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Supplementary Materials for

Cryo-EM Structure of the B Cell Co-receptor CD19 Bound to the Tetraspanin CD81

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Other Supplementary Materials for this manuscript includes the following:

Materials and Methods

Expression and Purification of Coltuximab F_{ab} for Cryo-EM

The heavy and light chains of the Coltuximab antibody were co-expressed in Expi293F cells (Thermo Fisher, Catalog number A14527). 300 mL of Expi293F cells maintained in Expi293 expression media were grown to a density of 2.8 x 10^6 cells/mL and then transiently co-transfected with heavy and light chain DNA at a 1:3 (w:w) ratio (0.24 mg total DNA) and FectoPro transfection reagent (Polyplus) at a 1:1 (w:w) DNA/FectoPro ratio. 20 hours after transfection, 5 mM valproic acid sodium salt (Sigma-Aldrich) and 2.7 mL of 45% D-(+)-Glucose solution (Sigma-Aldrich) were added to the cultures. Transfected cells were cultured for 5 days to produce protein and then the media was collected and separated from the cells by centrifugation at 4000 *g* for 15 minutes at 4 °C. The cultured media was loaded onto protein A resin (Millipore). The resin was washed with 100 mL 20 mM HEPES pH 7.4, containing 150 mM NaCl and then bound protein was eluted in 15 mL 100 mM glycine buffer pH 3.0. Elution fractions were immediately neutralized with 1 M HEPES pH 8. Eluted protein was buffer exchanged in a centrifugal filter into 20 mM HEPES pH 7.4, containing 150 mM NaCl and then 3C protease was added at a 1:1 w/w ratio and incubated overnight at 4°C. The purity of the eluted protein and efficiency of cleavage was assessed on an SDS-PAGE Coomassie-stained gel. The sample was then applied to Protein A resin to remove residual free Fc. Flow through from the protein A resin was then concentrated with a centrifugal filter, and the purified F_{ab} was isolated on an S200 size exclusion column in 20 mM HEPES pH 7.4, containing 150 mM NaCl, 0.05% GDN and 0.005% CHS. The purity of fractions corresponding to the F_{ab} peak were assessed on an SDS-PAGE Coomassie-stained gel and then pooled, concentrated to 1 mg/mL, flash frozen in liquid nitrogen, and stored at -80 °C.

Expression and Purification of CD19-CD81-Coltuximab Complex

The CD19-CD81 fusion protein was expressed in Expi293F cells. 300 mL of Expi293F cells maintained in Expi293 expression media were grown to a density of 2.8 x 10^6 cells/mL and then transiently transfected with 0.24 mg of DNA and FectoPro transfection reagent (Polyplus) at a 1:1 DNA/FectoPro ratio. 24 hours after transfection, the cells were fed 5 mM Valproic acid sodium salt (Sigma-Aldrich) and 5.5 mL of 45% D-(+)-Glucose solution (Sigma-Aldrich). Transfected cells were cultured for an additional 24 hours. Cells were harvested by centrifugation at 4000 *g* for 15 minutes at 4 °C and then flash frozen and stored at -80 °C until purification.

After the frozen cells were thawed, cells were lysed by osmotic shock in 20 mM HEPES pH 7.4, 2 mM magnesium chloride, 2 mg/mL iodoacetamide (Sigma Aldrich) and 1:100,000 (v:v) benzonase nuclease (Sigma Aldrich). Lysed cells were centrifuged at 18,000 *g* for 20 min. CD19-CD81 was then extracted from the pellet using a glass dounce tissue grinder in 75 mL solubilization buffer containing 20 mM HEPES pH 7.4, 250 mM NaCl, 10% (v/v) glycerol, 1% (w/v) n-Dodecyl-b-D-Maltoside (DDM - Anagrade; Anatrace), 0.1% (w/v) cholesterol hemisuccinate (CHS; Steraloids) and 2 mg/ml iodoacetamide. The sample was stirred for 2 hours at 4°C and then centrifuged at 20,000 rpm for 30 minutes.

