Supporting Information

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SI Materials and Methods

C57BL/6, C3H, and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were ordered from the Jackson Laboratory and housed and maintained by the Department of Laboratory Animal Medicine at the University of California, Los Angeles (UCLA). Protocols for all animal studies were reviewed and approved by the UCLA Chancellor's Animal Research Committee.

Determination of V_H and V_L Sequences from Parental Hybridomas. The YTS 169.4.2.1 (YTS169) hybridoma was obtained from the Therapeutic Immunology Group at Oxford University and cultured in Iscove's modified Dulbecco's medium (American Type Culture Collection) plus 10% FBS and penicillin/streptomycin (Pen/Strep) (1). The 2.43 hybridoma was obtained from the American Type Culture Collection (TIB-210) and cultured in DMEM (Mediatech, Inc.) plus 10% FBS and Pen/Strep. V_H and V_L sequences were obtained by RT-PCR using primers published by Dübel et al. (2). RNA was isolated from hybridomas grown in culture using the Quick-RNA MicroPrep kit (Zymo Research) according to the manufacturer's instructions. Freshly isolated RNA was immediately used for RT-PCR using a combination of primers as reported by Dübel et al. (2) for the heavy-chain FR1 region (Bi3, Bi3b, and Bi3c) and the kappa-chain FR1 region (Bi6, Bi7, and Bi8). The heavy-chain C_H1 primer used was 5′-CGG AAT TCA GGG GCC ATG GGA TAG AC. The kappa-chain constant domain primer used was 5′-CGG AAT TCG GAT GGT GGG AAG ATG GA. RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) according to the manufacturer's instructions before Tris-acetate-EDTA–agarose gel extraction, ligation using a TOPO TA Cloning kit (Invitrogen), and DH5α (Invitrogen) transformation. Colonies were selected for isolation of plasmid DNA using a Miniprep Kit (Invitrogen) for subsequent sequencing at the UCLA sequencing core facility. Sequences were analyzed with BLAST for V_H or V_L homology. Sequences were verified by three identical recovered sequences from at least two different experiments. For the hybridoma 2.43, the obtained V_H and V_L sequences were confirmed with trypsin digest-MS of the purified parental antibody, which was performed at the UCLA core facility. The YTS169 hybridoma was engineered to antibody fragments without further V_H and V_L validation.

Design and Construction of Anti-mCD8 Minibodies. The 2.43 and YTS169 minibody (Mb) constructs were synthesized by GeneArt (Invitrogen) to contain a Kozak sequence, followed by the mouse Ig kappa secretion signal, V_H, an 18 GlySer-rich amino acid linker (GSTSGGGSGGGSGGGGSS), V_L, murine IgG2a hinge, the murine IgG2a C_H3 domain, and a C-terminal hexahistidine sequence (Fig. 1*A*). The Mb cassette contains the N-terminal XbaI and C-terminal EcoRI restriction sites for subcloning into the mammalian expression vector pEE12 (Lonza).

Expression and Purification of Engineered Mb Antibody Fragments. A total of 2×10^6 NS0 mouse myeloma cells were transfected with 10 μg of FspI (New England Biolabs) linearized vector DNA by electroporation (Multiporator; Eppendorf) and selected in glutamine-deficient DMEM as previously described (3). The supernatants of individual clones were screened for expression by nickel-nitrilotriacetic acid (Ni-NTA) HisSorb Plates (Qiagen) using the goat anti-mouse IgG2a-alkaline phosphatase conjugate for detection (1:2,500; Santa Cruz Biotechnologies). Expression was also confirmed for clones positive for expression by ELISA by SDS/PAGE, followed by Western blot analysis using the same goat anti-mouse IgG2a-alkaline phosphatase conjugate (1:2,500) and developed with the NBT/BCIP Color Development substrate kit (Promega). The highest producing clones were expanded and brought to terminal culture for expression of secreted proteins.

