

Mapping the Extracellular Molecular Architecture of the pAg-Signaling Complex with α -Butyrophilin Antibodies

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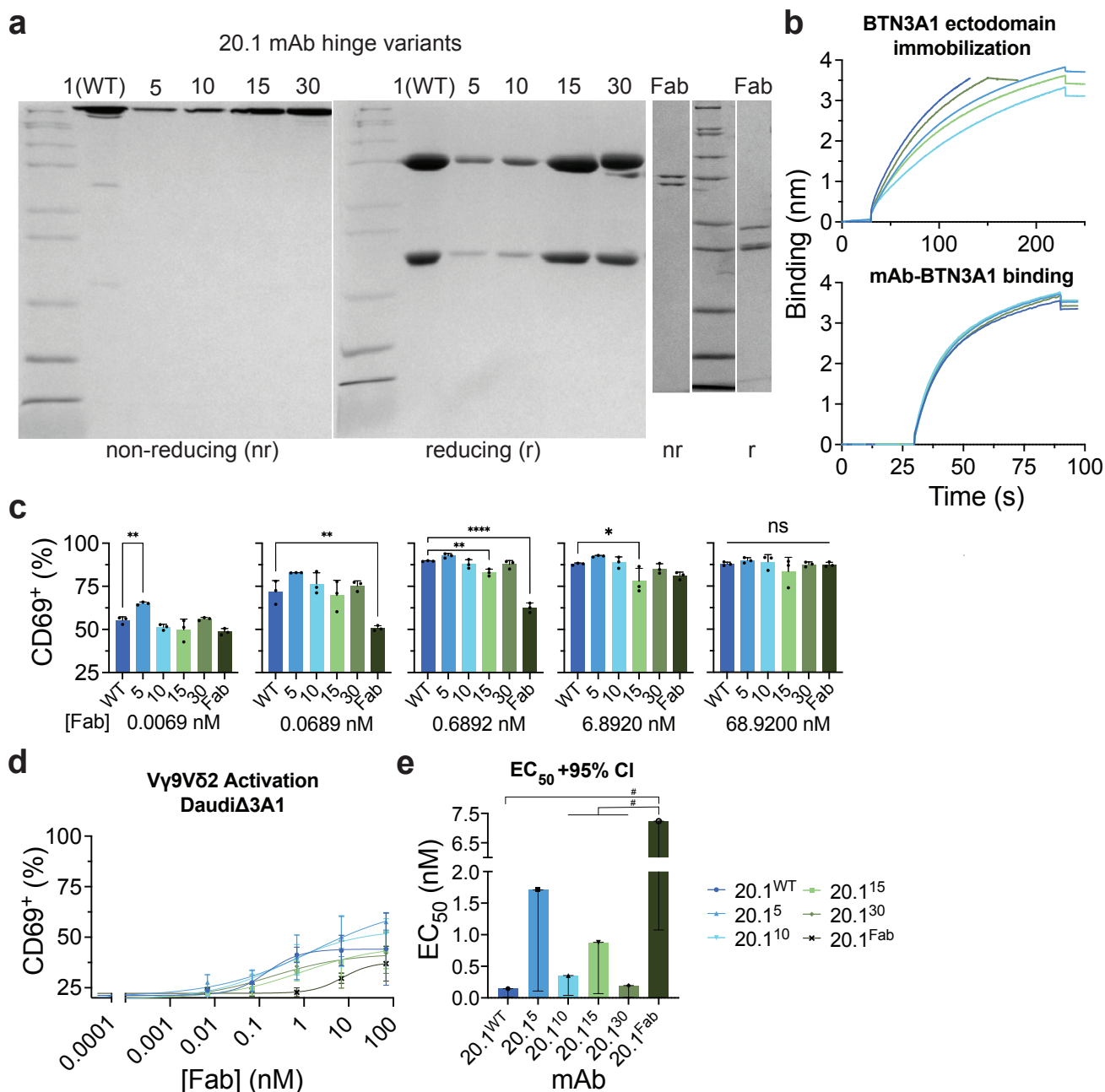
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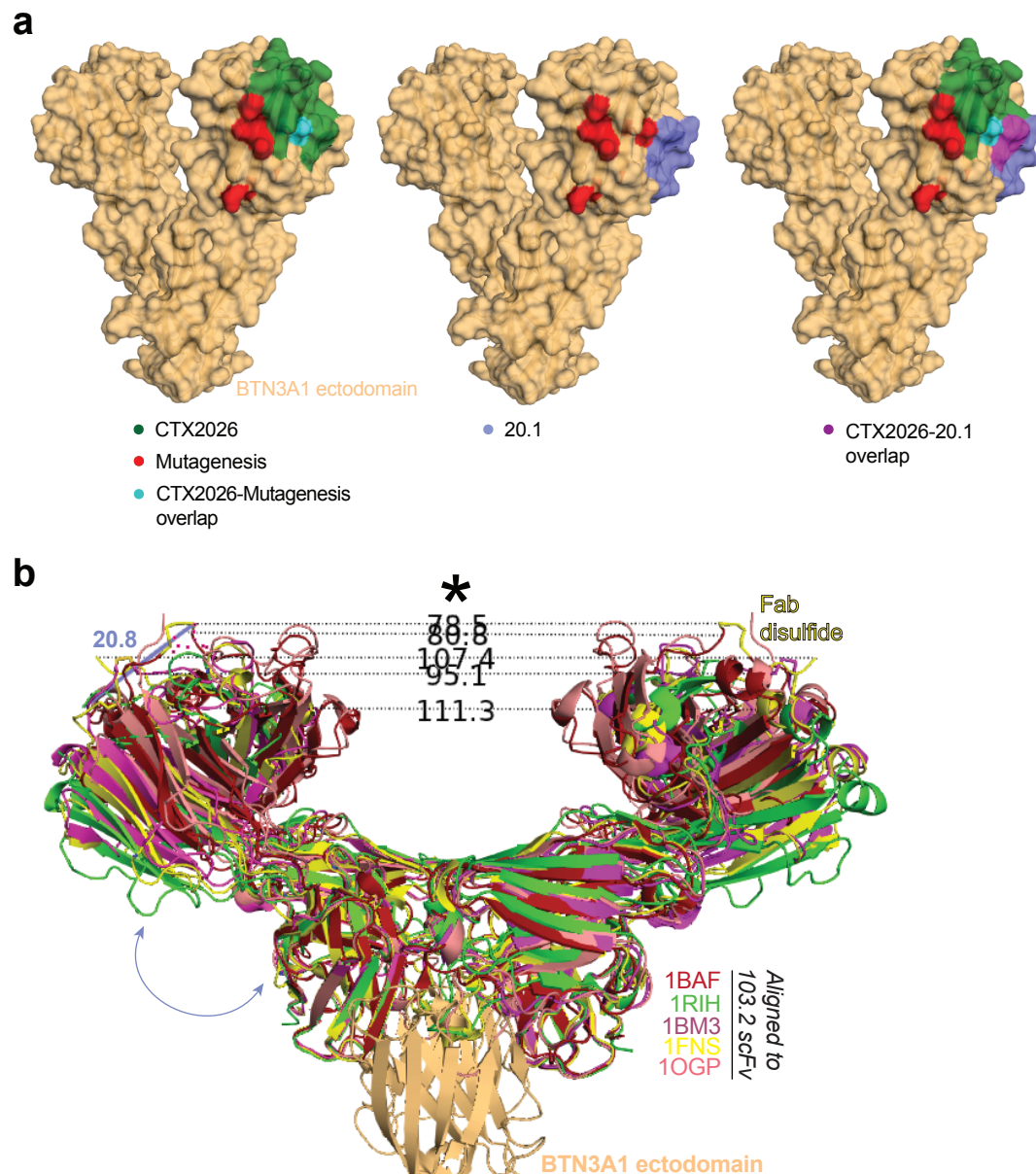
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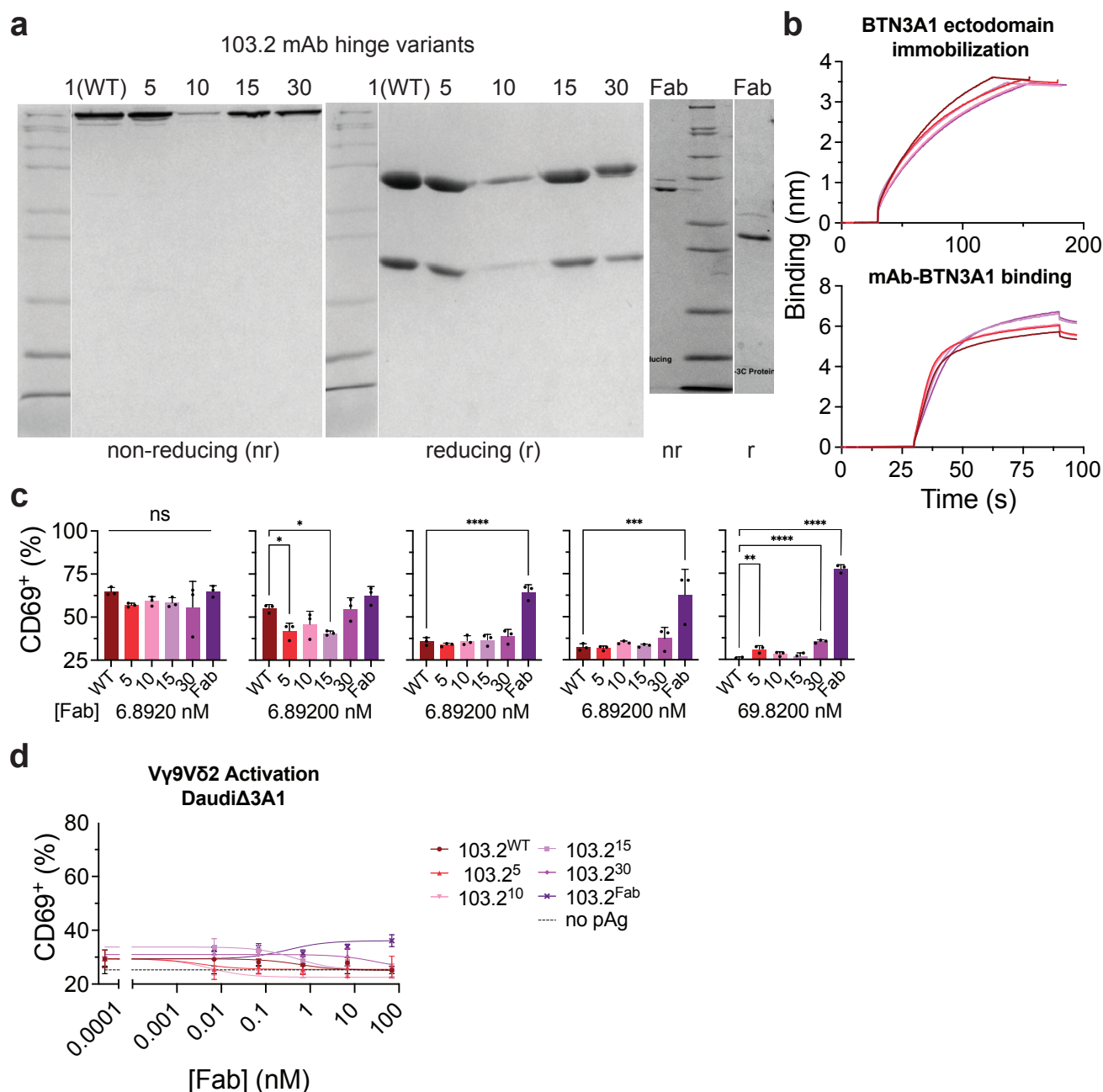