Supernatant supplemented with 2 mM calcium chloride was filtered through a glass microfiber prefilter and loaded by gravity flow onto 2 mL of M1 anti-FLAG antibody affinity resin. The resin was washed with 50 mL of buffer containing 2 mM $CaCl_2$, 250 mM NaCl, 20 mM HEPES pH 7.4, 1% glycerol, 0.1% DDM, and 0.01% CHS, then 50 mL of buffer containing 2 mM $CaCl_2$,150 mM NaCl, 20 mM HEPES pH 7.4, 0.1% glycerol, 0.1% DDM, and 0.01% CHS, and then 50 mL of

buffer containing 2 mM $CaCl_2$,150 mM NaCl, 20 mM HEPES pH 7.4, 0.05% GDN, and 0.005% CHS. 1 mg of Coltuximab F_{ab} was then added to anti-FLAG resin with bound CD19-CD81, and the resin was again washed with 50 mL buffer containing 2 mM CaCl2,150 mM NaCl, 20 mM HEPES pH 7.4, 0.05% GDN, and 0.005% CHS. Bound protein was eluted in 12 mL buffer containing 5 mM EDTA,150 mM NaCl, 20 mM HEPES pH 7.4, 0.05% GDN, and 0.005% CHS. The elution was concentrated in a 100k MWCO centrifugal filter, and then immediately purified by size exclusion chromatography (SEC) on a Sephadex S200 column (GE Healthcare) in buffer containing 150 mM NaCl, 20 mM HEPES pH 7.4, 0.05% GDN, and 0.005% CHS. Immediately after the F_{ab}-CD19-CD81 complex eluted from the S200 column, a single fraction was concentrated to 1.8 mg/mL using a 100k MWCO centrifugal filter and then applied to grids. Purity and monodispersity of the sample for cryo-EM was evaluated by SDS-PAGE and SEC (Figure S1).

Cryo-EM Grid Preparation

Samples for cryo-EM were prepared on Quantifoil® holey carbon film-coated 400 mesh copper grids (Electron Microscopy Sciences, R1.2/1.3 and R2/1). Grids were glow-discharged before the application of 3.5 µL of the sample (1.8 mg/mL). After blotting for 4.5-5.5 s with a blot force of 15-16, the grids were plunge-frozen in liquid ethane using a FEI Vitrobot Mark IV (FEI, Hillsboro) with 100% chamber humidity at 22°C.

Cryo-EM Data Collection and Processing

Data were collected in four separate sessions, with data from sessions 1-3 processed together initially ("data set 1") and then later merged with data collected in the fourth session ("data set 2"). Samples were imaged on a FEI Titan Krios at 300 kV with a Gatan Quantum Image Filter with K3 Summit direct detection camera in counting mode with a total exposure dose of around 55 electrons. Fifty frames per movie were collected at a magnification of 105,000x, corresponding to 0.825 Å per pixel. Micrographs were collected at defocus values ranging from −1.0 to −2.5 µm, and the movie frames were motion-corrected and dose-weighted by MotionCor2 (*48*) and contrast transfer function (CTF) parameters were estimated by CTFFIND4 (*49*). For data set 1, particles were picked on-the-fly using crYOLO, giving 1,784,973 initial particles (*50*). An initial starting model was generated ab-inito in Relion from a small data set collected previously on a Talos Arctica. Following multiple rounds of 2D and 3D classification (*51*), 3D refinement of 453,510 particles led to a reconstruction at 4.7 Å in Relion 3.0.8. Bayesian polishing was carried out on these particles in Relion 3.1, and then the particle stack was imported into cryoSPARC v2 (*52*). An additional three rounds of heterogenous classification were performed, and then particles were merged with particles from data set 2.

For data set 2, particles were picked on-the-fly using crYOLO, giving 1,013,972 initial particles (*50*). Following multiple rounds of 2D classification in Relion 3.0.8 (*51*), particles were merged with the 453,510 data set 1 particles from the 4.7 Å reconstruction. Following three rounds of 3D classification, 3D refinement led to a reconstruction at 4.2 Å in Relion 3.0.8. Bayesian polishing was carried out on data set 2 in Relion 3.1, and then the data set 2 particle stack was imported into cryoSPARC v2 (*52*). Polished particles were merged with particles from data set 1, and an additional two rounds of heterogenous classification were performed, followed by non-uniform refinement, resulting in a final reconstruction at 3.8 Å from 244, 583 particles. The map was post-

processed in DeepEMhancer, using the half maps as inputs. The data processing workflow can be found in Figure S2.

