

SUPPLEMENTAL DATA

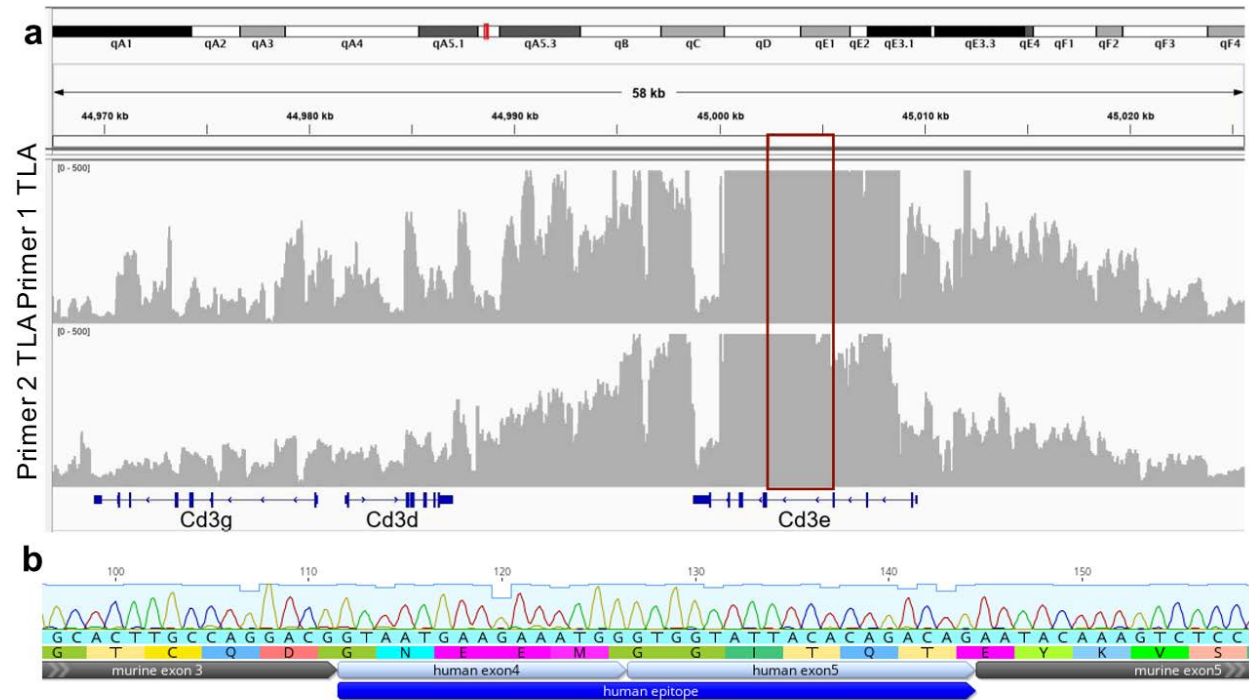


Figure S1. Sequence characterization of hCD3E-epi mice. (A) Splenocytes of the hCD3E-epi mice were collected and DNA was purified for TLA sequencing analysis. Reads were mapped back to the humanized sequence of the CD3e gene. Coverage across the humanized CD3e gene and wildtype CD3d and CD3g locus is shown in grey across different sequence amplifiers with one located within the humanized region and one located outside the humanized region in murine germline sequence. Humanized region marked in red box. Sequencing reads are contiguous across the humanized Cd3e locus and across the neighboring Cd3d and Cd3g locus without unexpected rearrangements. (B) Sanger sequencing of endpoint RT-PCR of T cells purified from the thymus of 20-week old mice across the humanized region of the CD3E-epi mice. Neighboring exons and humanized epitope are annotated below the confirmed reference sequence.

