8+ T cells of Balb/c WT or granzyme B-deficient mice were incubated in the plates with PBS or the anti-CD3 solution for 48 hours before harvested (each group; n = 4). (B) Granzyme B enzyme assay for the lysates from cytotoxic CD8+ T cells for 8 hours after the 10 µg/mL of the probe and 1 or 10 µmol/L of the granzyme B-inhibitor addition (each group; n = 4). Open bars indicate the baseline fluorescence; closed bars indicate the WT T cell groups; gray bars indicate the granzyme B-deficient T cell groups. The P-value refers to comparison with the control of each group. (C) Ex vivo FRI for the abdominal organs of the WT and granzyme B-deficient mice 24 hours after the probe administration (each group; n = 4). The control mice received PBS (vehicle). The right figures show the mean signal intensities of organs. Open bars indicate the control group; closed bars indicate the WT group; gray bars indicate the granzyme B-deficient group. The P-value refers to comparison with the control of WT group.* P < 0.05, † P < 0.001, ‡ P < 0.005, § P < 0.0001.
3. Supplemental Table

Supplemental Table I. Primer sequences (5' to 3') for RT-PCR analysis.

Abbreviation; IFN-γ, Interferon-gamma, IL-2, Interleukin-2, IL-6, Interleukin-6, TNF-α; Tumor necrosis factor-alpha, VCAM-1, vascular cell adhesion molecule-1, ICAM-1, intercellular adhesion molecule-1, GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

4. Supplemental References


**Supplemental Figure I**

**A**
Granzyme B

GAPDH

Cardiac troponin-I

![Granzyme B levels](image1)

Ratio compared to GAPDH in cardiomyocytes

![Granzyme B vs GAPDH](image2)

**B**
Granzyme B protein levels in medium (ng/mL)

![Granzyme B in medium](image3)

**C**
Granzyme B activity (count)

![Granzyme B activity](image4)

**D**
Co-cultured with T cells
Granzyme B colocalization

![Granzyme B colocalization](image5)
Supplemental Figure II

Perforin mRNA levels
Ratio compared to GAPDH in hearts

Interferon-γ mRNA levels
Ratio compared to GAPDH in hearts

Granzyme B mRNA levels
Ratio compared to GAPDH in hearts

TNF-α mRNA levels
Ratio compared to GAPDH in hearts

Interleukin-2 mRNA levels
Ratio compared to GAPDH in hearts

Interleukin-6 mRNA levels
Ratio compared to GAPDH in hearts

Caspase-3 mRNA levels
Ratio compared to GAPDH in hearts

Caspase-8 mRNA levels
Ratio compared to GAPDH in hearts

Bid mRNA levels
Ratio compared to GAPDH in hearts

Fas mRNA levels
Ratio compared to GAPDH in hearts

VCAM-1 mRNA levels
Ratio compared to GAPDH in hearts

ICAM-1 mRNA levels
Ratio compared to GAPDH in hearts
Supplemental Figure III

**A**

Granzyme B protein levels compared to GAPDH in T cells

- Granzyme B levels (in medium (ng/mL))
- Ratio compared to GAPDH in T cells

**B**

Granzyme B protein levels in medium (ng/mL)

- Comparative graph showing changes over time (anti-CD3 and Dex)

**C**

Granzyme B in OT-1 CD8+ T cells

- Granzyme B levels compared to GAPDH in cardiomyocytes
- Ratio of Granzyme B to GAPDH in cardiomyocytes

**D**

Granzyme B levels in medium (ng/mL)

- Comparative graph showing changes over time (T cells and Dex)

**E**

Granzyme B colocalization

- Comparative graph showing changes over time (Dex and CMy-Tg)
Supplemental Figure IV

**A**

<table>
<thead>
<tr>
<th></th>
<th>H&amp;E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>20x</strong></td>
<td><img src="image" alt="H&amp;E image" /></td>
</tr>
<tr>
<td><strong>100x</strong></td>
<td><img src="image" alt="H&amp;E image" /></td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th></th>
<th><em>ex vivo FRI (Heart section)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>White</strong></td>
<td><img src="image" alt="White image" /></td>
</tr>
<tr>
<td><strong>RGB</strong></td>
<td><img src="image" alt="RGB image" /></td>
</tr>
</tbody>
</table>