Model building was carried out using both non-uniform refinement and deepEMhancer maps overlayed in Coot. The atomic coordinates of the CD19 ectodomain (PDB: 6AL5), Coltuximab (PDB: 6ANI), and full length CD81 (PDB: 5TCX) were manually fitted into the density map using Chimera to generate a starting model (*53*). Residues 1-89 (TM1-TM2), 90-112 and 202-224 (TM3-TM4) and 113-201 (large extracellular loop) of CD81 were docked into the density separately to allow for fitting. The coordinates were then manually rebuilt using Coot (*54*). The transmembrane region of CD19 was modeled as a polyalanine helix, and side chains within the CD81 transmembrane domain were truncated to Cß. All models were refined in Phenix real-space refine with secondary structure restraints against the 3.8 Å map from non-uniform refinement in cryoSPARC. The final models were evaluated by MolProbity. Statistics of the map reconstruction and model refinement are presented in Supplementary Table 1. Structural biology applications used in this project (other than CryoSPARC) were compiled and configured by SBGrid (*55*).

Preparation of Fluorescein labeled Coltuximab, Denintuzumab, or Inebilizumab F_{ab}

The heavy and light chains of Coltuximab, Denintuzumab, or Inebilizumab were co-expressed in Expi293F cells. 300 mL of Expi293F cells maintained in Expi293 expression media were grown to a density of 2.8 x 10^6 cells/mL and then transiently co-transfected with heavy and light chain DNA at a 1:3 (w/w) ratio (0.24 mg total DNA) and FectoPro transfection reagent (Polyplus) at a 1:1 (w:w) DNA/FectoPro ratio. 20 hours after transfection, 5 mM valproic acid sodium salt (Sigma-Aldrich) and 5.5 mL of 45% D-(+)-Glucose solution (Sigma-Aldrich) were added to the cultures. Transfected cells were cultured for 5 days to produce protein and then the media was collected and separated from the cells by centrifugation at 4000g for 15 minutes at 4°C. The cultured media was loaded onto protein A resin (Millipore). The resin was washed with 100 mL 20 mM HEPES pH 7.4, containing 150 mM NaCl and then bound protein was eluted in 10 mL 100 mM citrate buffer pH 3.0. Elution fractions were immediately neutralized with 1 M citrate pH 6.

To generate F_{ab} fragments, 2 mL immobilized ficin agarose resin (Thermo Fisher Scientific) was washed twice with 10 mL 100 mM citrate buffer pH 6.0 containing 5 mM EDTA and 25 mM cysteine. The antibody elution with 5 mM EDTA and 25 mM cysteine was added to the ficin resin and rotated at 37°C for 3.5 hours. The supernatant was recovered and the immobilized ficin was washed five times with 10 mL 20 mM HEPES pH 7.4, containing 150 mM NaCl. The elution was concentrated in a 30k MWCO centrifugal filter, and then immediately purified by size exclusion chromatography (SEC) on a Sephadex S200 column (GE Healthcare) in buffer containing 150 mM NaCl, 20 mM HEPES pH 7.4. The purity of fractions corresponding to the F_{ab} peak were assessed on an SDS-PAGE Coomassie-stained gel and then pooled, concentrated to 2 mg/mL, flash frozen in liquid nitrogen, and stored at -80°C.

20-fold molar excess of NHS-fluorescein (Thermo Fisher Scientific) was added to the purified F_{ab} and then incubated at 4°C overnight with rocking. Un-reacted NHS-fluorescein was removed with a Zebaspin Desalting Column (7K cutoff).