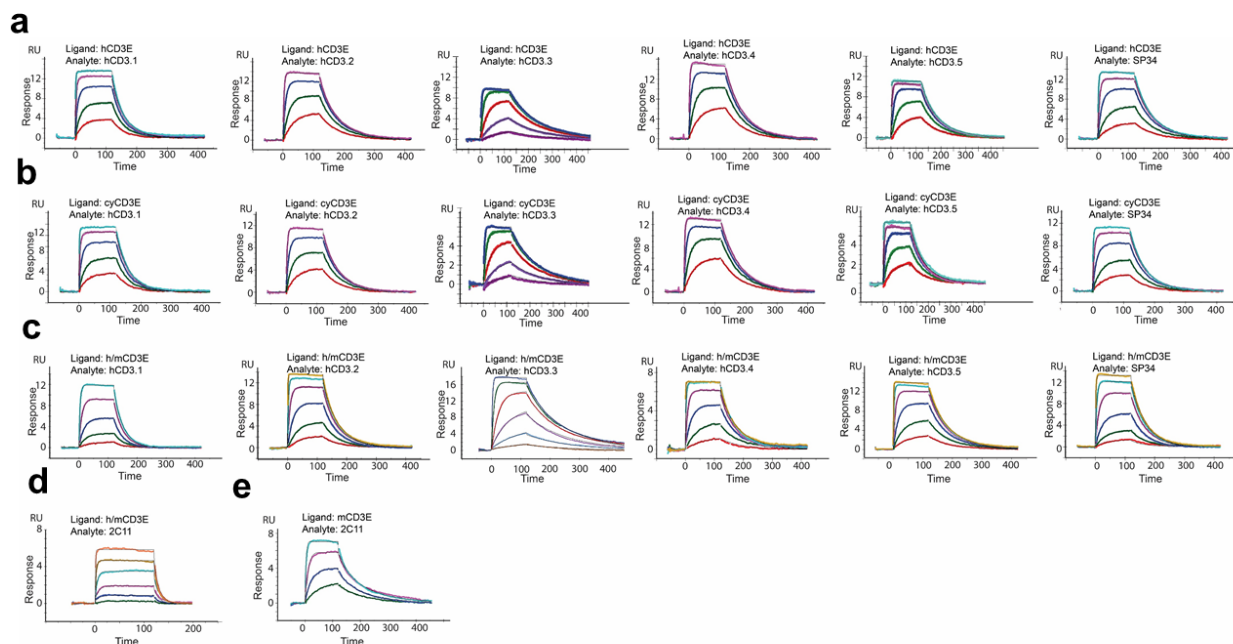


Figure S2. Binding of anti-CD3 Fabs to CD3 ϵ / δ variants. SPR binding signals of Fabs to immobilized (A) mFc-tagged human CD3 ϵ / δ , (B) mFc-tagged cyno CD3 ϵ / δ , (C-D) hFc-tagged human/murine chimeric CD3 ϵ / δ , and (E) hFc-tagged murine CD3 ϵ / δ . The signals shown in (A-B) use Fab concentrations corresponding to the following color code: 200 (cyan), 66.7 (light purple), 22.2 (blue), 7.4 (green), 2.5 (red), 0.82 (purple), and 0.27 (dark purple) nM. The data in (C) includes the following Fab concentrations: 1000 (yellow), 333 (cyan), 111 (light purple), 37 (blue), 12.3 (green), 4.1 (red), 1.37 (purple), 0.46 (light blue), and 0.15 (brown) nM. The data in (D-E) includes the following Fab concentrations: 3000 (orange), 1000 (yellow), 333 (cyan), 111 (light purple), 37 (blue), 12.3 (green) and 4.1 (red) nM.