Supplementary Figure 1. 20.1 mAb hinge variants and 20.1 Fab expression and validation. (A) Non-reducing (left) and reducing (middle) SDS-PAGE gels showing 20.1 hinge variants hinge variants mAbs expressed in Expi293 cells and purified using Protein-G resin. Non-reducing and reducing (right) SDS-PAGE gels showing 20.1 Fab expressed in High-Five insect cells and purified using Protein-Ga1 resin (Kossiakoff Lab, Univ. of Chicago). (B) Biotinylated BTN3A1 immobilization on streptavidin biosensor assessed by BLI, colored by 20.1 mAb hinge variant subsequently tested (top). Relative 20.1 mAb hinge variant affinity for immobilized, biotinylated BTN3A1 probed at 1.3 μ M by BLI (bottom) (representative of 3 independent experiments). (C) Activation of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with DaudiΔAAVS1 cells pre-incubated with 20.1 mAb hinge variants or 20.1 Fab at specific concentrations from Fig. 1C. Mean + SD (n=3). Dunnett test pairwise comparison to 20.1WT: ****p<0.0001, ***p<0.0002, **p<0.0021, *p<0.0332, nsp>0.1234. (D) Activation of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with DaudiΔ3A1 cells pre-incubated with 20.1 mAb hinge variants or 20.1 Fab at increasing concentrations or control (Buffer). CD69 expression as a marker of T cell activation was assessed by flow-cytometry (gated on isotype antibody). Means + SD (n=3). (E) EC₅₀ of agonism and 95% confidence intervals for 20.1 mAb hinge variants or 20.1 Fab with DaudiΔ3A1 cells was calculated by non-linear regression with variable slope - four parameters (R²= 0.6856, 0.8411, 0.8189, 0.7924, 0.6761, 0.7110, respectively). # = significant difference in EC₅₀ value determined by non-overlapping CI.

