Supporting Information

Use of ^{18}F‑ 2-Fluorodeoxyglucose to Label Antibody Fragments for Immuno-Positron Emission Tomography of Pancreatic Cancer

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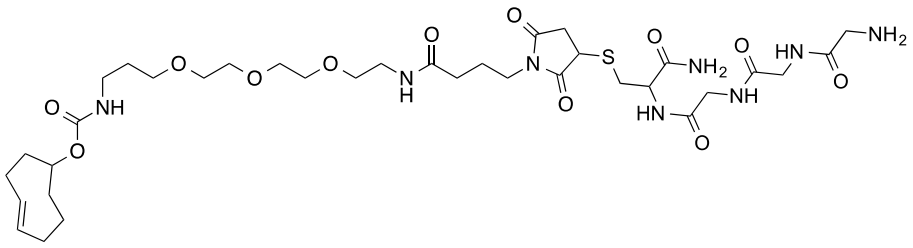
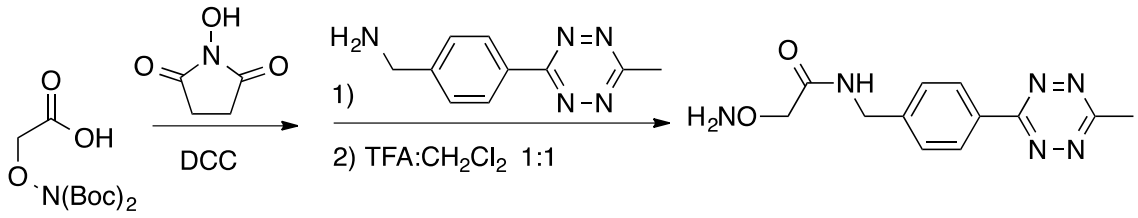
Synthesis of $(Gly)_3$-TCO.



The tetrapeptide GGGC was synthesized by standard solid phase peptide synthesis. Maleimide-TCO (from ClickChemistryTools) was dissolved in 0.05 M $NaCO_3$ buffer pH 8.3. The tetrapeptide GGGC was added and left to stir at room temperature for 2 h until LC-MS indicated near-complete conversion to the product. The solution was filtered and purified by reverse phase-HPLC with a semi-preparative column (Phenomenex, C_{18} column , Gemini, 5 µm, 10x250 mm) at a flow rate of 5.0 mL/min.; solvent A: 0.1% TFA in H_2O, solvent B: 0.1% TFA in CH_3CN. $(G)_3$-TCO eluted at 30–35% solvent B. Fractions containing pure product were collected and lyophilized. LC-MS calculated for $C_{35}H_{59}N_8O_{12}S$ $[M+H]^+$ 815.40, found 815.41.

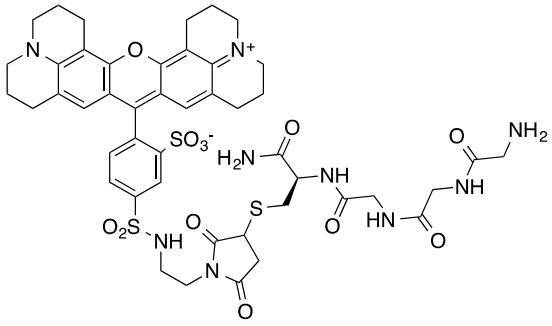
Synthesis of Aminooxy-tetrazine.



Bis-Boc-aminooxy acetic acid (0.5 g, 1.7 mmol) was dissolved in THF (25 mL) in a 50 mL flask and the solution was cooled to 0 °C in an ice bath. N,N′-Dicyclohexylcarbodiimide (DCC) (0.39 g, 1.9 mmol) was added slowly to the solution followed by the addition of N-hydroxysuccinimide (NHS) (0.22 g, 1.9 mmol). The reaction was stirred at 0 °C for an additional h and then overnight at room temperature until TLC showed complete conversion to the product. The solution was filtered to remove the insoluble N,N′-Dicyclohexylurea (DCU) byproduct and