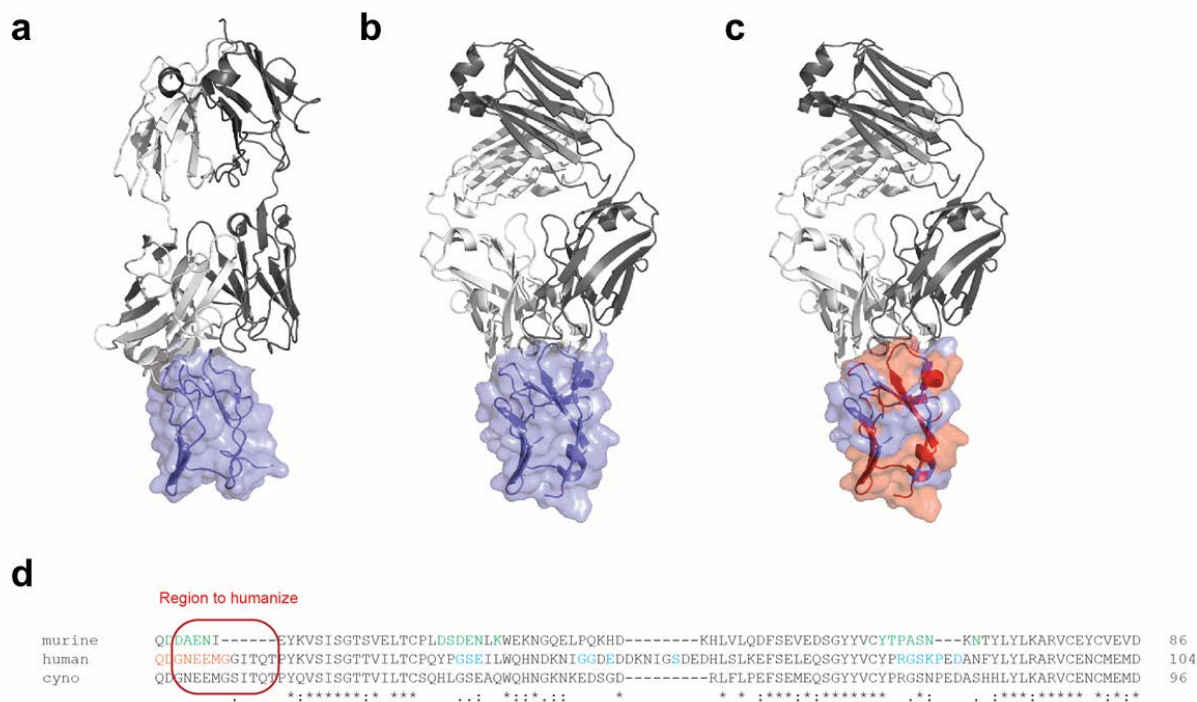
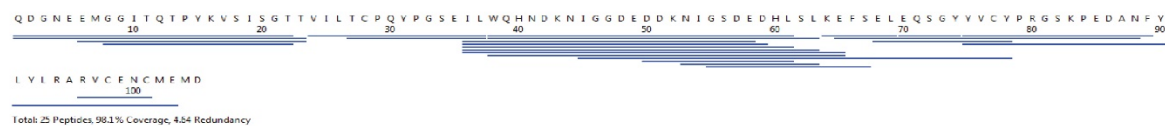


Figure S3. Characterization of the 2C11 and OKT3 epitopes based on structural data. (A) 2C11 in complex with murine CD3ε (PDB ID 3R08). (B) OKT3 in complex with human CD3 ε/δ (PDB ID 1SY6). Only CD3ε is shown. Human and murine CD3ε were aligned, and map to a similar region on CD3. (C) OKT3 in complex with human CD3ε/δ (PDB ID 1SY6) with residues conserved between human and cyno highlighted in red. OKT3 epitope maps to highly conserved region between human and cyno CD3ε. (D) A sequence alignment of human, murine, and cyno CD3ε. The contact residues for hCD3.1-5 (based on HDX) on highlighted in orange. The contact residues for 2C11 (based on the crystal structure) are highlighted in green. The contact residues for OKT3 (based on the crystal structure) are highlighted in blue.

hCD3 epsilon chain



hCD3 delta chain

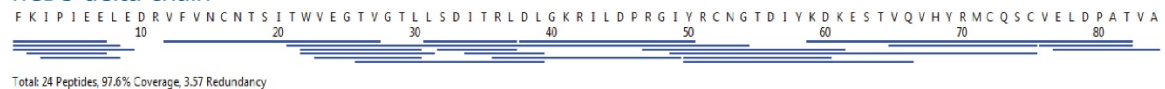


Figure S4. HDX-MS hCD3 ϵ chain sequence coverage.

