Supporting Information

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SI Materials and Methods

Strains and Growth Conditions. *Mycobacterium tuberculosis* (Mtb) strain Erdman (ATCC35801), H37Rv (ATCC25618), CDC1551 (CSU TVTRM Contract), bacillus Calmette-Guérin (Pasteur), and derivatives were grown in Middlebrook (M) 7H9 broth (Difco) supplemented with 0.5% glycerol, 10% oleic acid albumin dextrose complex (OADC), and 0.05% Tween 80 (M-OADC-TW broth). The H37Rv *blaC* mutant was kindly provided by Martin S. Pavelka (University of Rochester). Viable counts were determined for all bacterial cultures and all experimental conditions using Middlebrook 7H9 supplemented with 10% OADC and 15 g/L Bacto agar (M-OADC agar) or on 7H11 agar. Both *Staphylococcus aureus* (MRSA, kindly provided by Greg A. Somerville, University of Nebraska, Lincoln) and *Pseudomonas auruginosa* strain PA01 (kindly provided by Thomas K. Wood, Texas A&M University) were grown until an OD_{600} of 0.5 in Luria Broth (LB) and CFU were determined by plating dilutions on LB agar. When necessary, media were supplemented with 50 μg/mL hygromycin and 25 μg/mL kanamycin. Stocks were prepared by growth standing at 37 °C until an OD_{600} of 0.5 and stored in aliquots at −80 °C until used. Therapeutic treated cultures were inoculated with 10^6 bacteria and grown in either the presence or the absence of rifampin (10 μg/mL) plus isoniazid (10 μg/mL) before detection of bacteria with CNIR substrate.

Macrophage Infection Assays. J774A.1 cells were seeded at 2.5×10^5 cells/well in 24-well dishes or 1×10^5 cells/well in 8-well chamber slides and incubated overnight at 37 °C in DMEM. The media was changed just before infection with 0.2 mL of media that contains 10^6 bacteria, a multiplicity of infection (MOI) of ≈10 (bacteria/cell). The bacteria were incubated with the cells for 30 min at 37 °C and washed twice with warm PBS, and the media was replaced with DMEM plus 1% FBS. The cells in triplicate wells were either prepared for microscopy or scraped for FACS analyses. Viability was >98% and was not impacted by the presence of any substrates for the duration of experiments.

Flow Cytometry. J774A.1 cells were seeded in six-well plates with 1×10^6 cells/well in 2 mL of DMEM with 10% FBS and incubated at 37 °C with 5% CO_2 overnight. After removing the cell culture medium, GFP expressing CDC1551 strains were added to the six-well plates at an MOI of 1, 5, and 20 (bacteria per macrophage) in DMEM with 10% FBS in 200 μl. After 30 min coincubation, the cells were washed twice with prewarmed PBS to remove extracellular bacteria. Amikacin at a final concentration of 200 μg/mL was added to the medium and incubated for 2 h to kill extracellular bacteria. The cells were washed twice with PBS, and the medium was replaced with DMEM plus 10% FBS with 20 nM CNIR substrate. After various times of coincubation with the substrate at 37 °C in 5% CO_2, cells were washed twice with PBS and stained with 10 μg/mL DAPI in PBS by incubation for 5 min at room temperature. The cells were then scraped from the wells with a rubber policeman, fixed with ice cold 75% ethanol for 30 min, and stored at −20 °C until flow cytometry.

Synthesis of β-Lactamase Imaging Probes. 7-Amino-3-chloromethyl cephalosporanic acid benzhydryl ester was a gift from Otsuka Chemical (Osaka, Japan). CyDye monoreactive NHS esters were obtained from Amersham Biosciences. All of the other starting materials were obtained from Sigma-Aldrich. Commercially available reagents were used without purification, unless indicated otherwise. The solvents were dry according to standard protocols. All of the other chemicals were reagent grade or better. The synthesized compounds were characterized using ^{1}H NMR (Bruker ARX 300), and matrix-assisted laser desorption/ionization mass spectrometric analysis was performed at the Mass Spectrometry Facility of Stanford University.