Soluble Mbs were purified from cell culture supernatants using Ni-NTA affinity chromatography (GE Healthcare) using an ÄKTA purifier FPLC (GE Healthcare). Supernatants were loaded onto the column in the presence of 10 mM imidazole and eluted with a gradient of 10–500 mM imidazole. The purified proteins were then dialyzed against PBS using Slide-A-Lyzer dialysis cassettes [molecular weight cutoff (MWCO) = 10,000; Thermo Scientific] and concentrated with a Vivaspin 20 (MWCO = 10,000; Sartorius). Final protein concentrations were determined by measuring UV absorbance at 280 nm. Purified proteins were analyzed by SDS/PAGE under nonreducing conditions. Native structural size was determined by Superdex 200 (GE Healthcare) size exclusion chromatography (SEC) using PBS as the running buffer.

Production of Soluble CD8αβ Fusion Protein. PCR was used to amplify the soluble domains of both CD8α (residues Lys1–Ser124 of mature CD8α, Lyt2.2$^+$) and CD8β (residues Leu1–Val117 of mature CD8β) and to fuse them via a 29-aa α-helical linker (AGSADDARKDAGSKDDARKDDARKDGSSA). This linker is similar to the linker previously described for soluble CD8αβ (sCD8αβ) fusion (4), except for the GS amino acids at linker positions 12 and 13 (underlined) due to the insertion of a BamHI site for cloning purposes. CD8α was amplified to contain an N-terminal AgeI site and a C-terminal sequence corresponding to AGSADDARKDAGS. CD8β was amplified to contain the N-terminal amino acid sequence GSKDDARKDDARKDGSSA and a C-terminal NotI site. The following primers were used:

sCD8α-Forward (F): 5′-CACACAGAGCTCACCGGTAAGCCACAGGCACCCGAAC
sCD8α-Reverse (R): 5′-TGTGTGGGATCCCGCATCCTTTCTTGCATCGTCGGCAGATCCTGCAGAGTTCACTTTCTGAAG
sCD8β-F: 5′-CACACAGGATCCAAAGATGACGCAAGGAAGGACGATGCTAGGAAGGATGGATCTTCCGCACTCATTCAGACCCCTTCG
sCD8β-R: 5′-TGTGTGTCTAGACGCGGCCGCAACCACAGTCAGCTTCGTC

Briefly, PCR products of sCD8α and sCD8β were gel-purified, digested with BamHI, and ligated using T4 DNA ligase. Ligations were used as the template for PCR using the sCD8α-F and sCD8β-R primers. The PCR product was restriction enzyme-digested with AgeI and NotI and ligated into the predigested pEE12-2.43 Mb cassette constructed above for the addition of a C-terminal His-Tag. Sequence verification, NS0 electroporation, clonal selection, protein production, and Ni-NTA purification were performed as described above. SEC using a Superdex 75 column (GE Healthcare) was required for final purification.

Affinity Measurements. Before surface plasmon resonance (SPR) analysis, solution-phase binding of the Mbs to the recombinant sCD8αβ was confirmed using SEC. Equimolar amounts of sCD8αβ and Mb were incubated together in PBS for 5 min before Superdex 200 SEC analysis. Binding was confirmed by both earlier elution times in SEC and SDS/PAGE analysis of the elution fractions from SEC. SPR analysis was performed on a Biacore 3000

(Precision Antibody, Inc.). Briefly, Mbs were captured using goat anti-mouse IgG Fc, and sCD8αβ antigen was flowed over the chip at 100, 50, 25, 12.5, 6.25, and 0 nM. The equilibrium dissociation constant (K_D) was calculated from the observed association (k_a) and dissociation (k_d) rate constants.

FITC Conjugation. The 2.43 Mb was incubated with a 40-fold molar excess of FITC (Molecular Probes) at pH 8.5 for 4 h at 4 °C. Excess FITC was removed using a PD-10 column in PBS (GE Healthcare). Protein was concentrated using a 0.5-mL spin filter (MWCO = 3 kDa; Amicon), and the conjugation efficiency was evaluated using a NanoDrop (Thermo Scientific) to calculate the ratio of moles of fluorescein to moles of protein.