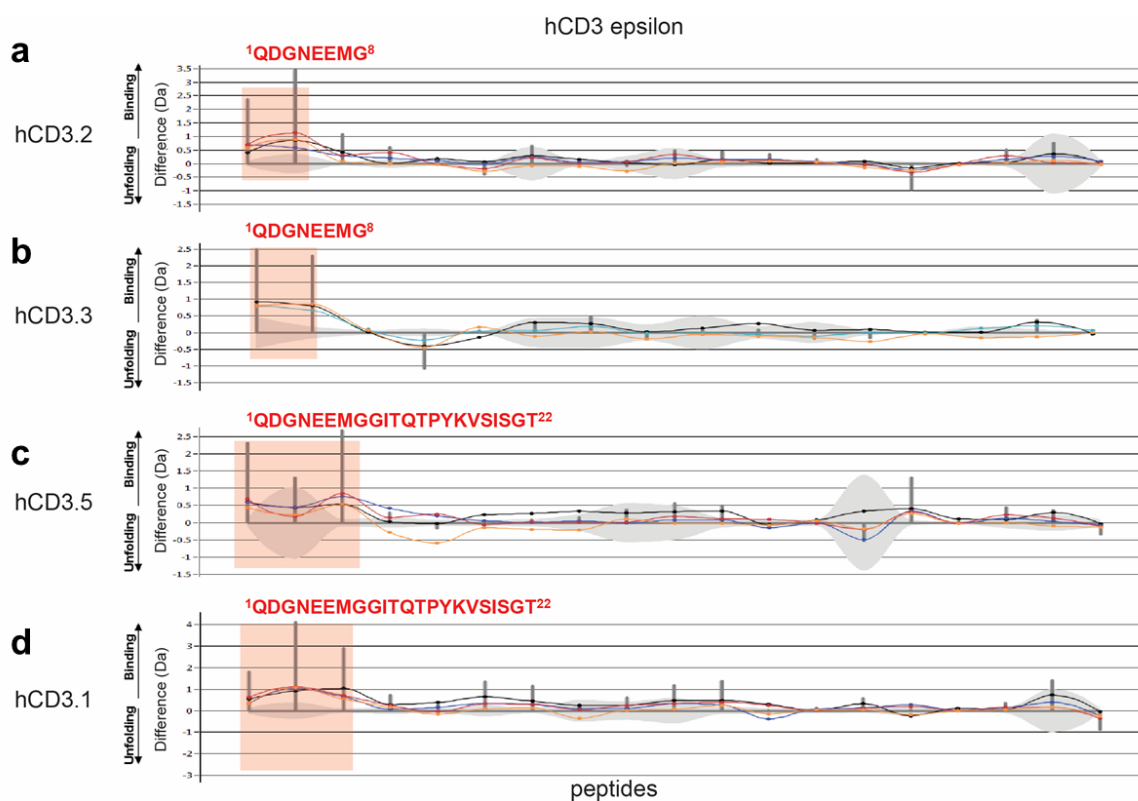


Figure S5. Differential HDX of the CD3 ϵ -subunit in CD3 vs. mAb/CD3 complex. Deuterium uptake differences across four exchange time points, 20 sec (yellow), 1 min (red), 10 min (blue) and 240 min (black), were summed and shown as the gray bars. Error band with 1σ is shown in gray. Red box indicates the primary epitope of each antibody.