Analytical reverse-phase high-performance liquid chromatography (HPLC) was performed on Acclaim C-18 columns (250 × 4.6 mm) at a flow rate of 1.0 mL/min, and semipreparative HPLC was performed on a similar column (250 × 10 mm) at 3 mL/min. Two solvent systems, A (water with 0.1% TFA) and B (Acetonitrile with 0.1% TFA), were used to elute the products and monitored by UV-visible absorbance at 280 and 650 nm, respectively. HPLC elution was performed with linear gradients of 0.1% trifluoroacetic acid (TFA) in water (solution A) and 0.1% TFA in acetonitrile (solution B). The linear gradient started from 80% solution A and 20% solution B, changed to 20% solution A and 80% solution B in 25 min, to 0% solution A and 100% solution B in the following 5 min, and then back to 80% solution A and 20% solution B in the next 5 min.

Mouse Infections and Imaging. Five- to 7-wk-old female BALB/c mice were obtained from Jackson Laboratories. There were four animals in each experimental group, and all experiments have been repeated at least three times independently with consistent results. Quantitative data shown in all figures are means and SDs for all groups. Animals were assigned randomly to experimental groups, allowed to acclimatize to the facilities for 1 wk, and fed commercial chow with low chlorophyll content and tap water ad libitum. To determine bacterial loads, portions of lungs and spleens were homogenized in PBS and dilutions were plated on 7H11 selective media to determine numbers of viable bacteria. For s.c. infections, appropriate concentrations of bacteria in 50 μL of saline were inoculated beneath the skin. Bacterial numbers were confirmed at 24 h postinoculation by harvesting the region of the skin and homogenization in PBS followed by dilution and plating on 7H11. Antibiotic treatment was carried out on four mice per group infected by the intratracheal route with 10^6 bacteria using 10 μg/g rifampin and 10 μg/g isoniazid in combination delivered intraperitoneally in saline daily beginning immediately postinfection. All animal studies were approved by the institutional animal use committee and comply with established guidelines for care and use of animals.

Approximately 5 nmol of each imaging substrate was administered i.v. via the tail vein to each mouse, including uninfected controls. Photographic images were directly overlaid with matching fluorescent images for all mice. Structured light images were taken in cases where fluorescence-mediated molecular tomography was used. Wavelength-resolved spectral imaging was carried out to image CNIR substrates in mice. In the case of CNIR4, the excitation wavelength was 605 nm and emission was collected in 20-nm increments from 640 to 740 nm, and in CNIR5, CNIR9, and CNIR10 the excitation wavelength was 640 nm and emission was collected in 20-nm increments from 680 to 780 nm. Each acquisition was taken for 3 s with f-stop 1 and medium binning. Four mice were imaged for each condition, including in the presence and the absence of therapeutic treatment, and the means for each region of interest were determined.