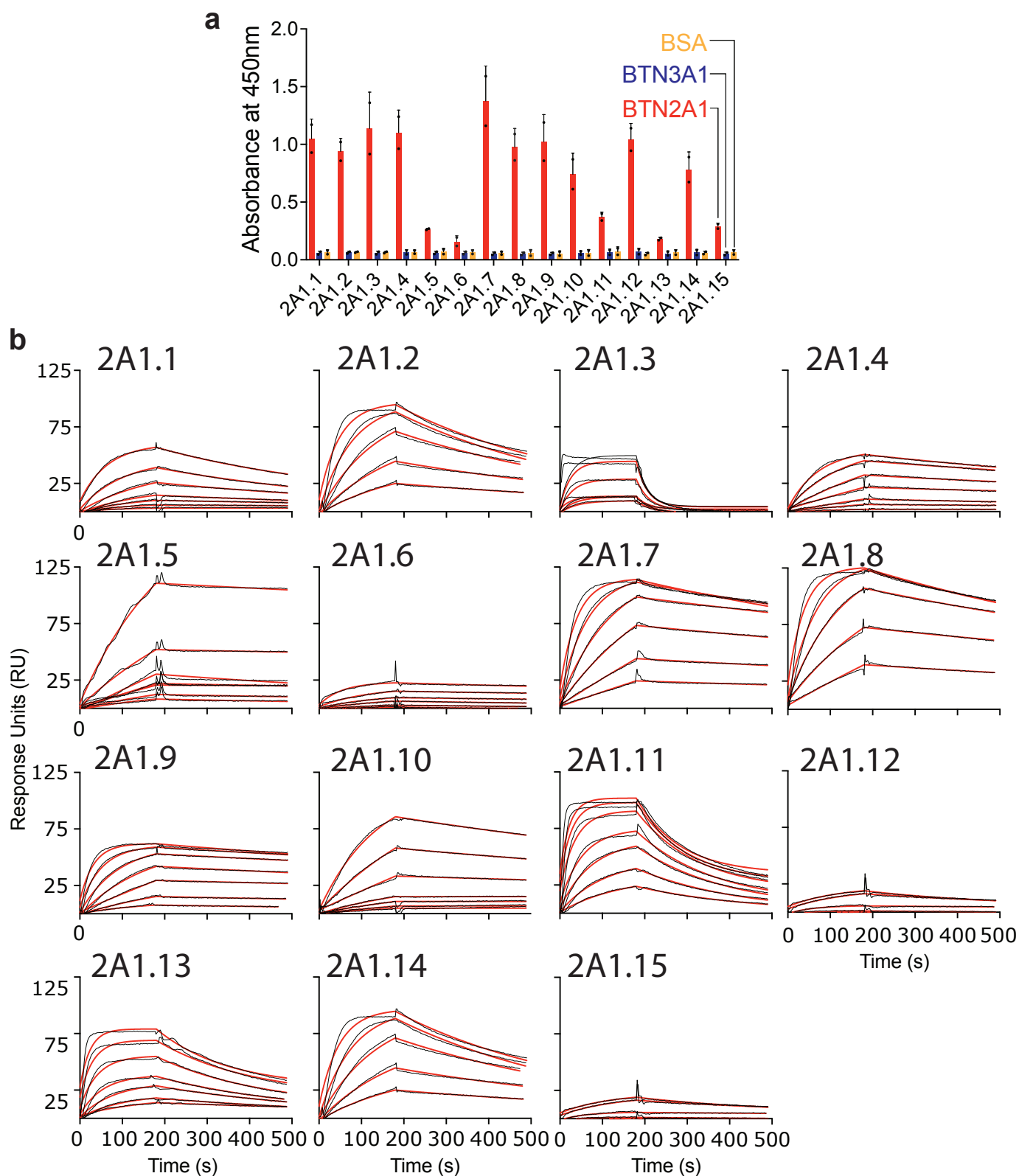


Supplementary Figure 2. Structural analyses of the mechanisms by which the 20.1 and 103.2 mAb modulate Vγ9Vδ2 T cell activation.

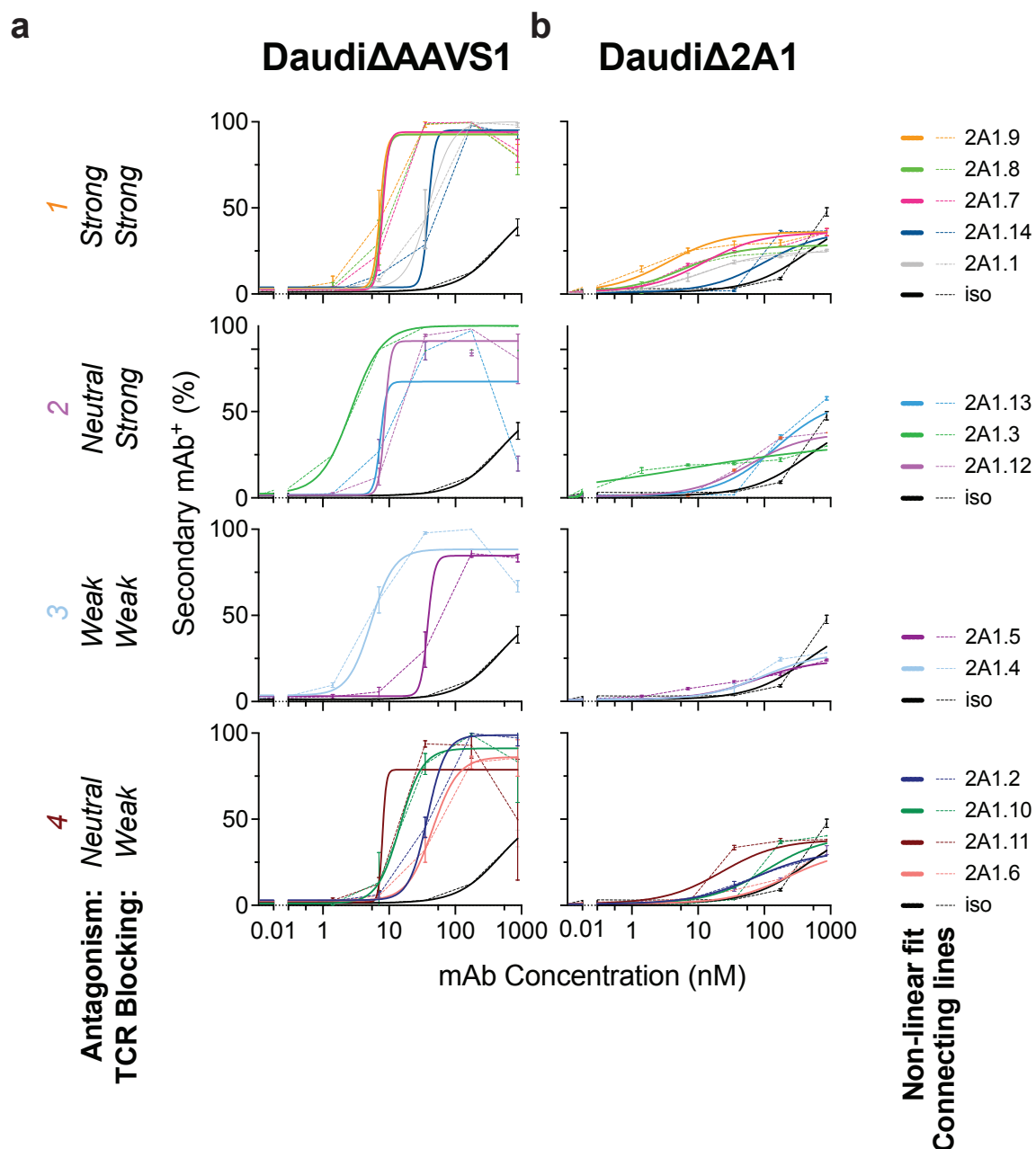
(A) Residues of which mutagenesis abrogates pAg signaling (red), epitopes for CTX2026 Fab (green), 20.1 scFv (blue), CTX2026-20.1 overlap (purple), and where the CTX2026 and pAg-abrogating mutagenesis overlap (teal). (B) Alignment of PDB structures of mulgG1 Fabs (PDB ID: 1BAF, 1RIH, 1BM3, 1FNS, OGP) with 103.2 scFv in complex with BTN3A1 (PDB ID: 4F9P) REF. Distances (*, black) were measured between duplicate Fabs aligned to each 103.2 scFv and calculated between CA of cysteine residues forming disulfide bonds with respective Fab light-chain in Pymol. Maximum flexibility between Fab IgV and IgC domains was calculated by measuring the distance between the CA of cysteine residues forming disulfide bonds with respective Fab light-chain of PDB ID: 1RIH and 1BAF (arrow, periwinkle).



Supplementary Figure 3. 103.2 mAb hinge variants and 103.2 Fab expression and validation. (A) Non-reducing (left) and reducing (middle) SDS-PAGE gels showing 103.2 mAb hinge variants hinge variants mAbs expressed in Expi293 cells and purified using Protein-G resin. Non-reducing and reducing (right) SDS-PAGE gels showing 103.2 Fab expressed in High-Five insect cells and purified using Protein-Ga1 resin (Kossiakoff Lab, Univ. of Chicago). (B) Biotinylated BTN3A1 immobilization on streptavidin biosensor assessed by BLI, colored by 103.2 mAb hinge variant subsequently tested (top). Relative 103.2 hinge variant affinity for immobilized, biotinylated BTN3A1 probed at 1.3 μ M by BLI (bottom) (representative of 3 independent experiments). (C) Activation of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with Daudi Δ AVS1 cells pre-incubated with 103.2 mAb hinge variants or 103.2 Fab at specific concentrations from Fig. 2C. Mean + SD (n=3). Dunnett test pairwise comparison to 103.2 WT: ****p<0.0001, ***p<0.0002, **p<0.0021, *p<0.0332, nsp>0.1234. (D) Inhibition of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with Daudi Δ 3A1 cells pre-incubated with 5 μ M HMBPP and 103.2 mAb hinge variants or 103.2 Fab at increasing concentrations or controls (Buffer +/- HMBPP). CD69 expression as a marker of T cell activation was assessed by flow-cytometry (gated on isotype antibody). Means + SD (n=3).