**Graph A**

- **Myocarditis grade**
  - **CMy-Tg**
  - **WT**

**Graph B**

- **Fluorescent intensity**
  - **CMy-Tg**
  - **WT**
Supplemental Figure V

A

<table>
<thead>
<tr>
<th></th>
<th>T cells = 2 × 10⁶</th>
<th></th>
<th>T cells = 3.5 × 10⁶</th>
<th></th>
<th>T cells = 5 × 10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dex</td>
<td>in vivo FMT-CT (2D)</td>
<td></td>
<td>in vivo FMT-CT (3D)</td>
<td></td>
<td>in vivo FMT-CT (2D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMy-Tg</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Granzyme B activity
(Heart: T cells = 2 × 10⁶)

Granzyme B activity
(Heart: T cells = 3.5 × 10⁶)

Granzyme B activity
(Heart: T cells = 5 × 10⁶)

C

R² = 0.591
P < 0.001

Myocarditis grade
Supplemental Figure VI

A

<table>
<thead>
<tr>
<th>T cells = 3.5 × 10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>in vivo FMT (2D)</strong></td>
</tr>
<tr>
<td>CMY-Tg</td>
</tr>
<tr>
<td>6h</td>
</tr>
<tr>
<td>24h</td>
</tr>
<tr>
<td>48h</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Granzyme B activity (Heart: T cells = 3.5 × 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMy-Tg WT 6h</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Fluorescent fold increase in plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe (hours) 0 4 8 12 16 20 24 28</td>
</tr>
</tbody>
</table>

* Indicates significant difference.
Supplemental Figure VII

A) Granzyme B protein levels in T cells compared to GAPDH

B) Granzyme B activity in cell lysate (fold increase)

C) ex vivo FRI (Abdominal organs)
Supplemental Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granzyme B</td>
<td>TGC TCT GAT TAC CCA TCG TCC</td>
<td>GCC AGT CTT TGC AGT CCT TTA TT</td>
</tr>
<tr>
<td>Perforin</td>
<td>CAA TGG CAA GTA TGT GGT GGT</td>
<td>CAG TGA GAT GGT TTC CCG AGT</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TCT GGA GGA ACT GGC AAA AG</td>
<td>TTC AAG ACT TCA AAG AGT CTG AGG</td>
</tr>
<tr>
<td>IL-2</td>
<td>GCA CCC ACT TCA AGC TCC A</td>
<td>AAA TTT GAA GGT GAG CAT CCT G</td>
</tr>
<tr>
<td>IL-6</td>
<td>GCT ACC AAA CTG GAT ATA ATC AGG A</td>
<td>CCA GGT AGC TAT GGT ACT CCA GAA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TCT TCT CAT TCC TGC TTG TGG</td>
<td>GGT CTG GGC CAT AGA ACT GA</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>ATG GGA GCA AGT CAG TGG AC</td>
<td>CGT ACC AGA GCG AGA TGA CA</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>CTT CGA GCA ACA GAA CCA CA</td>
<td>TTC TTC ACC GTA GCC ATT CC</td>
</tr>
<tr>
<td>Bid</td>
<td>CTG CCT GTG CAA GCT TAC TG</td>
<td>GTC TGG CAA TGT TGT GGA TG</td>
</tr>
<tr>
<td>Fas</td>
<td>TTC AGG ACA TGG TCC AGA AGG ACC</td>
<td>TGC TGG CAA AGA GAA CAC ACC AGG</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>TGC CGA GCT AAA TTA CAC ATT G</td>
<td>CCT TGT GGA GGG ATG TAC AGA</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>CCC ACG CTA CCT CTG CTC</td>
<td>GAT GGA TAC CTG AGC ATC ACC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGG GTG TGA ACC ATG AGA AG</td>
<td>GCT AAG CAG TTG GTG GTG C</td>
</tr>
</tbody>
</table>