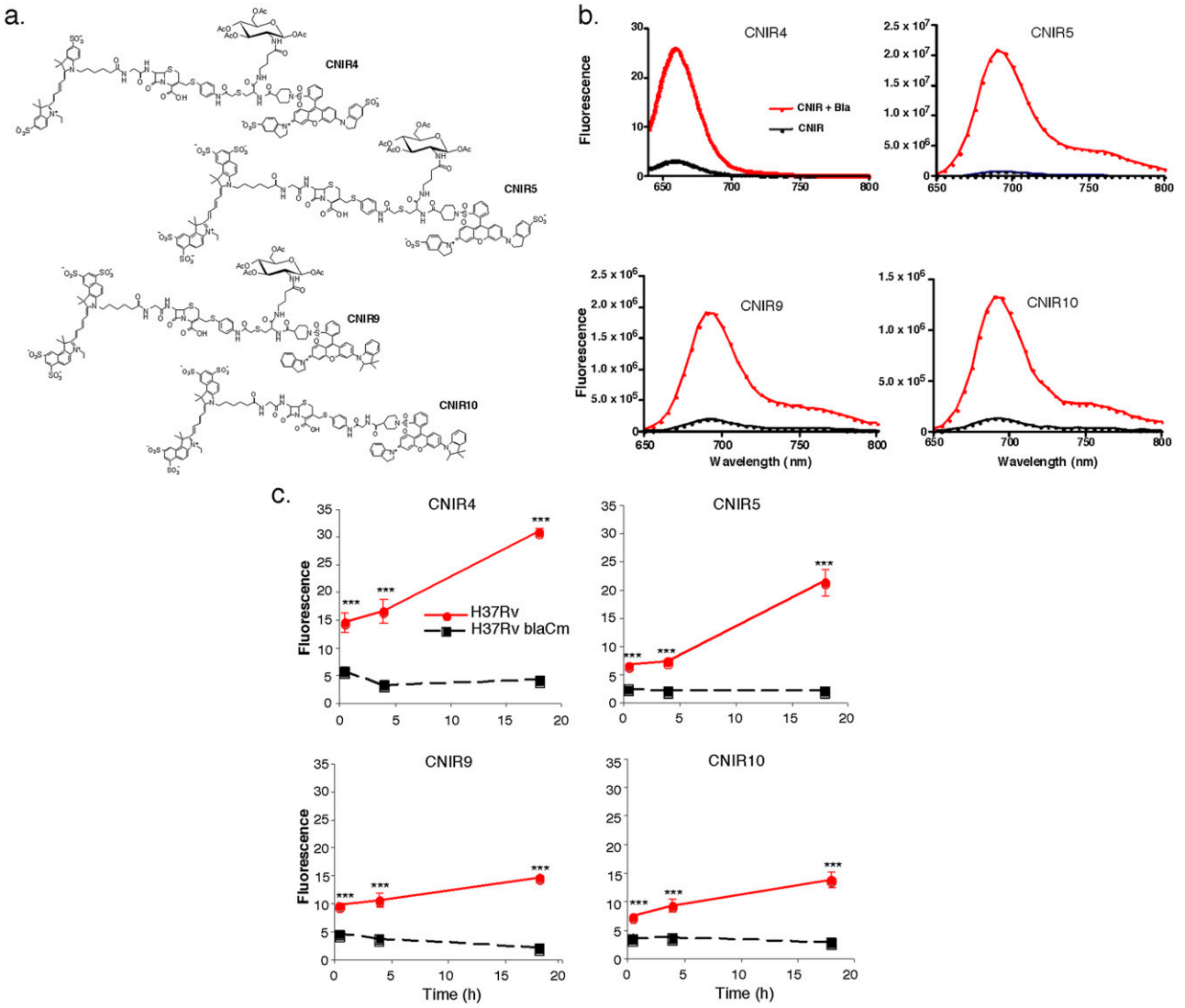

Fig. S1. Structures and fluorescence spectra of CNIR substrates. The quencher groups used are QSY21 or derivatives and the CNIR dyes are Cy5 (CNIR4) and Cy5.5 (CNIR5, CNIR9, and CNIR10). (*A*) Structures of CNIR4, CNIR5, CNIR9, and CNIR10. (*B*) Fluorescence emission spectra of CNIR4, CNIR5, CNIR9, and CNIR10 before (CNIR) and after (CNIR + Bla) hydrolysis with TEM-1 β-lactamase. (*C*) Kinetics of CNIR4, CNIR5, CNIR9, and CNIR10 fluorescence label incorporation directly into wild-type Mtb and the Mtb *blaC* mutant (blaCm). ***$P < 0.001$ significantly different from blaCm as calculated by ANOVA with the Bonferroni posttest.