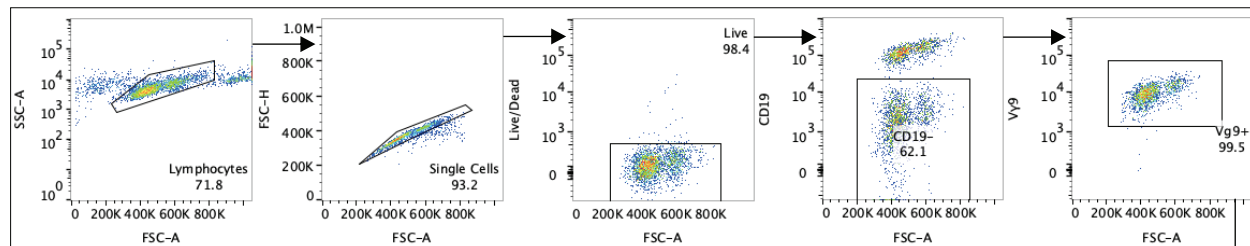


Supplementary Figure 4. Determination of α -BTN2A1 Fab specificity and biophysical affinity for Biotinylated BTN2A1 ectodomain. (A) α -BTN2A1 Fab expressed on bacteriophage binding to immobilized BTN2A1 ectodomain (red) or controls (BTN3A1 ectodomain (blue) or BSA (yellow)) as assessed by ELISA using Protein-L-HRP/TMB. Mean $\pm$ SD (n=2). (B) Biophysical affinity of α -BTN2A1 Fabs binding to immobilized BTN2A ectodomain assessed by SPR in 2-fold dilutions (grey). Non-linear regression - association then dissociation calculated using GraphPad Prism (red).

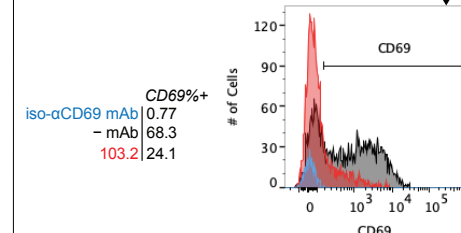
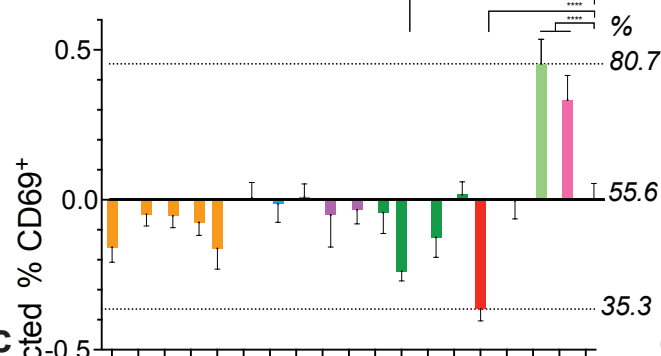


Supplementary Figure 5. Determination of α-BTN2A1 mAb cellular affinity for BTN2A1.

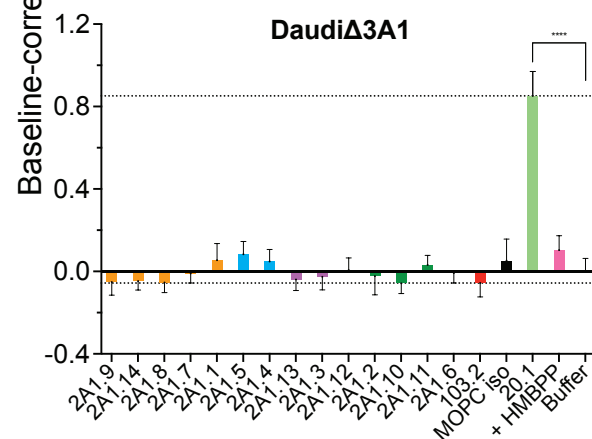
(A) α-BTN2A1 mAb binding to DaudiΔAAVS1 cells at increasing concentrations, separated by mAb class. 1° mAb binding to BTN2A1 ectodomain visualized using α-mulG1 2° antibody staining assessed by flow-cytometry (gated on no 1° mAb control). Mean + SD with connecting lines (dotted) (n=3), non-linear regression with variable slope - four parameters (solid). (B) α-BTN2A1 mAb binding to DaudiΔ2A1 cells as assessed in (A). Binding data is organized based on mAb physiological properties assessed in Fig. 3. Binding data is organized based on mAb physiological properties assessed in Fig. 3.

a**b**

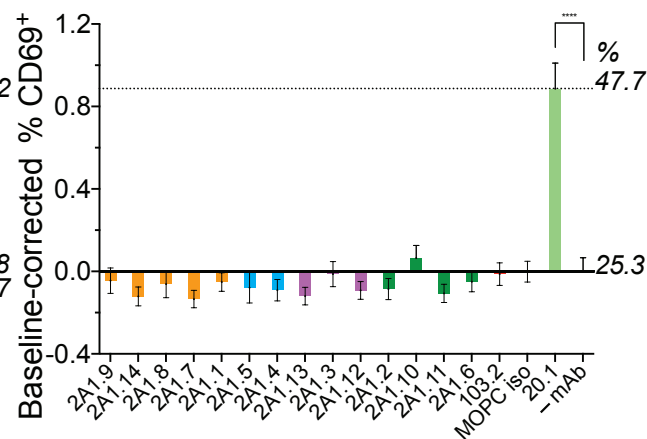
T Cell Activation - Agonism DaudiΔAAVS1

**c**

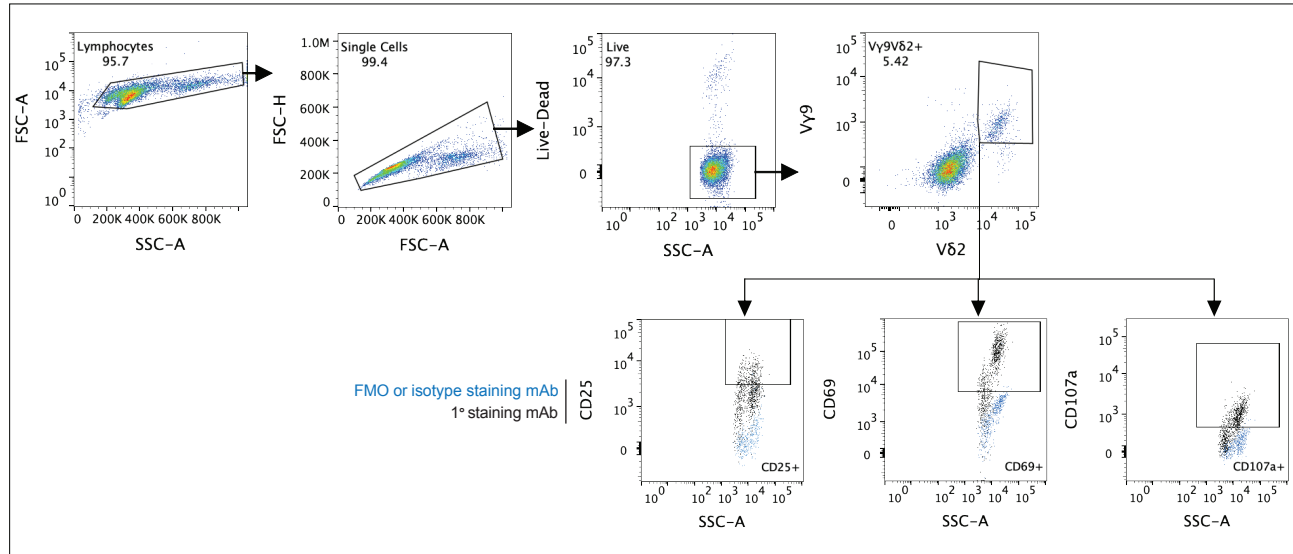
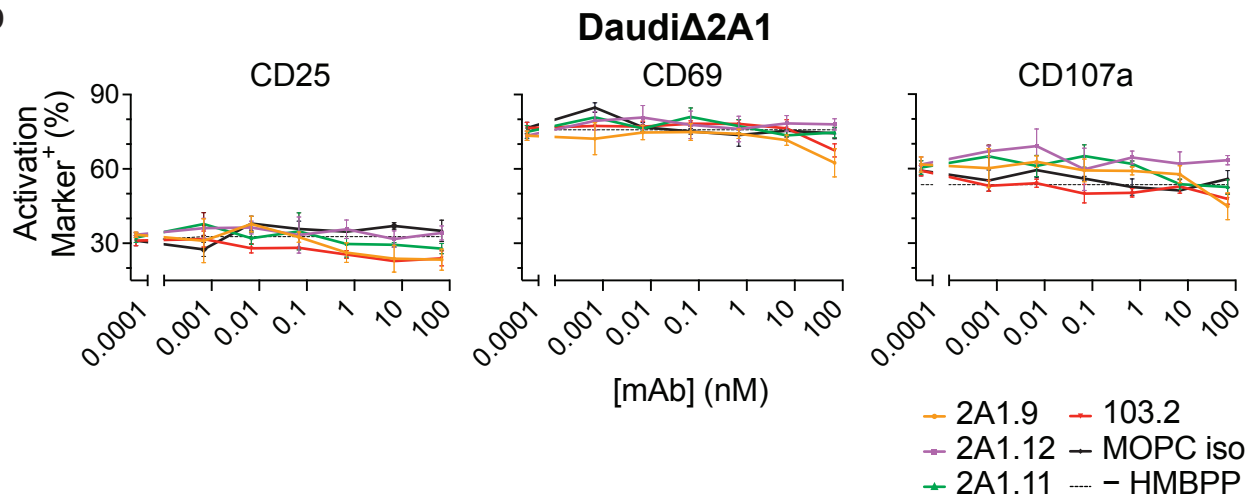
DaudiΔ3A1