Flow Cytometry. A total of 2×10^5 BW58, TK-1, or EL-4 cells (American Type Culture Collection) in 200 μL of PBS plus 1% FBS was incubated with 2 μg of the anti-CD8 Mb constructs for 45 min on ice. After two washes in PBS plus 1% FBS, they were subsequently stained with mouse goat anti-mouse IgG2a-phycoerythrin (PE; Santa Cruz Biotechnologies) for 45 min on ice in PBS plus 1% FBS. Following two additional washes in PBS plus 1% FBS, flow cytometry analysis was performed using a BD Biosciences LSR II at the UCLA flow cytometry core facility.

Flow cytometry on primary cells from Lyt2.2$^+$ C57BL/6 (B/6) and Lyt2.1$^+$ C3H mice was performed on single-cell suspensions from the spleen, bone marrow, peripheral blood, thymus, and lymph nodes. Organs were mashed over 75-μm filters and then 40-μm filters in RPMI plus 5% FBS to produce single-cell suspensions. Following RBC lysis using ammonium-chloride-potassium lysis buffer, the cells were stained for 1 h on ice with either Lyt2.2-specific fluorescein-conjugated 2.43 Mb or anti–CD8-fluorescein (clone 53-6.7; eBioscience) and anti–CD4-PE (clone GK1.5; eBioscience) or anti–CD45-allophycocyanin (clone 30-F11; eBioscience). Cells were then washed with PBS and analyzed using a BD FACSCanto (Becton Dickinson).

CD8 Depletion. Mice were treated for three consecutive days with 330 μg of anti-CD8 depleting antibody (clone 53-6.7 purchased from University of California, San Francisco Monoclonal Antibody Core) injected i.p. (165 μL in saline) or 250 μg of 2.43 Mb injected i.v. (125 μL in saline). Two to three days posttreatment, single-cell suspensions from the spleen, peripheral blood, thymus, and lymph nodes were isolated and stained as described above for CD8 depletion analysis.

SCN-NOTA Conjugation. All solutions were made metal-free (MF) using Chelex 100 (1.2 g/L; BioRad). The 2.43 and YTS169 Mbs were dialyzed against MF-PBS overnight using Slide-A-Lyzer MINI dialysis units (Thermo Scientific). Proteins at 1–2 mg/mL were then incubated with an 80-fold molar excess of S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA; Macrocyclics) for 4 h at 4 °C. The pH was adjusted to 8.5 using 1 M MF-NaOH. Excess p-SCN-Bn-NOTA was removed by PD-10 desalting columns that were preequilibrated MF-PBS. Eluted protein was concentrated with Amicon Ultra centrifugal filters (MWCO = 0.5 mL and 10 kDa; Millipore) that had been washed twice with MF-PBS.

^{64}Cu Radiolabeling. [^{64}Cu]$CuCl_2$ was obtained from the Division of Radiological Sciences, Washington University School of Medicine. Five microliters of 250 mM ammonium acetate (pH 7.0) was added to ∼37 MBq (∼1 mCi) $^{64}CuCl_2$ before the addition of 60–80 μg of NOTA-conjugated Mb at 1.4 mg/mL in saline. Protein was incubated for 30–45 min at 42 °C, followed by a challenge of 5 mM EDTA for 5 min at room temperature. Radiolabeling efficiency was measured using instant thin-layer chromatography (ITLC) strips (Biodex Medical Systems) with saline as the mobile phase. The ITLC strip was cut in half, and sections were counted using a Wizard 3″ 1480 Automatic Gamma Counter (PerkinElmer). Radiochemical purity was assessed by ITLC using saline as the mobile phase. Protein was purified using BioRad6 Spin columns equilibrated with PBS if the radiochemical purity was <95%.

The percentage of functional ^{64}Cu-NOTA Mb postradiolabeling was measured by incubating 30–50 ng of radiolabeled Mb with $>40 \times 10^6$ antigen-positive (BW58) or antigen-negative (EL-4) murine lymphoma cells in PBS plus 1% FBS for 1 h. Cells were centrifuged, the amount of activity in supernatant vs. the pellet was counted in a gamma counter, and the immunoreactive fraction was calculated as (% cell bound activity/total activity) * 100.

