Supporting Information

Toward *In vivo* Imaging of Heart Disease Using a Radiolabeled Single-Chain Fv Fragment Targeting Tenascin-C

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Supplementary Experimental Procedures

Buffers. PBS: 50 mM sodium phosphate buffer (pH 7.3) containing 9.0 g/L NaCl; M-PBS: PBS containing 20 g/L skim milk; G-PBS: PBS containing 1.0 g/L gelatin; T-PBS: PBS containing 0.050 (v/v) % Tween 20.

ELISA for Detection and Characterization of ScFv-4F10 (Figure 3A in the text). Microwells in 96-well microplates (no. 3590) (Costar) were coated with a solution of a Tnc (prepared and purified as reported previously)¹ (10 µg/mL) in 0.10 M carbonate buffer (pH 8.6) (100 µL/well) overnight at 4°C. After washing 3 times with PBS, the wells were blocked with M-PBS at 37°C for 1 h. The wells were washed again, and then crude periplasmic extract or purified scFv-4F10 protein, diluted with G-PBS (100 µL/well), was added along with free Tnc dissolved in G-PBS (100 µL/well). After the solutions were mixed and incubated at 37°C for 1 h, they were aspirated off and the wells were washed 3 times with T-PBS. Next, anti-FLAG M2 antibody (Sigma), diluted with G-PBS (5.0 µg/mL) (100 µL/well), was added and incubated at 37°C for 1 h. The wells were washed and horseradish peroxidase (POD)–conjugated rabbit anti-mouse IgG antibody (Fc-specific) (Jackson ImmunoResearch), diluted with G-PBS (100 µL/well), was added and incubated at 37°C for 1 h. Following washing, the bound POD activity in each well was determined colorimetrically using *o*-phenylenediamine as the hydrogen donor.^{2,3}

Determination of Binding Affinity and Kinetics of ScFv-4F10 to Tnc. Surface plasmon resonance (SPR) analysis was performed using a Biacore 2000 (Biacore) to determine the association and dissociation rate constants (k_a and k_d) of scFv-4F10. Five different concentrations of scFv (54, 107, 214,

428, 857 nM), diluted in 50 mM phosphate-buffered saline (pH 7.3) containing 0.15 M NaCl, and 0.020% gelatin, were applied to a flow cell equipped with a sensor tip that immobilized the Tnc, followed by washing with the same buffer. The flow rate of the solutions was set at 20 μ L/min.

Synthesisof1-[4-(5-maleimidopentyl)amidobenzyl]diethylenetriaminepentaaceticacid(EMCS-Bz-DTPA).4To a chilled (-15° C) solution of N-(carboxypentyl)maleimide5(282 mg, 1.34mmol) in tetrahydrofuran (THF, 1 mL) was added N-methylmorpholine (135 mg, 1.34 mmol) andisobutyl chloroformate (183 mg, 1.34 mmol) at the same temperature. After 2 min, a THF solution (1 mL)

N,N,N',N'',N''-pentakis[(*tert*-butylcarbonyl)methyl]-1-[(4-aminophenyl)methyl]diethylenetriamine [NH₂-Bz-DTPA(tBu)₅]⁶ (950 mg, 1.22 mmol) was added. The reaction mixture was stirred for 30 min at -15° C and for 2 h at room temperature. After removing the solvent *in vacuo*, the residue was purified by column chromatography on silica gel using a mixture of ethyl acetate and hexane (1:1) as an eluent, to produce EMCS-Bz-DTPA(tBu)₅ (355 mg, 30 %).

EMCS-Bz-DTPA(tBu)₅ (117 mg, 0.12 mmol) was treated with a mixture of trifluoroacetic acid (1.9 mL) and anisole (0.1 mL) for 3 h. After removing the solvent, diethyl ether was added to precipitate EMCS-Bz-DTPA as a pale yellow solid (77 mg, 93%). ¹H-NMR (DMSO): δ 1.21-1.25 (2H, q, J = 6.8 Hz, CH₂), 1.48-1.58 (4H, m, CH₂), 2.23-2.26 (2H, t, J = 7.2 Hz, NHCO-CH₂), 2.43-2.95 (8H, m, N-CH₂, Bz-CH₂), 3.12-3.58 (13H, m, CH₂-COO, CH, CH₂-N-maleimide), 6.99 (2H, s, *maleimide*), 7.10-7.13 (2H, d, J = 8.4 Hz, *aromatic*), 7.46-7.48 (2H, d, J = 8.4 Hz, *aromatic*), 9.77 (1H, s, NH). FAB-MS: *m*/*z* 692 (M+H)⁺.

Determination of reactive SH groups in scFv-Cys molecules before and after DTPA conjugation (Figure 4A in the text). ScFv-Cys was biotinylated with a reagent that contains a maleimide group (Biotin Labeling Kit–SH; Dojindo Molecular Technologies), and the product (~50 ng) in G-PBS (100 μ L) was incubated at 37°C for 1 h in the microwells coated with Tnc (1.0 μ g/well). After washing the wells 3 times with T-PBS, the POD-conjugated streptavidin (0.010 μ g) in T-PBS (100 μ L) was incubated in the wells at 37°C for 1 h. The wells were washed similarly and the bound POD activity was measured as described above over the course of a 10-min reaction.

Comparison of the binding activity to Tnc between the scFv-Cys and scFv-DTPA (Figure 4B in the text). ScFv-Cys and scFv-DTPA (each 200 ng) in G-PBS (100 μ L) were incubated at 37°C for 1 h in the microwells coated with Tnc (1.0 μ g/well). After washing the wells 3 times with T-PBS, POD-conjugated anti-FLAG M2 antibody (0.50 μ g) in T-PBS (100 μ L) was incubated in the wells at 37°C for 1 h. The wells were washed and the bound POD activity was determined as described above.