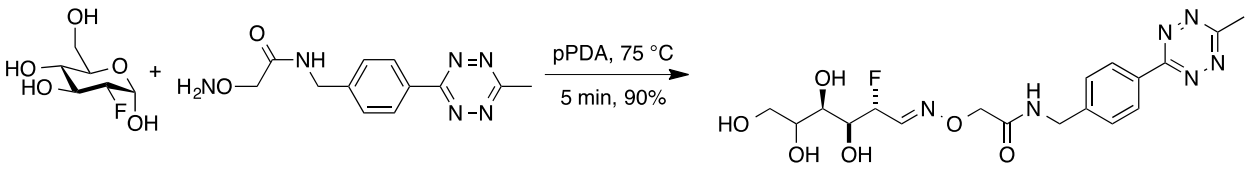
the solvent was removed *in vacuo*. The product was used without further purification. Amino-tetrazine (0.2 g, 1.0 mmol) was dissolved in DMF (10 mL) in a 25 mL flask. The crude product from the previous step, NHS activated bis-boc-aminooxy-acetic acid (0.58 g, 1.5 mmol), was added to the solution and the reaction was stirred at room temperature for 3 h when TLC analysis showed complete conversion to the product. Next, the solvent was removed *in vacuo* and a mixture of 1:1 TFA:CH_2Cl_2 (10 mL) was added to the flask. The reaction was allowed to proceed for 2 h at room temperature when TLC showed complete conversion to the product. The solvent was removed *in vacuo* and the product was redissolved in a mixture of 1:1 DMF:H_2O (1 mL). The solution was filtered and purified by reverse phase-HPLC with a semi-preparative column (Phenomenex, C_{18} column , Gemini, 5 µm, 10x250 mm) at a flow rate of 5.0 mL/min; solvent A: 0.1% TFA in H_2O, solvent B: 0.1% TFA in CH_3CN. Aminooxy-tetrazine eluted at 25–30% solvent B. Fractions containing pure product were collected and lyophilized. LC-MS calculated for $C_{12}H_{15}N_6O_2$ [M+H]+ 275.12, found 275.10.

Synthesis of $(Gly)_3$-Texas Red.



The tetrapeptide GGGC was synthesized by standard solid phase peptide synthesis. Maleimide-Texas Red (from Vector lab) was dissolved in 20 mM $NaHCO_3$ buffer (pH 8.3). The tetrapeptide GGGC was added and left to stir at room temperature for 3 h until TLC (1:2 Hex:EtOAc v/v) indicated near-complete conversion to the product. The solution was filtered and purified by reverse phase-HPLC with a semi-preparative column (Phenomenex, C_{18} column , Gemini, 5 µm, 10x250 mm) at a flow rate of 5.0 mL/min; solvent A: 0.1% TFA in H_2O, solvent B: 0.1% TFA in CH_3CN. $(G)_3$-Texas Red eluted at 40–45% solvent B. Fractions containing pure product were collected and lyophilized. LC-MS calculated for $C_{46}H_{54}N_9O_{12}S_3[M+H]^+$ 1020.305, found 1020.310.

Catalyzed oxime ligation between 2-Fluorodeoxyglucose and aminooxy-tetrazine.