CD19 Antibody Competition Assay

Expi293F cells were transfected with the CD19-CD81 fusion protein as described above. Cells were harvested by centrifugation at 4000g for 15 minutes at 4°C, and then immediately seeded at 100,000 cells per well in a 96 well plate. Cells were washed twice with 100 µL of ice-cold PBS per well. Cells were then incubated 100 µM with of unlabeled Denintuzumab or Inebilizumab (or in positive control condition, no antibody) on ice for 30 minutes. Cells were washed twice with 100 µL of ice-cold PBS per well, and Coltuximab, Denintuzumab, or Inebilizumab F_{ab} directly labeled with Fluorescein were added at 10 µg/mL for 20 minutes on ice. Cells were washed twice with 100 µL ice-cold PBS and then analyzed on a BD Accuri C6 Plus flow cytometer.

CD19 Export Assay

$CD81^{-/-}$ HEK293T cells (RRID CVCL_0063 for parental cells) were seeded at 100,000 cells/well in 24 well plates 12-18 hours prior to transfection. $CD81^{-/-}$ HEK293T cells were transfected using Lipofectamine 2000 with either with either 1.5 µg of empty pcDNA3.1(+) vector, 0.75 µg of CD19 DNA and 0.75 µg of empty pcDNA3.1(+) vector DNA (CD19 condition), 0.75 µg of CD19 DNA and 0.75 µg of CD81 DNA (CD19+CD81 condition), or 0.75 µg of CD19 DNA and 0.75 µg of mutant CD81 DNA. 36-48 hours after transfection, cells were harvested in phosphate buffered saline (PBS) supplemented with 3 mM EDTA, transferred to a 96 well V-bottom plate, and then washed twice with PBS. Cells were then incubated on ice for 20 minutes with 2 µg/mL Alexa 488-anti-CD19 (ThermoFisher, Catalog number MHCD1920, RRID AB_10373539) and APC-anti-CD81 (BioLegend, Catalog number 349510, RRID AB_2564021) in 20 mM HEPES buffer pH 7.4, containing 150 mM NaCl, and 0.1% BSA. Cells were washed two times with PBS and analyzed on a BD Accuri C6 flow cytometer.

Cloning of Constructs

CD19-CD81 Fusion Protein:

The CD19-CD81 fusion was cloned into pcDNA3.1(+) with an N-terminal haemagglutinin signal sequence followed by a FLAG epitope tag and a 3C protease cleavage site. Residues 20-329 of CD19 (ectodomain, transmembrane domain, and first 15 cytoplasmic amino acids) were connected to full length CD81 using a GGSx4 linker.

Coltuximab

The variable regions of the antibody heavy chain were subcloned into the pFUSE-hIgG1-Fc2 vector (Invitrogen). The variable region of the light chain and the human kappa constant sequence with an N terminal "MDWTWRILFLVAAATGAHS" signal sequence were cloned in the pD2610-v5 vector (ATUM). A 3C protease site flanked by a Gly-Gly-Ser-Gly linker inserted into the hinge region of the heavy chain, allowing for generation of the Coltuximab F_{ab} after cleavage with 3C protease for use in cryo-EM

Inebilizumab and Denintuzumab

The variable regions of each antibody heavy chain were subcloned into the pFUSE-hIgG1-Fc2 vector (Invitrogen). The variable region of the light chains and the human kappa constant sequence with an N terminal "MDWTWRILFLVAAATGAHS" signal sequence were cloned in the pD2610-v5 vector (ATUM).

CD81 Type VI Immunodeficiency Truncation Mutant

The DNA sequence “atgcgcgaggccg” was inserted directly after the codon corresponding to K187 in wild type CD81, resulting in the frameshift mutation “MREAGGLPPEDR” starting a position 188 and a premature stop codon at position 200.