Micro-PET Imaging. Two hundred-microliter doses containing 2.6–2.9 MBq (70–80 μCi, 8–10 μCi/μg) of ^{64}Cu-radiolabeled Mb were prepared in saline and injected i.v. into B/6, C3H, or NSG mice. At 4 h postinjection, mice were anesthetized using 2% isoflurane and micro-PET scans were acquired using an Inveon microPET scanner (Siemens), followed by micro-CT scans (ImTek). Micro-PET images were reconstructed using nonattenuation or scatter-corrected filtered back-projection, and AMIDE was used for image analysis and display.

Biodistribution. After micro-PET/CT imaging, mice were euthanized, the organs and blood were collected and weighed, and the activity was determined in a gamma counter. The percent-injected dose per gram of tissue was calculated using a standard containing 2% of the injected dose. Left and right axillary lymph nodes were pooled and counted for biodistribution studies.

Data Analysis. Data values are reported as mean ± SD. Statistical analysis was performed using a two-tailed Student *t* test. Ex vivo biodistributions of ^{64}Cu-NOTA-2.43 Mb in C3H, NSG, B/6 blocked, and B/6 depleted mice were compared with the WT B/6 mice individually. Micro-PET/CT images are displayed as 20- or 2-mm maximum intensity projections for coronal or transverse images, respectively.

1. Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H (1984) Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature* 312(5994): 548–551.
2. Dübel S, et al. (1994) Isolation of IgG antibody Fv-DNA from various mouse and rat hybridoma cell lines using the polymerase chain reaction with a simple set of primers. *J Immunol Methods* 175(1):89–95.
3. Yazaki PJ, et al. (2001) Mammalian expression and hollow fiber bioreactor production of recombinant anti-CEA diabody and minibody for clinical applications. *J Immunol Methods* 253(1-2):195–208.
4. Chang HC, et al. (2005) Structural and mutational analyses of a CD8alphabeta heterodimer and comparison with the CD8alphaalpha homodimer. *Immunity* 23(6): 661–671.