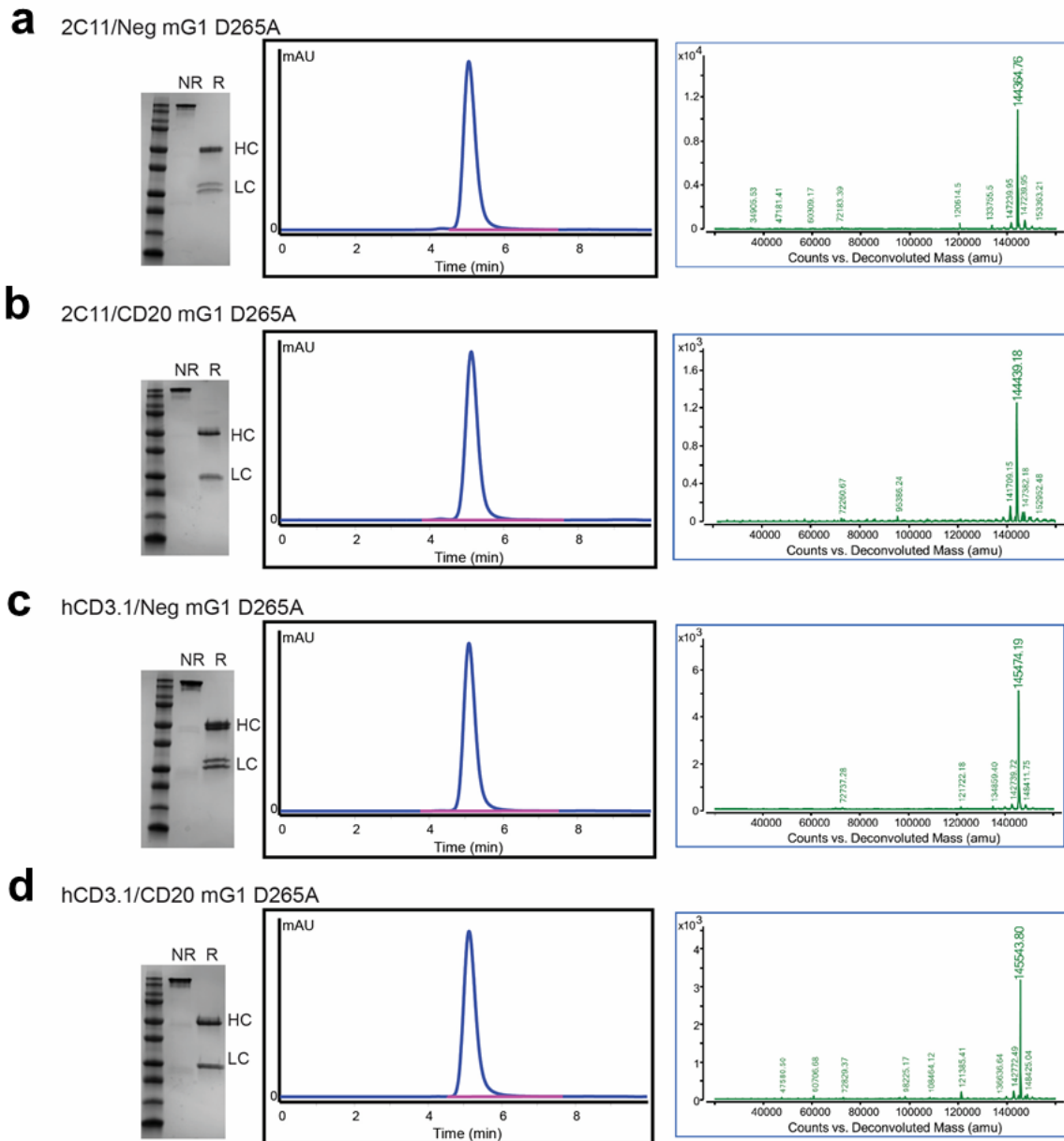