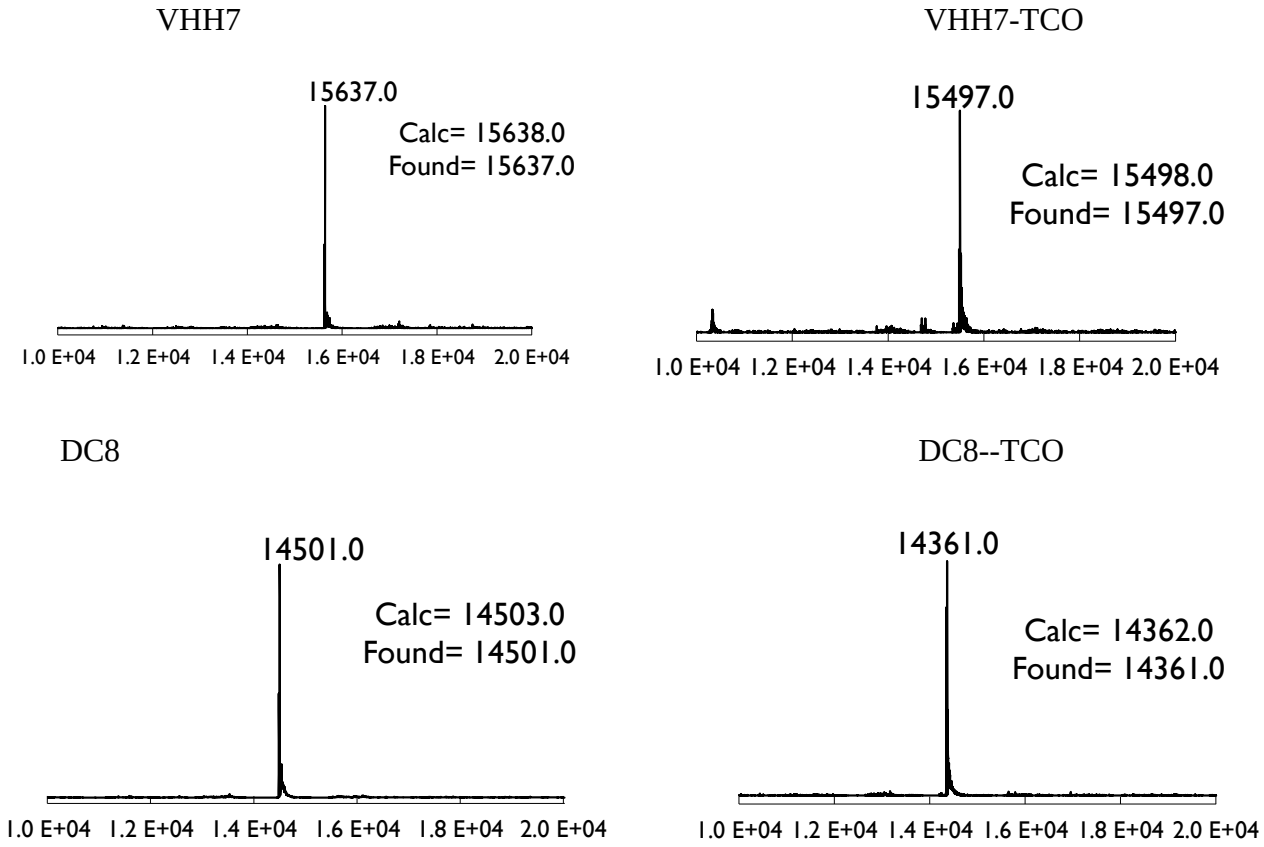


Reaction mixtures (50 µL) contained aminooxy-tetrazine (0.2-0.3 M), 2-Fluorodeoxyglucose (1 mM) and p-phenylenediamine (0.4-0.6 M). After incubation at 75 °C with agitation for ~5-10 min, reaction product was analyzed by LC-MS and HPLC, with yields generally >90%. LC-MS calculated for $C_{18}H_{24}FN_6O_6$ $[M+H]^+$ 439.17, found 439.17.

Enzymatic incorporation of substrates into proteins using sortase.

The penta-mutant sortase A, with an improved k_{cat}, was used.[1] Reaction mixtures (1 mL) contained Tris·HCl (50 mM, pH 7.5), $CaCl_2$ (10 mM), NaCl (150 mM), triglycine-containing probe (500 µM), LPETG-containing probe (100 µM), and sortase (5 µM).[2, 3] After incubation at 4 °C with agitation for 2 h, reaction products were analyzed by LC-MS. Yields were generally >90%. When the yield was below 90%, the reaction was allowed to proceed for an additional two hours, with addition of sortase to 10 µM and triglycine-containing probe to 1 mM. Ni-NTA beads were added to the reaction mixture with agitation for 5 min at 25 °C followed by centrifugation to remove sortase and any remaining unreacted His-tagged substrate. The final product was purified by size exclusion chromatography in PBS or Tris·HCl (50 mM, pH 7.5). The labeled protein was stored at -80 °C with 5% glycerol for up to six months.

LC-MS analysis of VHHs and their corresponding sortagged products.



Two-photon imaging.

Two-photon imaging was performed with an Olympus BX61 upright microscope (Olympus 25X 1.05 NA Plan Objective), fitted with a SpectraPysics MaiTai DeepSee laser. Images were acquired using 910 nm excitation and following filters: 2nd harmonic emission (from collagen) (460-510 nm) and GFP (495-540 nm), separated by a 505 nm dichroic mirror, and a third filter (575-630 nm) for the Texas Red signal. Images were acquired with 5 µm Z resolution with Olympus FluoView FC1000 software. Tile images were saved as JPEG files. Images in Figures 2G, 2F, 3H and Image 01 in the SI were processed to obtain a scale bar in Imaris v 7.4.0; no intensity or contrast adjustments were made.

Synthesis of ^{18}F –Tetrazine.

[^{18}F]-FDG (no carrier added, (n.c.a.)) in H_2O (8.6 ± 0.6 mCi), purchased from PETNET, was transferred to a reaction vessel (10 mL) and the solvent was removed *in vacuo*. After drying, DI water (20 μL) and aminooxy-tetrazine (25 μL, 0.35 M stock solution in DMSO) were added, and the solution mixture was transferred to a 0.5 mL tube containing pPDA (2.5 mg) and was heated to 70 ^{o}C for 10 min with constant agitation. The mixture was diluted with H_2O (1 mL) and subjected to preparative HPLC purification (17.5% MeCN in H_2O, 0.1% TFA at 4.8 mL/min using a Phenomenex, C_{18} column, Gemini, 5 μm, 10 x 250 mm. ^{18}F-Tetrazine was collected (t_R = 14.0 min) in 5–6 mL of solvent, diluted with H_2O (40 mL) and isolated by manual C18 solid phase extraction. Elution from the C18 cartridge with MeCN (4 x 200 μL) gave 2.3 ± 0.5 mCi, a 50.7 ± 7.3% decay-corrected radiochemical yield in an average time of 105 min (n = 4).