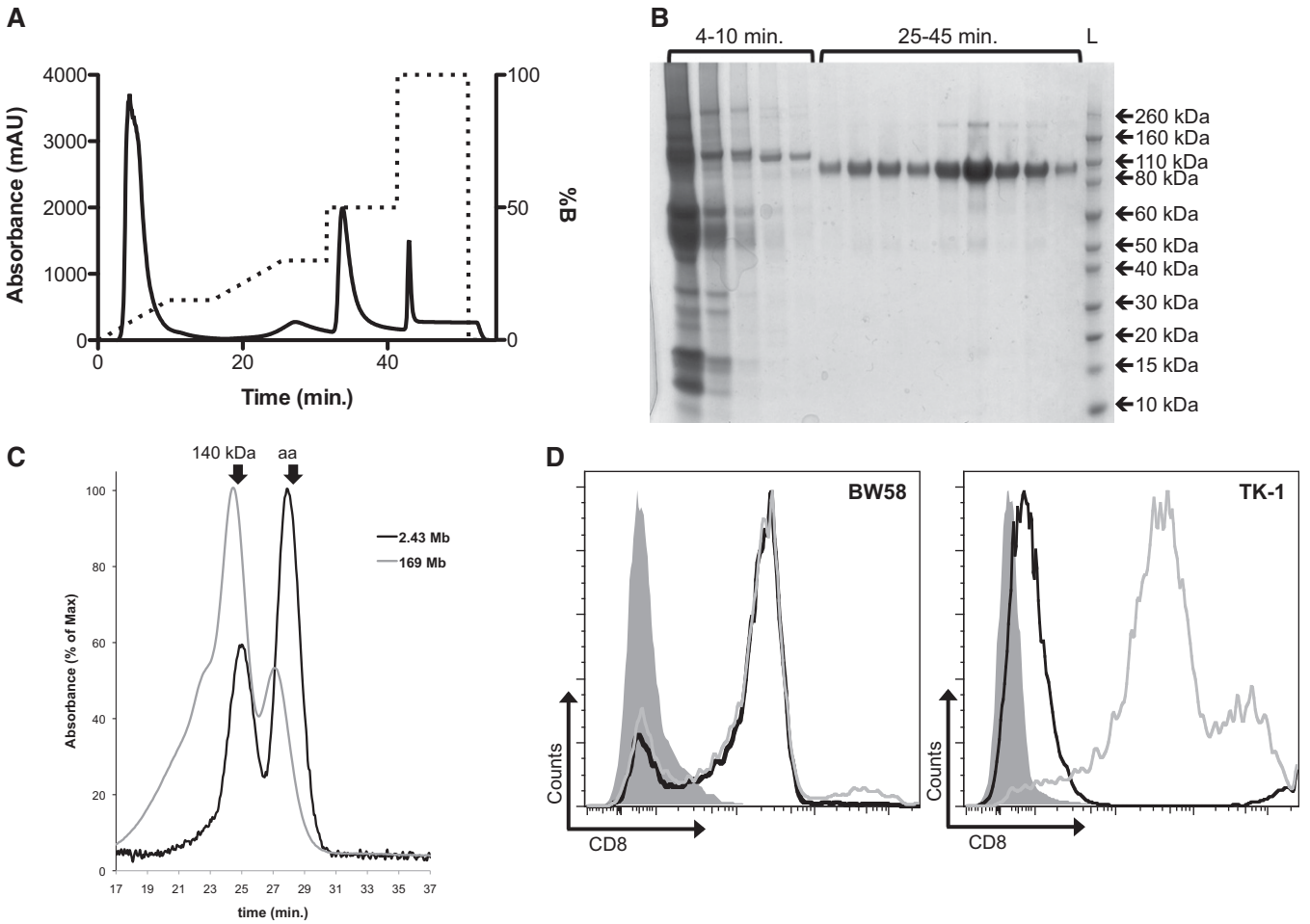


Fig. S1. Purification and characterization of 2.43 and YTS169 Mbs are shown. Mbs were purified from NS0 supernatant in one step using Ni-NTA immobilized metal affinity chromatography using imidazole elution (*A*) as shown by SDS/PAGE (*B*). mAU, milli-absorbance units. (*C*) SEC shows the presence of 81% ~80-kDa Mb for the 2.43 Mb and 23% ~80-kDa Mb for the YTS169 Mb. Max, maximum. (*D*, *Left*) Both the 2.43 Mb (black line) and YTS169 Mb (gray line) bind CD8 expressed on the Lyt2.2$^+$ BW58 murine thymoma cell line. (*D*, *Right*) Only the YTS169 Mb binds CD8 expressed on the Lyt2.1$^+$ TK-1 murine lymphoma cell line, however. The solid gray peak is cells only.

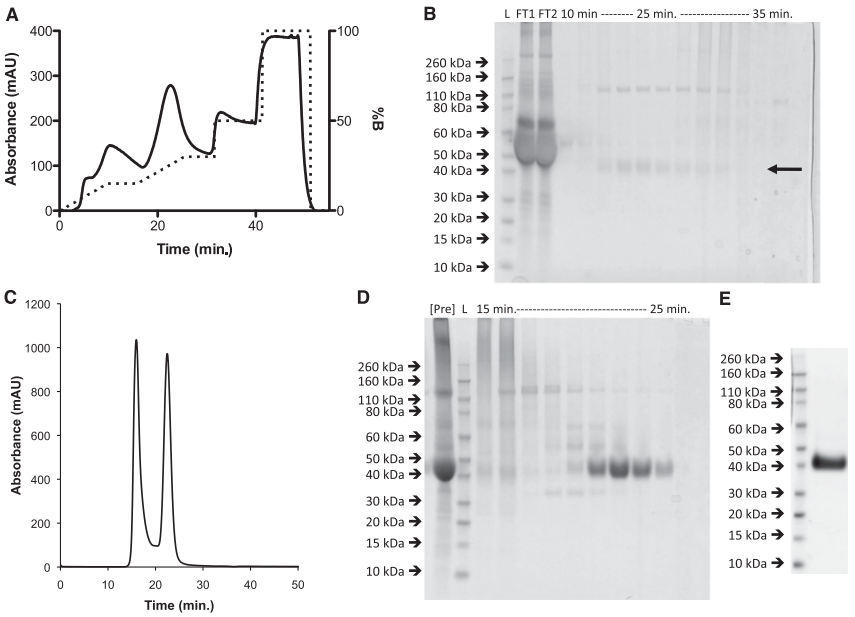


Fig. S2. Purification of the sCDαβ antigen in two steps. First, Ni-NTA immobilized metal affinity chromatography using imidazole elution (*A*) purified the sCD8αβ and some higher molecular-weight contaminants, as shown by SDS/PAGE (*B*). Second, SEC (*C*) was used to remove contaminants, as shown by SDS/PAGE (*D*). (*E*) Purified antigen is shown.