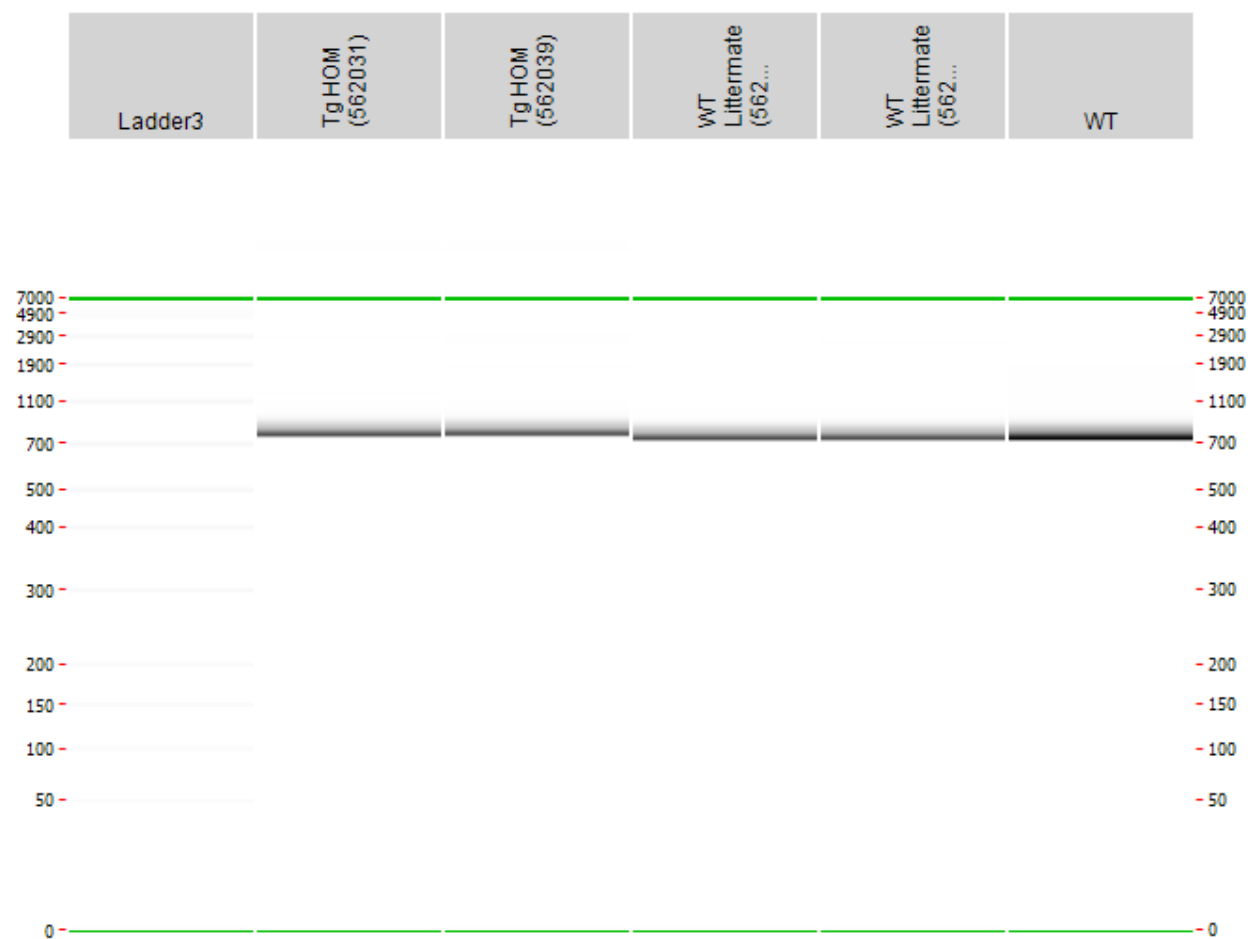