Synthesis and characterization of ^{18}F –VHHs.

In a typical reaction, a 1.5-mL centrifuge tube was loaded with VHH-TCO in 1xPBS (40 μL, 150 μM), 1xPBS (150 μL), and ^{18}F-Tetrazine in DMSO (1.4 ± 0.2 mCi (51.8 MBq), 100 μL). The tube was sealed and shaken at room temperature for 20 min. The mixture was analyzed by radio-TLC (ITLC, 100% MeCN, R_f ^{18}F-Tetrazine = 0.9, R_f ^{18}F-VHH = 0.0) showing 90% conversion to ^{18}F-VHH. The reaction mixture was loaded onto a PD-10 size-exclusion cartridge (GE Healthcare) and elution with 1xPBS provided 0.5 ± 0.2 mCi (18.5 MBq) of ^{18}F-VHH in 55.7% decay-corrected radiochemical yield.

PET-CT imaging.

All procedures and animal protocols were approved by the Massachusetts General Hospital subcommittee on research animal care. For all imaging experiments, mice were anesthetized using 1.5% isoflurane in O_2 at a flow rate of ~1 L/min. Mice were imaged with PET-computed tomography (CT) using an Inveon small animal scanner (Siemens, Munich, Germany). Each PET acquisition took approximately 30 minutes. High resolution fourier rebinned PET images were reconstructed by a 3D ordered subsets expectation algorithm using maximum a priori (OSEM3D/MAP) with 18 MAP iterations and 2 OSEM3D iterations into 0.796 x 0.796 x 0.861 mm images on a 128 x 128 x 159 image matrix. Peak sensitivity of the Inveon accounts for 11.1% of positron emission, with a mean resolution of 1.65 mm. More than 100 counts were

acquired per pixel, and the mean signal-to-noise ratio was greater than 20. CT images were acquired using an 80 kVp 500 mA x-ray tube over 360 projections on a 125mm detector. A modified feldkamp conebeam reconstruction algorithm (COBRA Exxim incorporated) was used to reconstruct the CT images into a 110 μm isotropic image matrix of 512 x 512 x 768. Reconstruction of data sets, PET-CT registration, and image analysis were performed using IRW software (Siemens). Two- and three-dimensional visualizations were produced using the DICOM viewer OsiriX (The OsiriX Foundation, Geneva, Switzerland).

Blood half-life measurement of ^{18}F-VHHs.

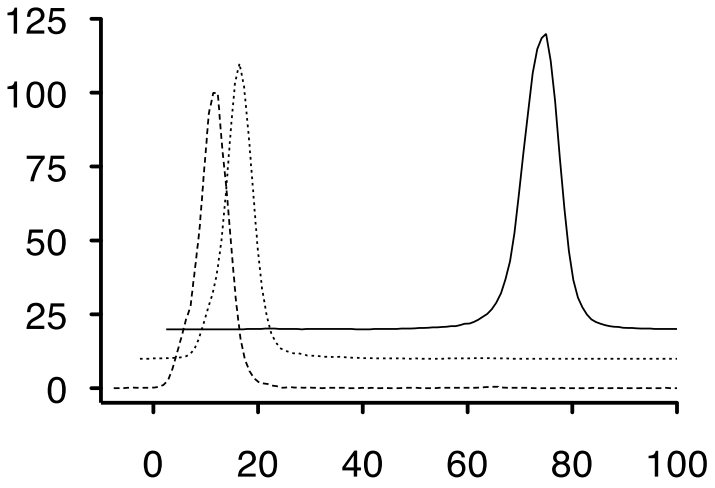
Mice were administered 30 ± 3 uCi of ^{18}F-VHH7 by intravenous tail-vein injection. Blood samples were obtained by retro-orbital puncture using tared, heparinized capillary tubes. Blood samples and capillaries were weighed and radioactivity was measured using a Perkin-Elmer Wallac Wizard 3" 1480 Automatic Gamma Counter. Values are expressed as percentages of the injected dose per gram of tissue were fit (least squares) to a two-compartment bi-exponential decay model was performed using GraphPad Prism 4.0c (Figure S3).

PET Standard Uptake Value Calculation.

Standard uptake value (SUV) is the derived ratio of tissue radioactivity concentration (Bq/mL) and the injected radioactivity per gram of the mouse's body weight. The calculation used the following equation: SUV = (region of interest radioactivity concentration)/(injected activity/mouse total mass).

Analysis of the purity of the radiolabeled VHHs.

The purity of the radiolabeled VHHs were assessed with TLC performed on silica impregnated glass sheets (ITLC plates, Pall Life Sciences). Plates for ^{18}F-VHHs were developed with 100% acetonitrile and analyzed using a Bioscan AR-2000 scanner operated by WinScan V3 software package.