**d**

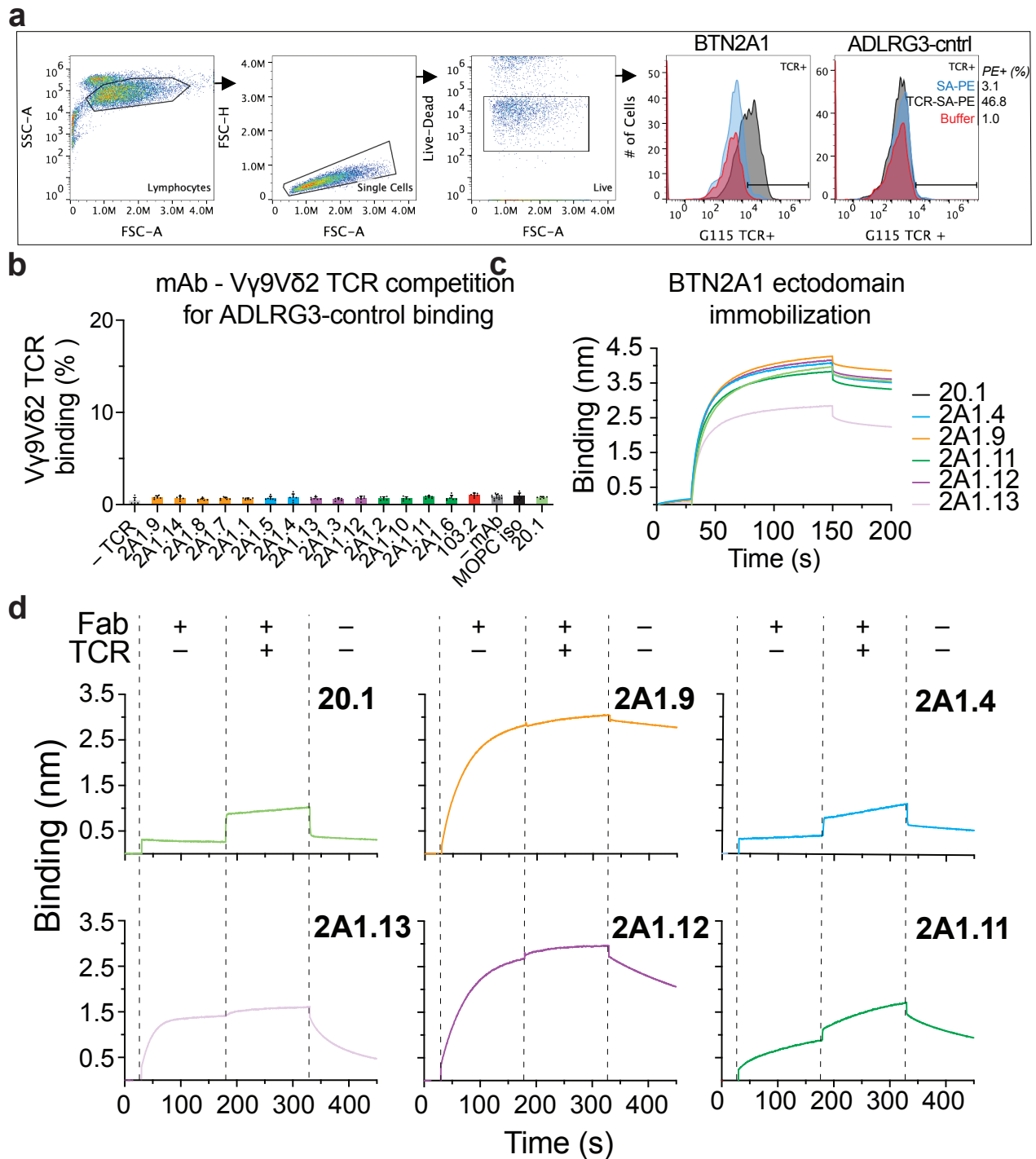
T Cell Activation - Antagonism DaudiΔ3A1



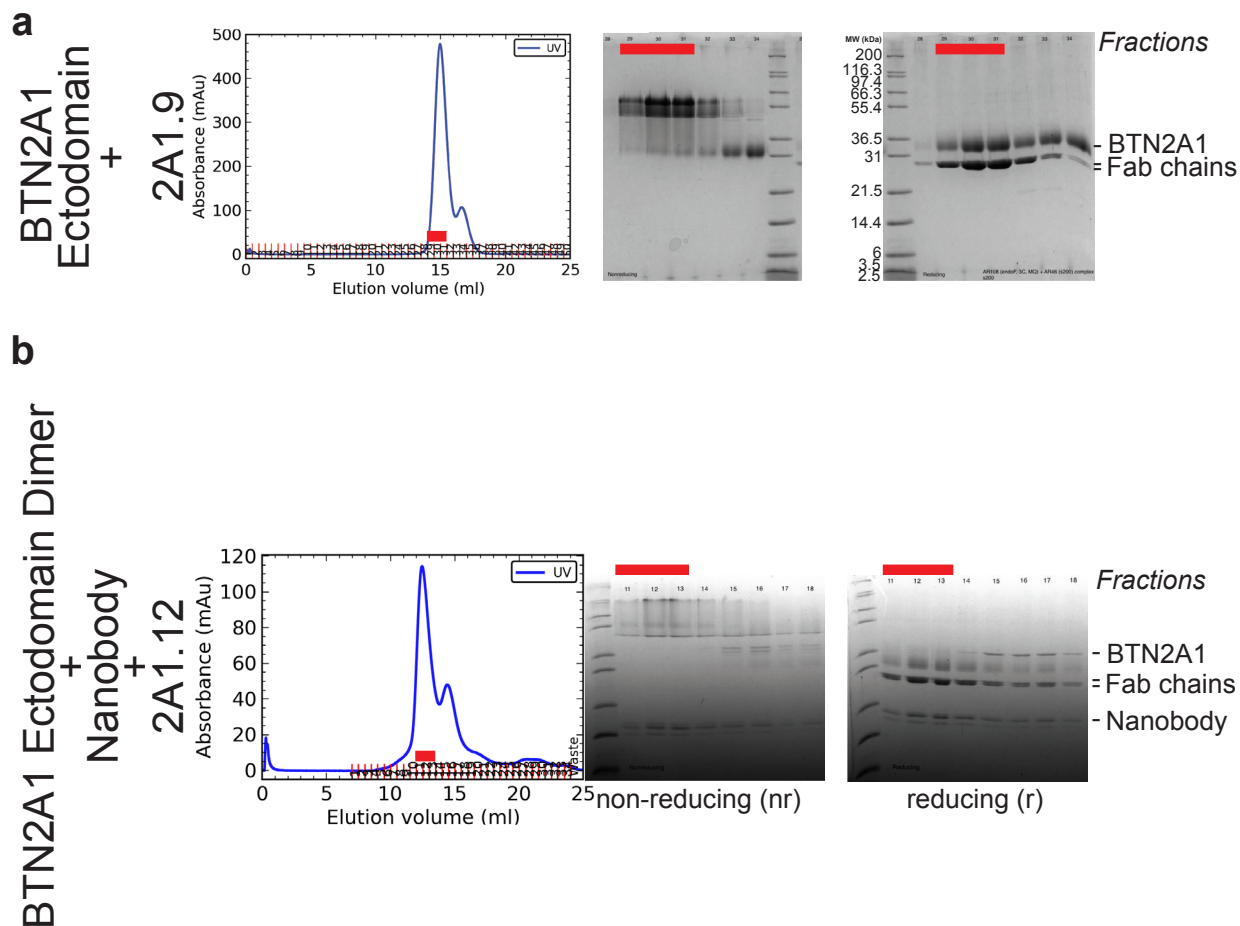
Supplementary Figure 6. Supporting Data for Jurkat Vγ9Vδ2 Activation Assays. (A) Flow-cytometry gating strategy for Jurkat T cell antagonism assays with Daudi target cells pre-incubated with 5 μM HMBPP. CD69 gate drawn to ~1% positive staining by isotype controls. (B) Activation of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with DaudiΔAAVS1 cells pre-incubated with 6.8nM (1 μg/mL) α-BTN2A1 mAb or controls (MOPC isotype, 20.1 mAb, 103.2 mAb, 5 μM HMBPP or Buffer). CD69 expression as a marker of T cell activation was assessed by flow-cytometry (gated on isotype antibody) and normalized to Buffer condition (Value-Baseline/Baseline). Dunnett test pairwise comparison to Buffer control: *p<0.0332, ****p<0.0001. (C) Activation of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with DaudiΔ3A1 cells pre-incubated with 6.8nM (1 μg/mL) α-BTN2A1 mAb or controls (MOPC isotype, 20.1 mAb, 103.2 mAb, Pamidronate or Buffer +/- HMBPP). CD69 expression as a marker of T cell activation was assessed by flow-cytometry (gated on isotype antibody) and normalized to Buffer condition (Value-Baseline/Baseline). Dunnett test pairwise comparison to Buffer control: ****p<0.0001. (D) Inhibition of activation of G115-TCR expressing Jurkat Jrt-3.3 cells after co-incubation with DaudiΔ3A1 cells pre-incubated with 5 μM HMBPP and 6.8nM (1 μg/mL) α-BTN2A1 mAb or controls (MOPC isotype, 20.1 mAb, 103.2 mAb or Buffer +/- HMBPP). CD69 expression as a marker of T cell activation was assessed by flow-cytometry (gated on isotype antibody) and normalized to Buffer + 5 μM HMBPP condition (Value-Baseline/Baseline). Dunnett test pairwise comparison to - mAb control: ****p<0.0001.