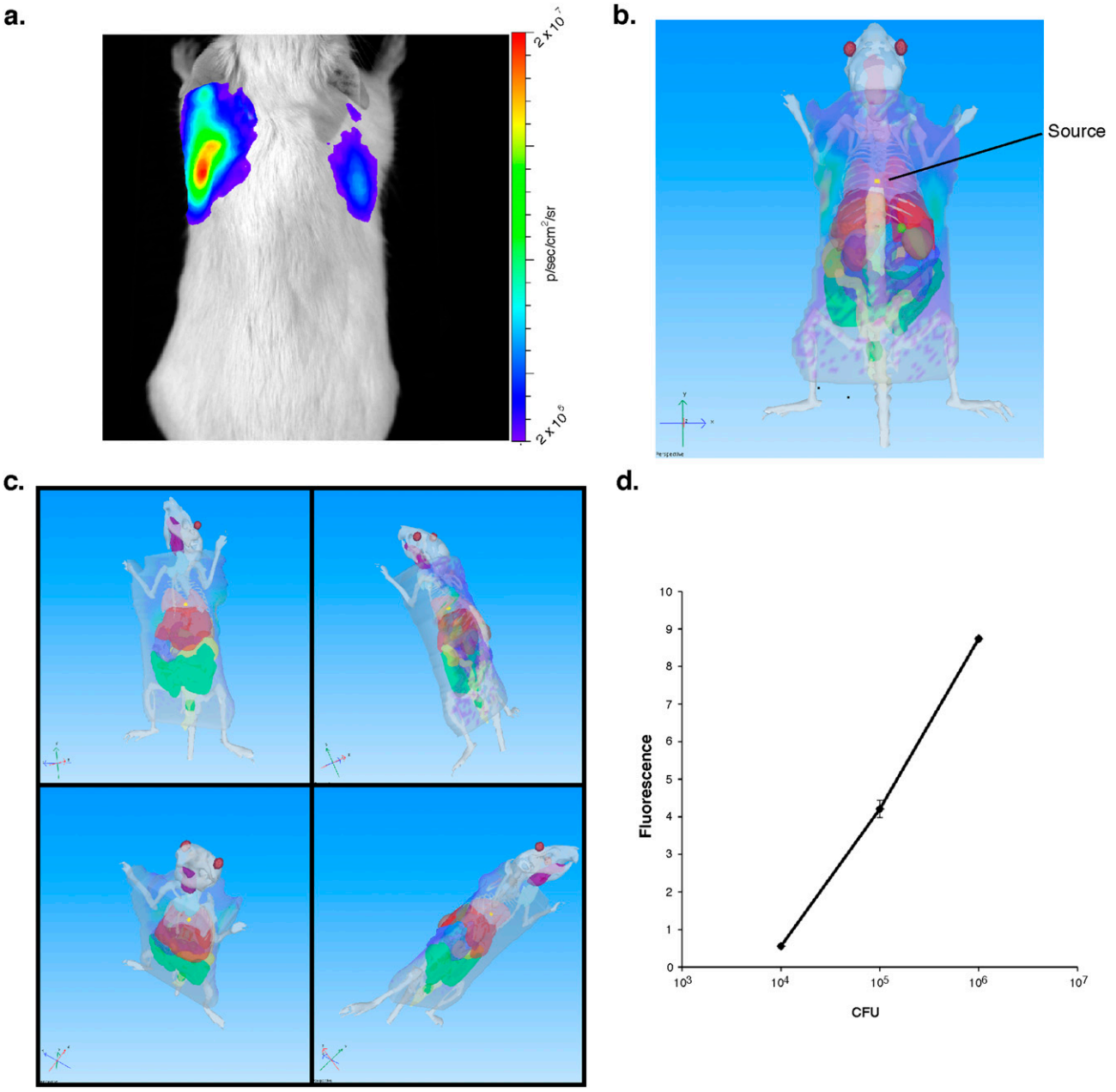

Fig. S2. Three-dimensional fluorescence-mediated molecular tomography (FMT) to quantify and localize the source of infection in the lungs after pulmonary infection. (*A*) Fluorescent signal from a mouse infected and imaged at 24 h postinfection using CNIR5. (*B*) Location of source of signal (yellow cube) as determined by FMT. (*C*) Multiple views of the same mouse as generated by 3D tomographic reconstruction and FMT analysis. (*D*) The relationship between FMT-calculated fluorochrome concentration and colony forming units (CFU) present in the lungs of mice (four mice per group) infected with different numbers of bacteria.