Figure S6. Analytical characterization of bispecifics. Non-reducing (NR) and reducing (R) SDS-PAGE, analytical-SEC, and LC/MS confirmed the purity and identity of (A) 2C11/Neg mG1 D265A, (B) 2C11/CD20 mG1 D265A, (C) hCD3.1/Neg mG1 D265A, and (D) hCD3.1/CD20 mG1 D265A.



Supplemental FigureS7: Full length uncropped gel from Figure 2 Panel B.

Anti-CD3 variant	VH	VL
hCD3.1	EVQLVESGGGLVQPGGSLRLSCAASG FTFSDYYMTWVRQAPGKGLEWVAFIR NRARGYTS DHNPSVKGRFTISRDNK NSLYLQMNSLRAEDTAVYYCARDRPS YYVLDYWGQGTTVTVSS	DIVMTQSPDSLAVSLGERATINCKSSQSL FNVRSRKNYLAWYQQKPGQPPKLLISWA STRESGVPDRFSGSGSGTDFTLTISLQA EDVAVYYCKQSYDLFTFGSGTKLEIK
hCD3.2	EVQLVESGGGLVQPGGSLRLSCAASG FTFNTYAMNWVRQAPGKGLEWVARIR SKYNNYATYYAASVKGRFTISRDDSKN SLYLQMNSLKTEDTAVYYCARHGNFG NSYVSWFAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCRSSTGA VTTSNYANWVQQKPGQAPRGLIGGTNKR APGTPARFSGSLLGGKAALTL SGVQPED EAEYYCALWYSNLWVFGGGTKLTVL
hCD3.3	EVQLVESGGGLVQPGGSLRLSCAASG FTFNKYAMNWVRQAPGKGLEWVARIR SKYNNYATYYADSVKDRFTISRDDSKN TAYLQMNNLKTEDTAVYYCVRHGNFG NSYISYWAYWGQGLTVTVSS	QTVVTQEPSLTVSPGGTVTLTCGSSTGA VTSGNYPNWVQQKPGQAPRGLIGGTKFL APGTPARFSGSLLGGKAALTL SGVQPED EAEYYCVLWYSNRWVFGGGTKLTVL
hCD3.4	QVQLVQSGAEVKKPGASVKVSCKASG FNIKDYMHWVRQAPGQRLEWMGWI DLENGNTIYDPKFQGRVTITRDTASTA YMESSLSRSED TAVYYCARDGYGRYF YDVWGQGLTVTVSS	DIVMTQSPDSLAVSLGERATINCKSSQSL LNSRTGKNYLAWYQQKPGQPPKLLIYWA STRESGVPDRFSGSGSGTDFTLTISLQA EDVAVYYCKQSYSRRTFGGGTKVEIK
hCD3.5	EVQLLES GGGLVQP GGSLRLSCAASG FTFSTYAMNWVRQAPGKGLEWVSRIR SKYNNYATYYADSVKGRFTISRDDSKN TLYLQMNSLRAEDTAVYYCVRHGNFG NSYVSWFAYWGQGLTVTVSS	QAVVTQEPSLTVSPGGTVTLTCGSSTGA VTTSNYANWVQEKPGQAFRGLIGGTNKR APGTPARFSGSLLGGKAALTL SGAQPED EAEYYCALWYSNLWVFGGGTKLTVL
SP34	EVQLVESGGGLVQPKGSLKLSCAASG FTFNTYAMNWVRQAPGKGLEWVARIR SKYNNYATYYADSVKDRFTISRDDSQS ILYLQMNNLKTEDTAMYYCVRHGNFGN SYVSWFAYWGQGLTVTVSA	QAVVTQESALTTSPGETVTLTCRSSTGAV TTSNYANWVQEKPDHLFTGLIGGTNKRA PGVPARFSGSLIGDKAALTITGAQTEDEAI YCALWYSNLWVFGGGTKLTVL
2C11	EVQLVESGGGLVQPGKSLKLSCEASG FTFSGYGMHWVRQAPGRGLESVAYIT SSSINIKYADAVKGRFTVSRDNAKNLLF LQMNILKSED TAMYYCARFDWDKNYW GQGTMTVTVSS	DIQMTQSPSSLPASLGDRV TINCQASQDI SNYLNWYQQKPGKAPKLLIYYTNKLADG VPSRFSGSGSGRDSSTFTISSLESEDIGSY YCQQYYNYPWTFGPGTKLEIK

Supplementary Table S1. Sequence information for anti-CD3 variants.