a**b**

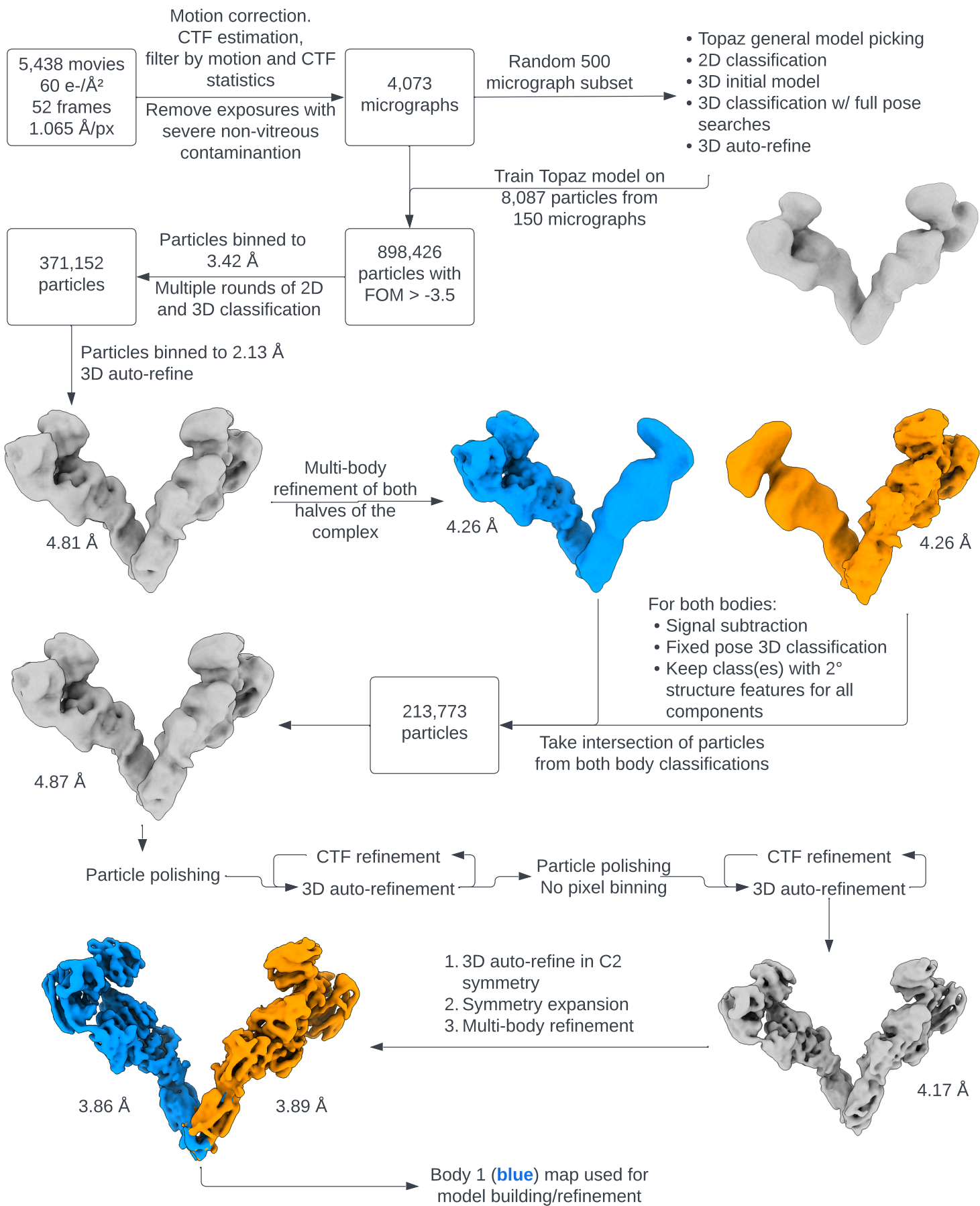
Supplementary Figure 7. Supporting Data for Primary Vy9Vδ2 Activation Assays. (A) Gating strategy for Primary Vy9Vδ2 T cell activation assays. CD25, CD107a, and CD69 gates drawn to ~1% positive staining by isotype (CD69, CD107a) or FMO (CD25) controls. (B) Inhibition of activation of Vy9Vδ2 T cells expanded from primary PBMCs after co-incubation with DaudiΔ2A1 cells pre-incubated with 5 μM HMBPP and increasing concentrations of α-BTN2A1 mAb or controls (MOPC isotype, 103.2 mAb or Buffer + HMBPP). CD25 (left), CD69 (middle) and CD107a (right) expression as markers of T cell activation were assessed by flow-cytometry (gated on isotype antibody). Mean + SD (n=3).