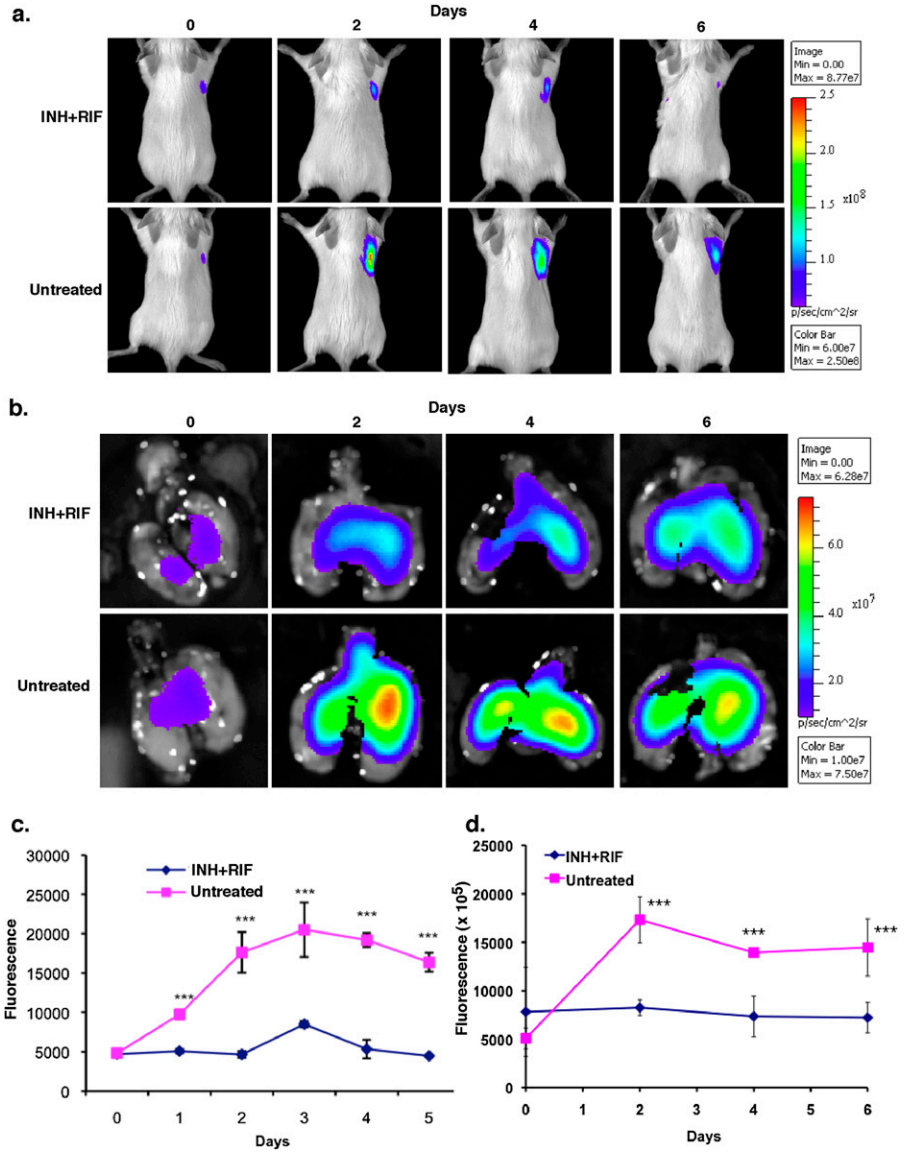

Fig. S3. Evaluation of therapeutic efficacy using CNIR imaging in vitro with whole bacteria (*C*) and in mice (*A*, *B*, and *D*). (*A*) Intratracheally infected mice with 10^6 bacteria were treated with isoniazid (INH) and rifampin (RIF) daily and imaged with CNIR5, and the fluorescent signal was compared with untreated mice. (*B*) Ex vivo images of the lungs demonstrate that loss of signal in live-mouse whole-body images correlates with signal in the lungs. (*C*) Impact of INH plus RIF treatment on fluorescent signal generated by 10^6 bacteria in culture using CNIR5. (*D*) Quantification of total fluorescent signal generated in treated versus untreated mice in *A*. ***$P < 0.01$: significantly different from untreated as calculated by ANOVA with the Bonferroni posttest.