Sequence of VHH7, DC1, DC8, DC14 and DC15.

VHH7:
Nucleic Acid:
CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGGCTGGGGACTCTCTGAG
ACTCTCCTGCGCAGCCTCTGGACGCACCTTCAGTCGCGGTGTAATGGGCTGGTTCCG
CCGGGCTCCAGGGAAGGAGCGTGAGTTTGTAGCAATCTTTAGCGGGAGTAGCTGGA
GTGGTCGTAGTACATACTATTCAGACTCCGTAAAGGGCCGATTCACCATCTCCAGAG
ACAACGCCAAGAACACGGTGTATCTGCAAATGAACGGCCTGAAACCTGAGGACACG
GCCGTTTATTACTGTGCAGCGGGATATCCGGAGGCGTATAGCGCCTATGGTCGGGAG
AGTACATATGACTACTGGGGCCAGGGGACCCAGGTCACCGTCTCCTCAGGATCCCTT
CCTGAAACTGGT

Peptide:
QVQLQESGGGLVQAGDSLRLSCAASGRTFSRGVMGWFRRAPGKEREFVAIFSGSSWSG
RSTYYSDSVKGRFTISRDNAKNTVYLQMNGLKPEDTAVYYCAAGYPEAYSAYGRESTY
DYWGQGTQVTVSSGSLPETGGHHHHHH

DC1:
Nucleic Acid:

CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAG
ACTCTCCTGTGCAGCCTCTGGACGCACCTTCAGTAGGTCAGCCATGGGCTGGTTCCG
CCAGGCTCCAGGGAAGGAGCGTGAGTTTGTAGCAACTATTAGTTGGAGTGGTGGTG
TCACATACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCA
AGAACACGGTGTATCTGCAAATGAACGGCCTGAAACCTGAGGACACGGCCGTTTAT
TACTGTGCAGCGGGATATCCGGAGGCGTATAGCGCCTATGGTCGGGAGAGTACATA
TGACTACTGGGGCCAGGGGACCCAGGTCACCGTCTCCTCAGGATCCCTTCCTGAAAC
TGGT

Peptide:
QVQLQESGGGLVQAGGSLRLSCAASGRTFSRSAMGWFRQAPGKEREFVATISWSGGVT
YYADSVKGRFTISRDNAKNTVYLQMNGLKPEDTAVYYCAAGYPEAYSAYGRESTYDY
WGQGTQVTVSSGGLPETGGHHHHHH

DC8:
Nucleic Acid:
CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGCCTGGGGGGTCTCTGAG
ACTCTCCTGTACAGCCTCTGGATTCACATTCAGTACTTACTACATGAGCTGGGTCCGC
AAGGCTCCAGGGAAGGGGCCCGAGTGGGTCTCAGTTATGAATAGTAGTGGTGGTGA
CACAAGGTATGCAGACTTCGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCA
AGAACACACTGTATCTCCAAATGAACAGCCTGAAACCTGAGGATACGGCCCTGTATT
ACTGTGCGCAAGGTAGATCAGATATATACCCAACCTTCACGCGGGGCCAGGGGACC
CAGGTCACCGTCTCCTCAGGAGGACTGCCGGAAACCGGC

Peptide:
QVQLQESGGGLVQPGGSLRLSCTASGFTFSTYYMSWVRKAPGKGPEWVSVMNSSGGD
TRYADFVKGRFTISRDNAKNTLYLQMNSLKPEDTALYYCAQGRSDIYPTFTRGQGTQVT
VSSGSLPETGGHHHHHH

DC14:
Nucleic Acid:
CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGGCTGGGGGCTCTCTGAG
ACTCTCCTGTGCAGCCTCTGGACGCACCTTCGGATATGCCTTGGGCTGGTTCCGCCA
GGCTCCGGGCAAGGAGCGTGAGTTTGTAACAGCTATTAACTGGAGTGGTGGTAGGT
TAGGCTATGCAGACTCCGTGAAGGGTCGATTCACCATCTCCAAAGACAACTACAAG
AACACGTTGTATCTGCAAATGAACGGCCTGAAACCTGAGGACACGGCCGTTTATTAC
TGTGCAGCCGGGAGGGGCTCGACCCGCTCTGATGACTATGACTACTGGGGCCAGGG
GACCCAGGTCACCGTCTCCTCAGGATCCCTTCCTGAAACTGGT

Peptide:
QVQLQESGGGLVQAGGSLRLSCAASGRTFGYALGWFRQAPGKEREFVTAINWSGGRLG
YADSVKGRFTISKDNYKNTLYLQMNGLKPEDTAVYYCAAGRGSTRSDDYDYWGQGTQ
VTVSSGSLPETGGHHHHHH