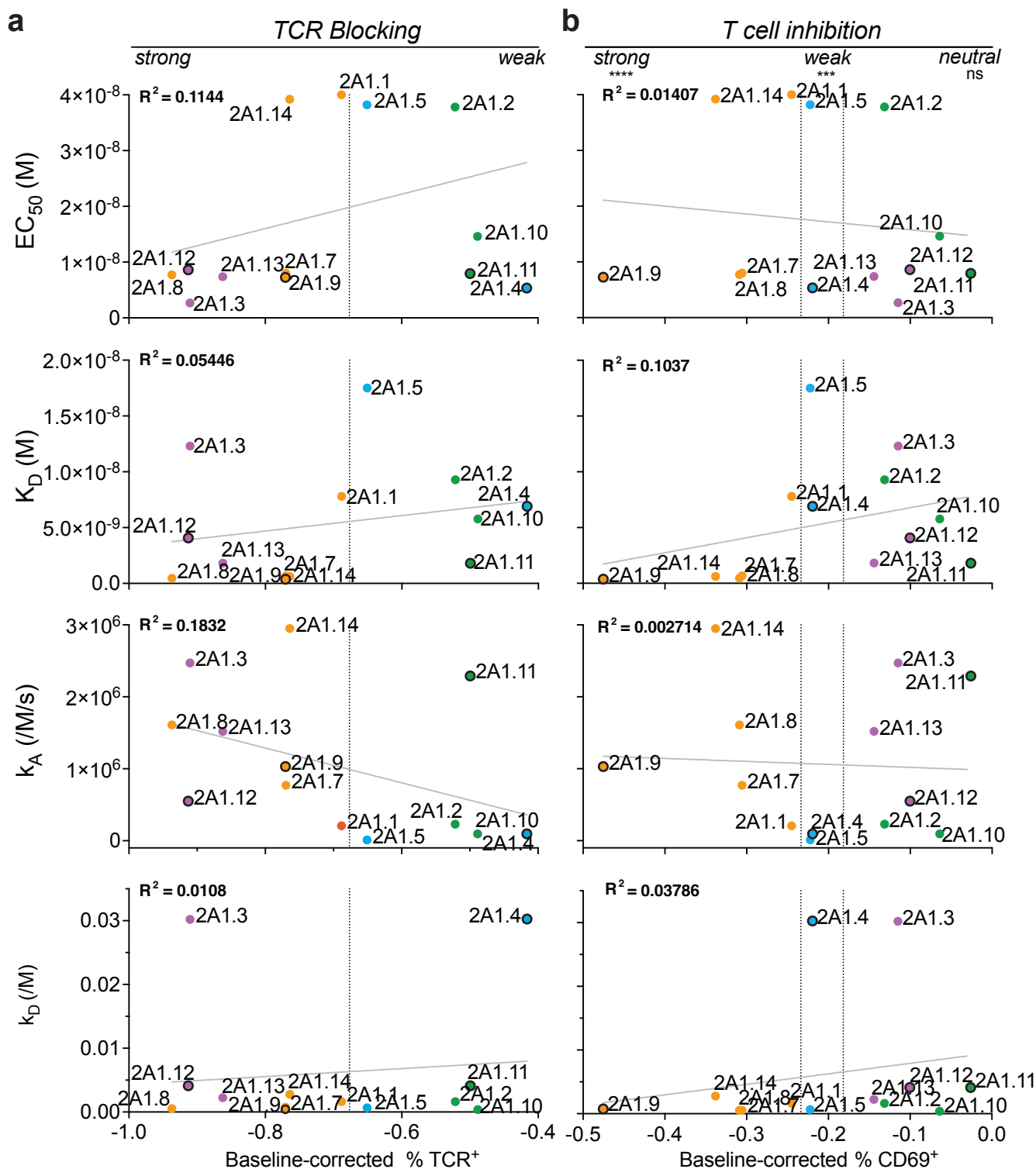


Supplementary Figure 8. Supporting Data for α -BTN2A1 mAb/Fab - V γ 9V δ 2 TCR competition Assays. (A) MAb and TCR competition for BTN2A1 binding was assessed on High-Five cells transfected with BTN2A1 or ADLRG3-control. Gating strategy is shown including V γ 9V δ 2 TCR Tetramer positive gate drawn to ~1% positive staining in – mAb (Buffer) condition. (SA-PE: Streptavidin-PE). (B) Competition between α -BTN2A1 mAbs and the V γ 9V δ 2 TCR for binding to BTN2A1 ectodomain was assessed in High-Five insect cells expressing full-length ADLRG3 control transmembrane protein stained with 150nM α -BTN2A1 mAbs or controls (20.1, 103.2, MOPC isotype or Buffer) followed by 120nM fluorescently-tagged V γ 9V δ 2-TCR tetramers (G115 clone). TCR tetramer staining was assessed by flow-cytometry (gated on no TCR). Mean + SD (n=6, 2 independent experiments). (C) Biotinylated BTN2A1 immobilization on streptavidin biosensor assessed by BLI, colored by reagent subsequently tested (representative of 2 independent experiments). (D) α -BTN2A1 Fab or control (20.1 Fab) competition with V γ 9V δ 2 TCR for binding to BTN2A1 ectodomain assessed by BLI. Immobilized Biotinylated BTN2A1 was exposed to 1. 66 μ M Fab followed by 2. 66 μ M Fab + 80 μ M TCR (G115 clone). 20.1 (top-left), 2A1.9 (top-middle), 2A1.4 (top-right), 2A1.13 (bottom-left), 2A1.12 (bottom-middle) and 2A1.11 (bottom-right) were assessed.



Supplementary Figure 9. Protein expression and purification for structural studies. (A) Chromatography trace of 2A1.9 Fab - BTN2A1 ectodomain complex purified over the S200 Size Exclusion Chromatography (left). SDS-PAGE gel showing complex elution fractions from S200 purification (right). (Fab: 48kDa, BTN2A1 ectodomain: 26kDa). (B) Chromatography traces of 2A1.12 Fab - BTN2A1 dimer - nanobody complex purified over the S200 Size Exclusion Chromatography (left). SDS-PAGE gels showing complexes elution fractions from S200 purification (right). (Fab: 48 kDa, BTN2A1 dimer: 51 kDa, Nanobody: 15 kDa). Mark12 Unstained protein standard was used to determine Molecular Weight (MW) for each gel. Red bars indicated fractions used for structural determination.





Supplementary Figure 11. Analysis of the relationship between reagent affinity and mAb physiological property. (A) Baseline-corrected TCR blocking from (Fig. 3B) plotted against mAb or Fab affinity properties including mAb EC_{50} of binding to DaudiΔAAVS1 cells, Fab K_D , Fab k_A and Fab k_D from (Supplementary Figure 4). (B) Baseline-corrected CD69 expression from (Fig. 3A) plotted against mAb or Fab affinity properties including mAb EC_{50} of binding to DaudiΔAAVS1 cells, Fab K_D , Fab k_A and Fab k_D from (Supplementary Figure 2). Simple linear regression was calculated with R^2 values indicated.

Supplementary Table 1: α -BTN2A1 Fab^{*}/mAb[‡] properties

	CDR-L3	CDR-H1	CDR-H2	CDR-H3	K _D (M) *	k _{on} (/M/sec) *	k _{off} (/sec) *	EC ₅₀ (M) ‡
2A1.1	SSSSLI	LYSSSI	SIYSSSGYTY	VYYQRGYIYSGF	7.794E-09	2.079E+05	1.620E-03	3.996E-08
2A1.2	ASDWPI	FSYSSI	SIYSYYGSTS	YYYARGYVYAF	9.280E-09	2.298E+05	2.132E-03	3.784E-08
2A1.3	ARSPLI	IYSSSI	SIYSYSGYTY	YYYYWGGVGDAL	1.227E-08	2.465E+06	3.024E-02	2.676E-09
2A1.4	SSSSLI	FSSSSI	SIYSSSGYTY	WQSYRGYPWPGF	6.907E-09	9.494E+04	6.557E-04	5.343E-09
2A1.5	SSSSLI	FSSSSI	SISSSSGSTS	GHLYWEYVQWGF	1.754E-08	1.147E+04	2.012E-04	3.818E-08
2A1.6	SSSSLI	FSSSSI	SISSSSGSTS	GHYYYWYYPGYGF	ND	ND	ND	4.556E-08
2A1.7	SSSSLI	FSSSSI	YIYPSSGSTS	YYYYRGYIDAM	6.730E-10	7.695E+05	5.363E-04	7.986E-09
2A1.8	SSSSLI	FSSSYI	SIYSYYGSTS	ETVYKGYVPAL	4.609E-10	1.612E+06	7.423E-04	7.723E-09
2A1.9	SSSSLI	LYSSSI	YIYPSSGYTS	YYYTRGYPDGM	3.727E-10	1.032E+06	3.844E-04	7.248E-09
2A1.10	SSSSLI	FSSSSI	SISSSSGSTS	WFYERGYVWAM	5.766E-09	9.566E+04	5.516E-04	1.462E-08
2A1.11	SSSSLI	FSSSSI	SISSSSGSTS	GHTYWMYYSGWGM	1.799E-09	2.291E+06	4.121E-03	7.936E-09
2A1.12	SSSSLI	FSSSSI	SIYSSSGYTY	IEYGRGYWDAF	4.072E-09	5.502E+05	2.241E-03	8.608E-09
2A1.13	SQGELI	FSSSSI	SISSSSGSTS	GHYYYYYYSGWGF	1.824E-09	1.517E+06	2.765E-03	7.388E-09
2A1.14	SKYMLI	FSYSSI	SISPYSSSTS	GHVYYHYYPGYGM	6.214E-10	2.948E+06	1.832E-03	3.922E-08
2A1.15	SSSSLI	FSSSSI	SISSSSGSTS	GHQYYYYYGWGF	ND	ND	ND	5.715E-10