Table S1. Nucleotide sequences of PCR primers used in this study.

Primer	Sequence $(5' \rightarrow 3';$ Restriction site ^{<i>a</i>})
V _L -IIa V _L -IIa-ext	GATGTTKTGATGACCCAAACT ACTAGTCGACGATRTTKTGATRACCCA (<i>Sal</i> I)
) GGAT <u>CCCGGG</u> CGGAAAATGTGTAGTTTGCAC (<i>Xma</i> I)
V_{H} -Rev1	ATTGTTATTACTCGC <u>GGCCCAACCGGCC</u> ATGGCCGAGGTCCTGCTGCAACAGTC (Sfi I)
$V_{\rm H}\text{-}{\rm For1}$	CCGCCGGATCCACCTCCGCCTGAACCGCCTCCACCTGCAGAGACAGAGACCAGAG
V _L -Rev1	CAGGCGGAGGTGGATCCGGCGGTGGCGGATCGGATGTTTTGATGACCCAAACTC
V_{L} -For1	GCTCAACTTTCTT <u>GTCGAC</u> TTTATCATCATCATCTTTATAATCTTTGATTTCCAGCTTGGTGC
	(Sal I)
V_{L} -For2	GCTCAAACTTTCTT <u>GTCGAC</u> TTATCAGCATGCTCCGCCCGCACGTTTGATTTCCAGTTTGGTT
	TTATCATCATCTTTATA (Sal I)

^{*a*} Underlined in the nucleotide sequences

V_{H} -sequence

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4F10-cDNA Cloned VH	GAG	GTC	CTG	CTG	CAA	CAG	TCT	GGA	CCT	GAG	CTG	GTG	AAG	CCT	GGG	GCT	TCA	GTG
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4F10-cDNA CTG GAA ATC AAA $FLAG \ tag$ Cloned VZ --- --- GAT TAT AAA GAT GAT GAT GAT AAA L E I K D D D K

Linker sequence

GGT GGA GGC GGT TCA GGC GGA GGT GGA TCC GGC GGT GGC GGA TCG G G G G S G G G S G G S G G S

Figure S1. Nucleotide and amino acid sequences of the V_H and V_L domains of the anti-Tnc antibody 4F10.

The upper and lower lines show the sequences of the cDNA and the scFv-4F10 construct, respectively. One silent mutation and one missense (Val3Leu) mutation were introduced in V_L of scFv-4F10, due to the use of degenerate primer in the PCR cloning steps. The complementarity-determining regions (CDRs)⁷ are shown in bold italic letters. The sequence of the linker that connects V_H and V_L is also shown.

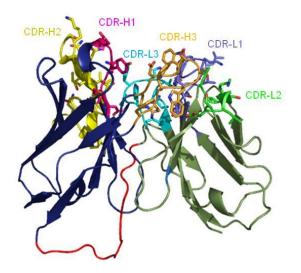


Figure S2. Protein modeling of scFv-4F10.

The protein ribbon structures of scFv-4F10 was generated using the SWISS-MODEL software.⁸ The β -sheet structures consisting of the amino acids of the framework regions (FRs) are drawn by bold arrows (dark blue and dark green arrows for V_H and V_L, respectively). Amino acid residues forming the CDRs⁷ (CDR-H1, -H2, -H3 in the V_H domain and CDR-L1, -L2, -L3 in the V_L domain) are indicated. The linker peptide is shown in red. This model structure has the characteristic V-domain architecture, *i.e.*, the CDR-composing amino acids form flexible loops that are raised above the surface of the rigid β -sheet scaffold.

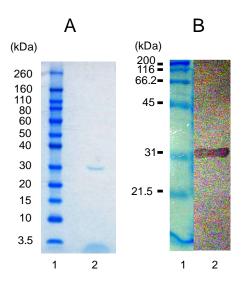


Figure S3. PAGE of scFv-4F10.

(A) SDS-PAGE of affinity-purified scFv-4F10 (Coomassie brilliant blue staining): lane **1**, M_r marker; **2**, scFv. (B) Immunoblotting of the affinity-purified scFv-4F10: lane **1**, M_r marker; **2**, scFv. The scFv was detected by sequential incubation with anti-FLAG M2 antibody followed by POD-conjugated rabbit anti-mouse IgG Fc-specific antibody. Bound POD activity was visualized using hydrogen peroxide and 4-chloro-1-naphthol as substrates.

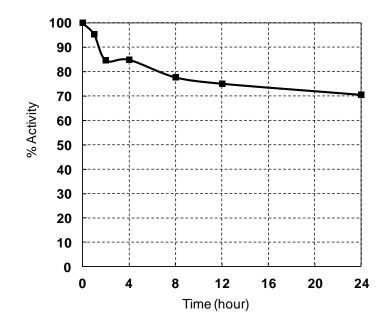


Figure S4. Retention of the binding activity of scFv-Cys against Tnc that has been incubated in plasma.

Normal rat plasma (Jackson ImmunoResearch) (200 μ L) containing scFv-Cys (1.0 μ g) was prepared in sextuplicate, and each of them was incubated at 37°C for 1, 2, 4, 8, 12, or 24 h. Then, an aliquot (40 μ g) was taken from the mixture, diluted 25-fold with PBS, and 100 μ L aliquot of the resulting solution (containing 200 ng/mL scFv, if this remained intact) was incubated in duplicate at 37°C for 1 h in microwells coated with a 10 μ g/mL Tnc solution as described above. The wells were washed 3 times with T-PBS, and the POD-conjugated anti-FLAG M2 antibody (0.50 μ g) in T-PBS (100 μ L) was incubated at 37°C for 1 h. After washing the wells, bound POD activity was determined as described in the text.

References (for Supporting Information)

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