DC15:
Nucleic Acid:

CAGGTGCAGCTGCAGGAGTCAGGGGGAGGATTGGTGCAGCCTGGGGCGTCTCTGAG
ACTCTCCTGCGTTTCTGCGTTCTCCTTAGATCATTATTCCGTAGCCTGGTTCCGCCAG
GCCCCAGGGAAGGAGCGTGAGGGGGTCTCATGTCACAGTCGGTCAAATAAAGCCAC
GGACTATGCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAGCGCCAAGC
GCACGGTGTATCTGCAAATGAACAACCTGAAACCAGAAGACACAGCCGTATATTAC
TGTGCAGTCAAACAATGGGGAATGTGCACTGGTGGTGTTTGGGGCCGTTCCCAGTAT
GACTACTGGGGCCAGGGGACCCAGGTCACCGTCTCCTCAGGATCCCTTCCTGAAACT
GGT

Peptide:
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YADSVKGRFTISRDSAKRTVYLQMNNLKPEDTAVYYCAVKQWGMCTGGVWGRSQYD
YWGQGTQVTVSSGSLPETGGHHHHHH

Supporting Figures.

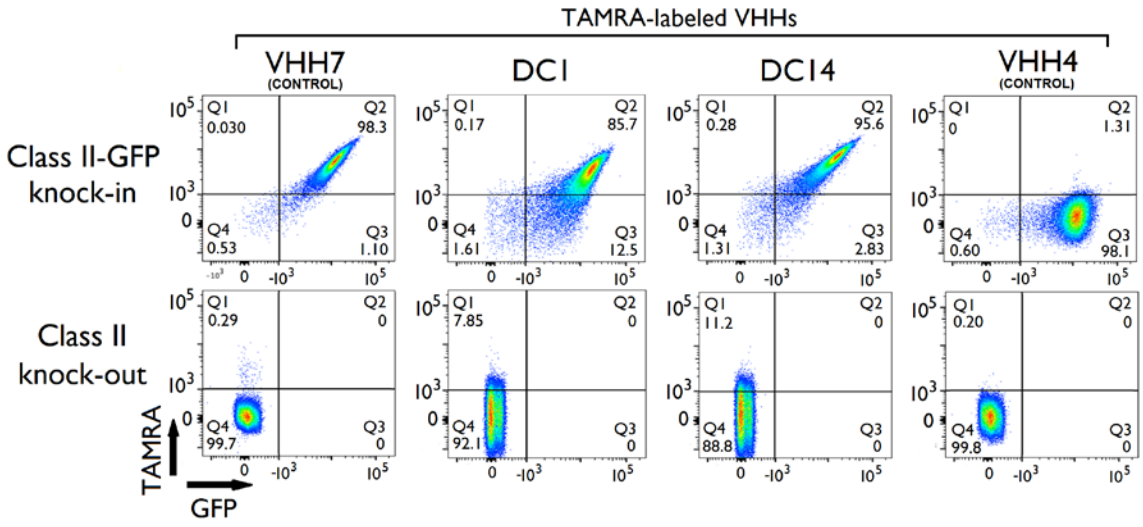

Figure S1. *DC1 and DC14 specifically recognize the mouse Class II MHC complex*: 10^6 splenocytes isolated from C57BL/6 class II-GFP knock-in and class II knock-out mice were stained with TAMRA-sortagged VHHs as indicated. Plots are gated on live, CD19+ cells. VHH7 has been previously demonstrated to recognize mouse MHC class II. DC1 and DC14 are novel VHHs isolated through staining of dendritic cells. VHH4 is specific for human MHC class II and does not recognize the murine homologue.