Supplementary Table 2: Crystallography Data Collection and Refinement Statistics

2A1.9 Fab + BTN2A1 C219S ectodomain	
PDB ID code	8VC7
Data Collection	
Radiation Source	SSRL 14-1
Wavelength (Å)	1.1950
Resolution range (Å)	39.46 - 2.76 (2.83 - 2.76)
Space group	P 21 21 21
Unit cell	44.915 200.976 206.684 90 90 90
Data Processing	
Total reflections	441063 (41679)
Unique reflections	49500 (4503)
Multiplicity	8.9 (9.3)
Completeness (%)	99.90 (100.00)
Mean I/sigma(I)	7.2 (1.6)
Wilson B-factor	42.85
R-merge	0.272 (1.513)
R-meas	0.289 (1.600)
R-pim	0.095 (0.516)
CC1/2	0.99 (0.663)
Data Refinement	
Reflections used in refinement	49406 (3511)
Reflections used for R-free	1998 (136)
R-work	0.2184 (0.3430)
R-free	0.2602 (0.3822)
Number of non-hydrogen atoms	9797
macromolecules	9727
ligands	70
solvent	0
Protein residues	1288
RMS(bonds)	0.009
RMS(angles)	1.14
Ramachandran favored (%)	96.86
Ramachandran allowed (%)	2.91
Ramachandran outliers (%)	0.24
Rotamer outliers (%)	3.83
Clashscore	6.12
Average B-factor	38.63
macromolecules	38.46
ligands	62.13

Supplementary Table 3: 2A1.9 Fab contacts with BTN2A1

2A1.9 Fab Residue	BTN2A1 residue	Type
CDR1 Light		
Ser30 (OG)	Asp33 (OD1)	HB
Ser30 (OG)	Glu32, Asp33, Gly52, Gly53	VdW
Ser31	Asp33	VdW
Ala32	Gly52	VdW
CDR2 Light		
Tyr49	Gly102, Arg103	VdW
Ser53	Gly102, Arg103	VdW
CDR3 Light		
Ser91 (O)	Arg56 (NH2)	HB
Ser91 (O)	Arg56	VdW
Ser92 (O)	Arg54 (NE)	HB
Ser92 (OG)	Gly52 (O)	HB
Ser92	Gly52, Arg54	VdW
Other Light		
Arg66 (NH1)	Asp33 (OD2)	HB, SB
Arg66 (NH1)	Asp33	VdW
CDR1 Heavy		
Tyr30	Gln42, Pro45	VdW
Ser31	Ser44	VdW
CDR2 Heavy		
Tyr50 (OH)	Glu58 (OE2)	HB
Tyr50 (OH)	Glu58	VdW
Tyr52 (OH)	Glu58 (OE2)	HB
Tyr52 (OH)	Glu58, Glu59	VdW
Ser54	Pro45	VdW
Ser55 (OG)	Glu58 (O)	HB
Ser55 (OG)	Glu58, Arg65	VdW
Tyr57	Thr57, Glu58, Arg65	VdW
CDR3 Heavy		
Tyr101	Phe43, Ser44, Glu59	VdW
Thr102	Phe43	VdW
Arg103 (NE)	Glu107 (OE1, OE2)	HB, SB
Arg103 (NH2)	Glu107 (OE1, OE2)	HB, SB
Arg103	Phe39, Phe43, Arg96, Tyr98, Glu107	VdW
Gly104 (N)	Tyr98 (OH)	HB
Gly104 (N)	Tyr98, Gln100, Tyr105	VdW
Tyr105 (OH)	Glu35 (OE1)	HB
Tyr105 (OH)	Lys 51 (NZ)	HB
Tyr105 (O)	Gln100 (NE2)	HB
Tyr105	Glu35, Phe43, Gln100	VdW
Asp107 (OD1, OD2)	Lys51 (NZ)	SB

[vdW: van der Waal contact (distance < 4 Å); H: hydrogen bond (distance < 3.5 Å) SB: salt bridge (distance < 4.5 Å, polar interaction between oppositely charged atoms)]

Supplementary Table 4: Epitopes on BTN2A1

TCR	Mutagenesis[25]	2A1.9	2A1.12	2A1.9 & TCR	2A1.12 & TCR	2A1.9 & 2A1.12	2A1.9&Mutagenesis
39	37	32	33	39	39	33	51
41	51	33	35	42	43	35	96
42	96	35	37	43	44	39	98
43	98	39	39	44	96	43	107
44	107	42	43	96	98	44	
96		43	44	98	105	51	
98		44	51	100	107	52	
100		45	52	105		54	
105		51	54	107		56	
106		52	56			58	
107		53	58			59	
109		54	59			65	
		56	62			96	
		57	65			98	
		58	96			102	
		59	98			103	
		65	102			105	
		96	103			107	
		98	105				
		100	107				
		102					
		103					
		105					
		107					

Numbers indicate BTN2A1 residue. & indicates shared binding epitope.

Supplementary Table 5: Cryo-EM Data Collection and Processing

Fab in Complex	2A1.12 Fab + Nanobody + BTN2A1 ectodomain
Data Collection	
Accelerating voltage (kV)	300
Nominal magnification	81,000X
Calibrated pixel size at magnification (Å)	1.065
Spot size	6
Condenser aperture (µm)	50
Camera mode	Counted super-resolution, 2x hardware binning; CDS
Energy filter width (eV)	20
Exposure dose (e-/Å ²)	60
Exposure movie frames	52
Target defocus range (µm)	-2.5 to -0.9

Supplementary Table 6: 2A1.12 Fab contacts with BTN2A1

2A1.12 Fab Residue	BTN2A1 residue	Type
CDR1 Light		
Val29	Gly52	VdW
Ser30	Asp33, Gly52	VdW
Ser31	Asp33	VdW
CDR2 Light		
Tyr49	Arg103	VdW
Ser53	Gly102, Arg103	VdW
CDR3 Light		
Ser91	Arg56	VdW
Ser92	Gly52, Arg54, Arg56	VdW
Ser94	Arg54, Glu58	VdW
Other Light		
Arg66	Asp33	VdW
Arg66 [NH1]	Asp33[OD2]	SB
Arg66 [NH2]	Asp33[OD2]	SB
CDR1 Heavy		
Ser31	Ser44	VdW
CDR2 Heavy		
Tyr52	Glu58	VdW
Ser54	Ser44, Glu62	VdW
Ser55	Glu58	VdW
Tyr57	Arg65	VdW
Tyr59	Phe43, Glu58	VdW
CDR3 Heavy		
Tyr101	Arg37, Ser44, Glu59	VdW
Gly102	Phe43	VdW
Arg103 [NE]	Glu107 [OE1]	SB
Arg103 [NH1]	Glu107 [OE1]	SB
Arg103 [NH1]	Glu107 [OE2]	SB
Arg103 [NH2]	Glu107 [OE1]	SB
Arg103	Phe39, Phe43, Arg96, Glu107,	VdW
Gly104	Tyr98, Tyr105	VdW
Tyr105	Glu35, Lys51	VdW
Asp107 [OD1]	Lys51 [NZ]	SB
Asp107	Lys51	VdW

[vdW: van der Waal contact (distance < 4 Å); H: hydrogen bond (distance < 3.5 Å) SB: salt bridge (distance < 4.5 Å, polar interaction between oppositely charged atoms)]