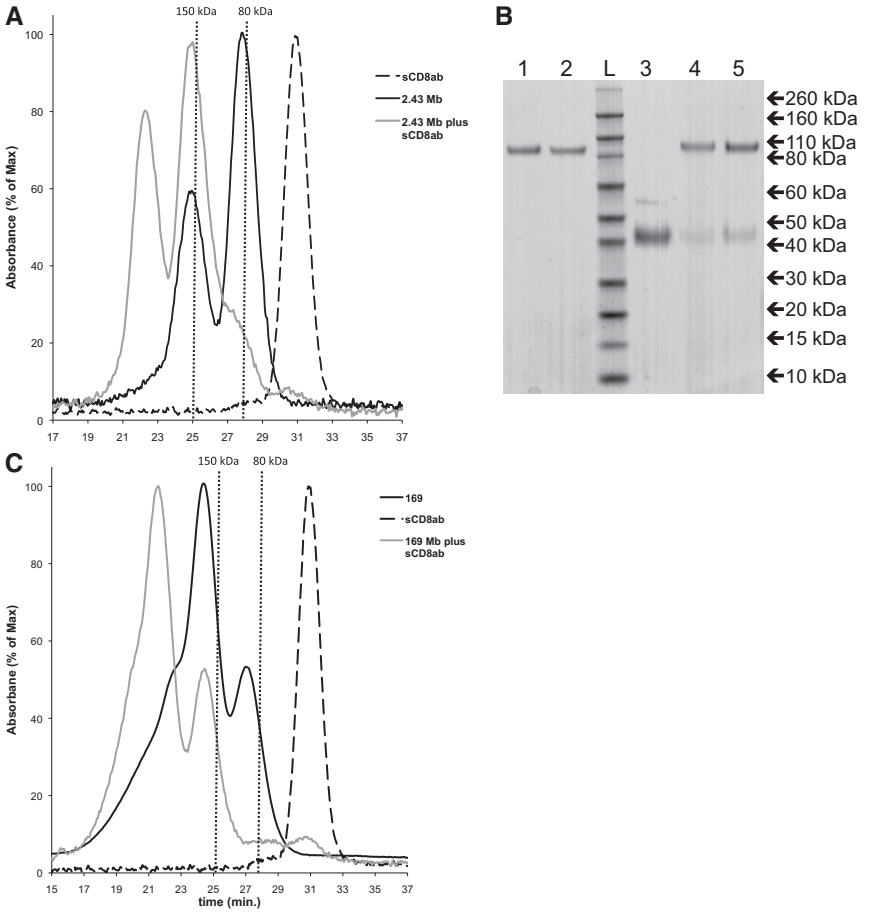
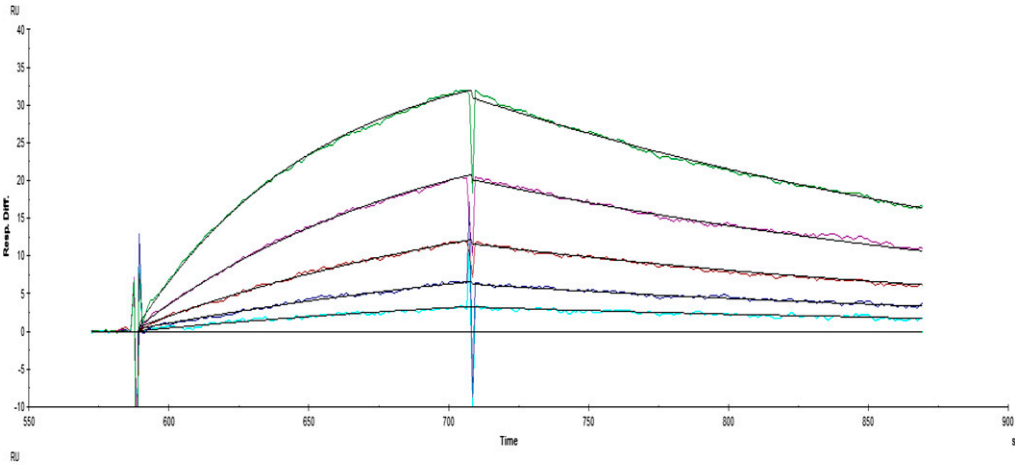
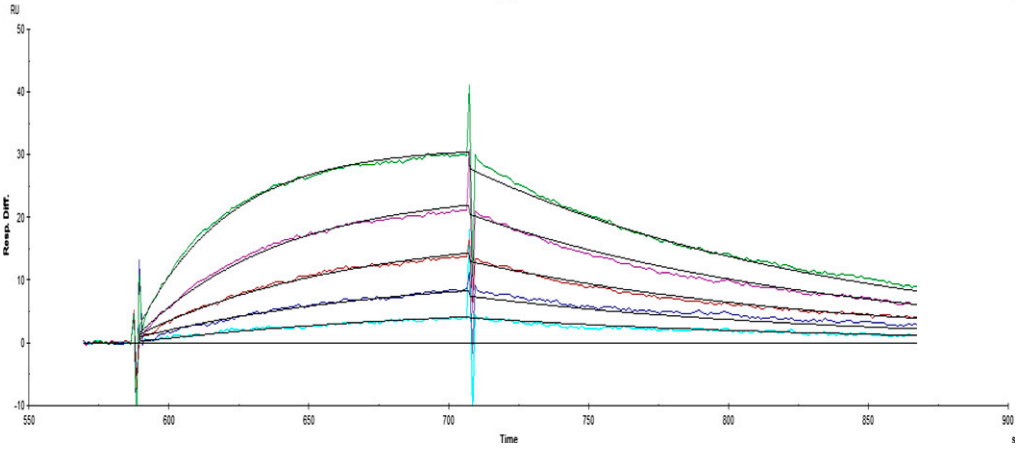