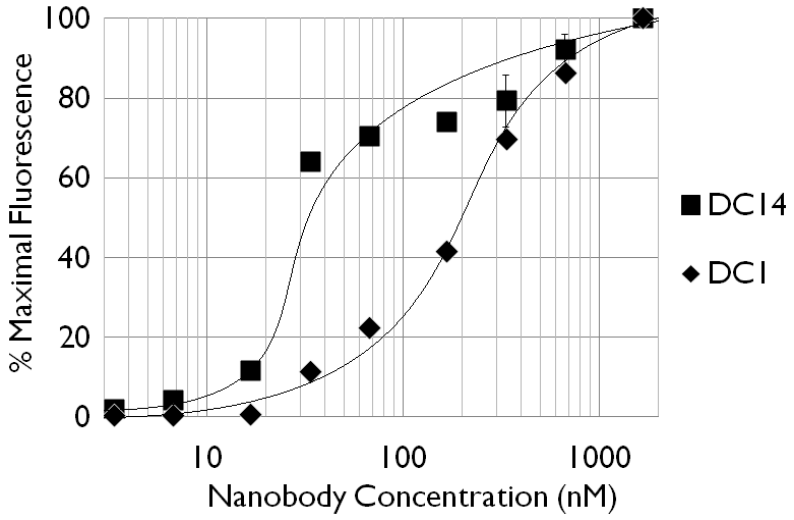

Figure S2. *DC14 is able to stain murine B-cells at concentrations too low for DC1 staining:* 10^6 splenocytes isolated from WT C57/BL6 mice were stained with the indicated concentrations of TAMRA-labeled VHH. Populations were gated on live, CD19+ cells, and the mean TAMRA fluorescence of each population is plotted. These data are derived from a single flow cytometry run but are consistent across numerous replicates (n=5).

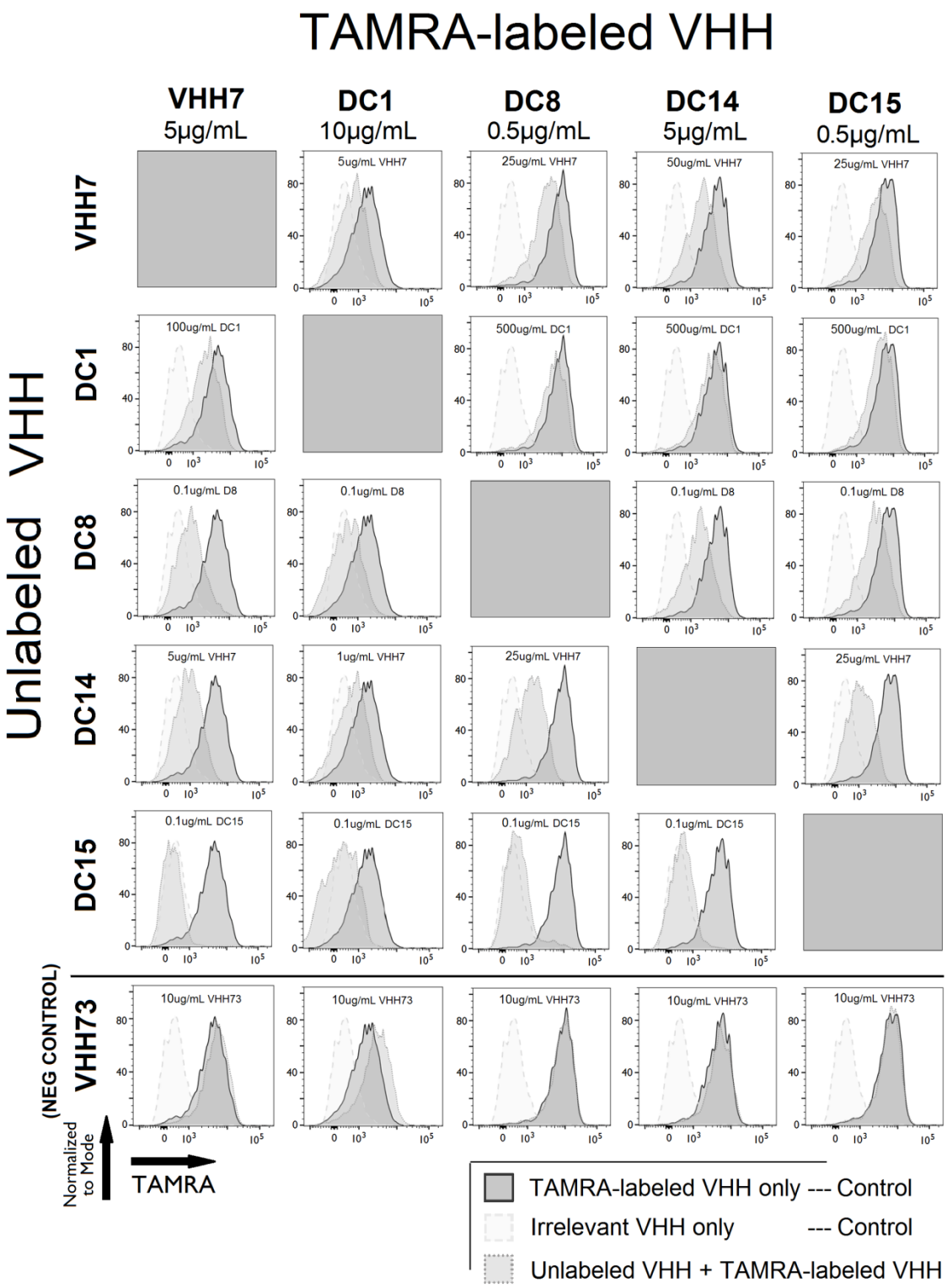
TAMRA-labeled VHH
VHH7
5μg/mL
DC1
10μg/mL
DC8
0.5μg/mL
DC14
5μg/mL
DC15
0.5μg/mL
Unlabeled VHH
VHH7
DC1
DC8
DC14
DC15
VHH73
(NEG CONTROL)
5ug/mL VHH7
25ug/mL VHH7
50ug/mL VHH7
25ug/mL VHH7
100ug/mL DC1
500ug/mL DC1
500ug/mL DC1
500ug/mL DC1
0.1ug/mL D8
0.1ug/mL D8
0.1ug/mL D8
0.1ug/mL D8
5ug/mL VHH7
1ug/mL VHH7
25ug/mL VHH7
25ug/mL VHH7
0.1ug/mL DC15
0.1ug/mL DC15
0.1ug/mL DC15
0.1ug/mL DC15
10ug/mL VHH73
10ug/mL VHH73
10ug/mL VHH73
10ug/mL VHH73
10ug/mL VHH73
Normalized to Mode
TAMRA
TAMRA-labeled VHH only --- Control
Irrelevant VHH only --- Control
Unlabeled VHH + TAMRA-labeled VHH

Figure S3. *VHH7, DC1, DC8, DC14, and DC15 compete for a common binding epitope*: 10^6 splenocytes isolated from WT C57/BL6 mice were co-stained with TAMRA-labeled VHH and a variable concentration of unlabeled VHH. The FACS plots indicate the minimum concentration of unlabeled VHH required for a 10% reduction in TAMRA fluorescence compared to a single-stained control. VHH73 is specific for murine T-cells and was used to co-stain lymphocyte populations as a negative control for binding interference.

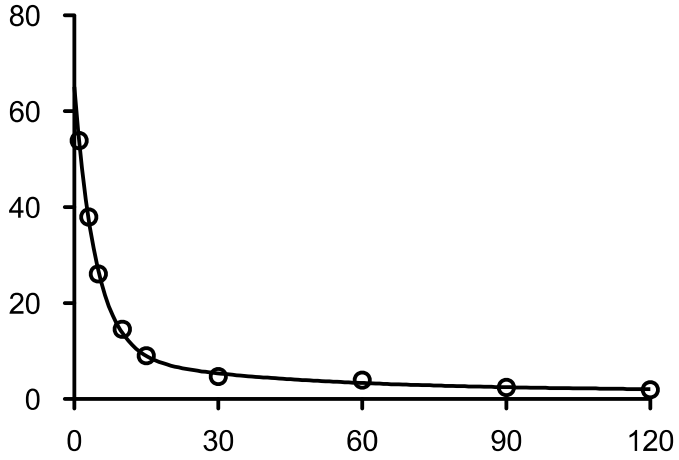

Figure S4. Blood half-life data for ^{18}F-VHHs. Data is reported as percent of injected dose per gram blood (%ID/g). Data was fitted to a two-compartment model (bi-exponential non-linear regression) to give a weighted blood half-life of 6.0 min.

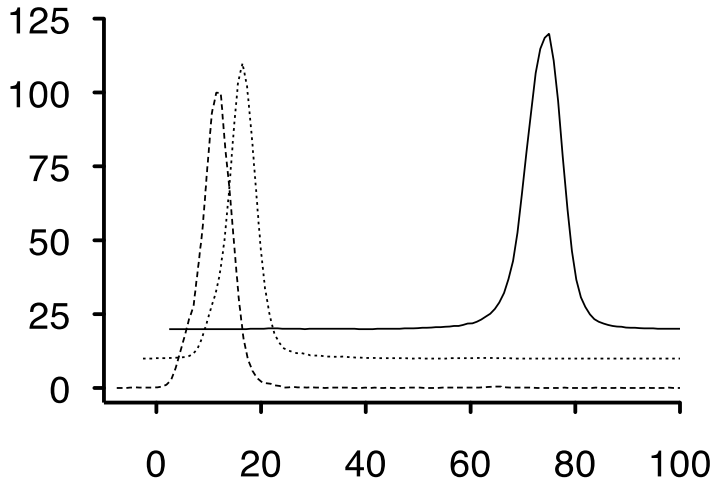

Figure S5. Radio-TLC analysis of ^{18}F- VHHs. Radio-TLC analysis of labeled VHHs was performed after size-exclusion chromatography demonstrating >98% radiochemical purity of all labeled VHHs.

References.

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(3) Witte, M. D., Cragnolini, J. J., Dougan, S. K., Yoder, N. C., Popp, M. W., and Ploegh, H. L. (2012) Preparation of unnatural N-to-N and C-to-C protein fusions. , *Proc. Natl. Acad. Sci. 109*, 11993–11998.
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