Fig. S3. Solution phase binding of 2.43 and YTS169 Mbs to sCD8αβ is demonstrated by SEC. The 2.43 Mb (*A*) or the YTS169 Mb (*C*) was mixed with equimolar amounts of sCD8αβ for 5 min before SEC analysis. (*B*) SDS/PAGE analysis of SEC elutions of 2.43 Mb alone at 25 and 27.5 min (lanes 1 and 2), sCD8αβ alone at 31 min (lane 3), and 2.43 Mb plus sCD8αβ at 22 and 25 min (lanes 4 and 5). Binding of 2.43 Mb to sCD8αβ is confirmed for both the 80-kDa Mb and the ∼160-kDa multimer in lanes 4 and 5.

A



B



C

Ligand	Analyte	k_a (1/Ms)	k_d (1/s)	R_{max} (RU)	[analyte]	K_A (1/M)	K_D (M)	χ^2
2.43	sCD8αβ	$1.2x10^5$	$4.1x10^{-3}$	48.9	0-100 nM	$3.0x10^7$	$3.4x10^{-8}$	0.05
YTS 169	sCD8αβ	$2.3x10^5$	$7.6x10^{-3}$	37.9	0-100 nM	$3.0x10^7$	$3.3x10^{-8}$	0.17

Fig. S4. Kinetic analysis of Mb binding to sCD8αβ antigen using SPR Biacore 3000 is shown. The 2.43 (*A*) or YTS169 (*B*) Mb was immobilized using goat anti-mouse IgG Fc capture. Soluble monovalent antigen was then flowed over the chip at 100 (green), 50 (magenta), 25 (red), 12.5 (dark blue), 6.25 (light blue), and 0 (black) nM. (*C*) Full kinetic analysis is shown. k_a, association rate constant; K_A, equilibrium association constant; k_d, dissociation rate constant; K_D, equilibrium dissociation constant; Resp. Diff., response difference; R_{max}, maximum resonance units.; RU, resonance units.