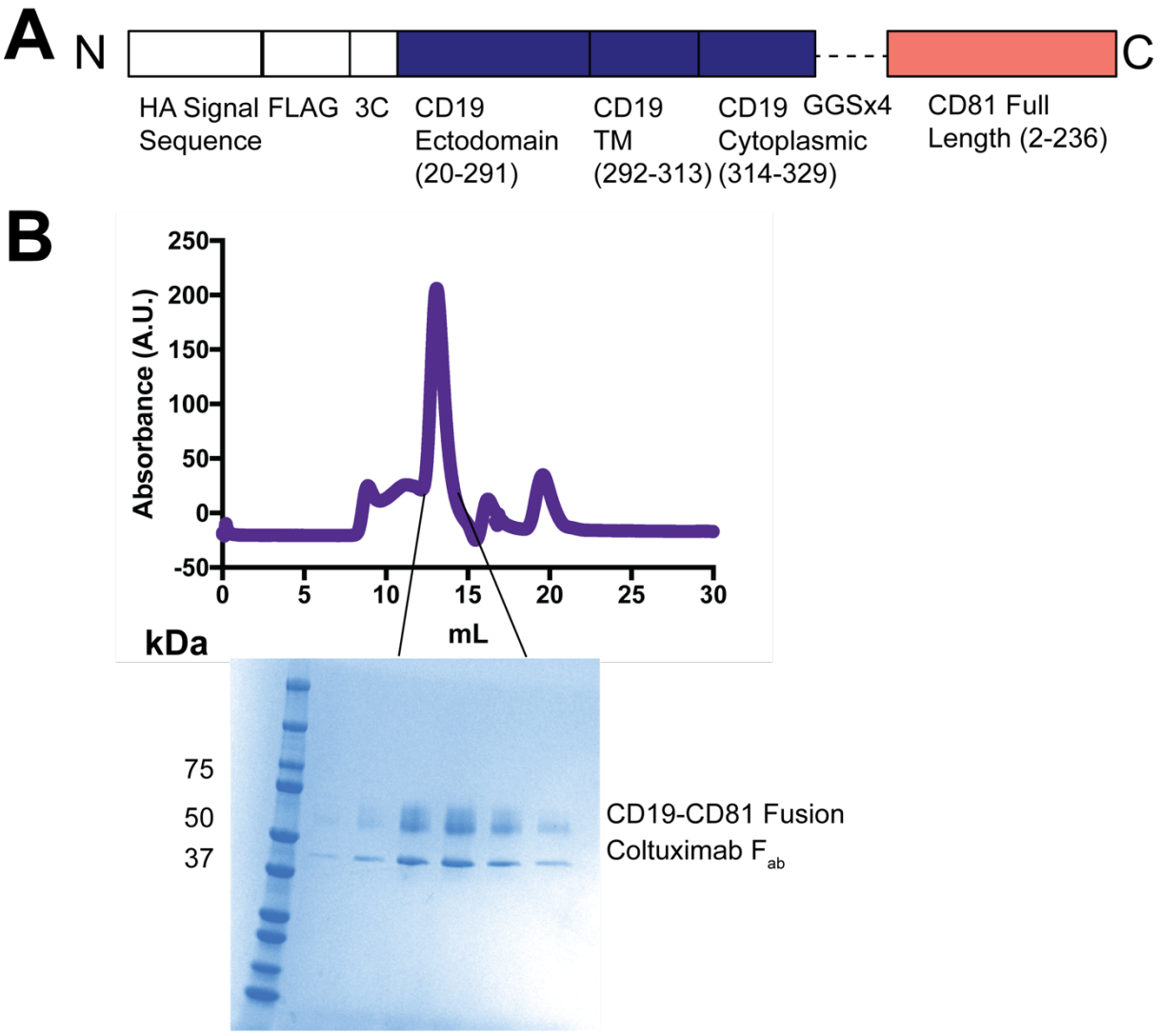

Fig. S1.

Biochemical characterization and cryo-EM analysis of CD19-CD81 complex. (A) Schematic of the construct used to produce the CD19-CD81 complex. Wild type CD19 (residues 20-329) was linked to full length, wild type CD81 using a Gly-Gly-Ser repeat. (B) Size exclusion profile of the purified CD19-CD81-F_{ab} complex in detergent buffer on a Superdex 200 column. An SDS-PAGE gel of the purified complex is shown below the chromatogram. The small peaks at approximately 15 mL and 20 mL correspond to free detergent micelle and FLAG peptide, respectively.

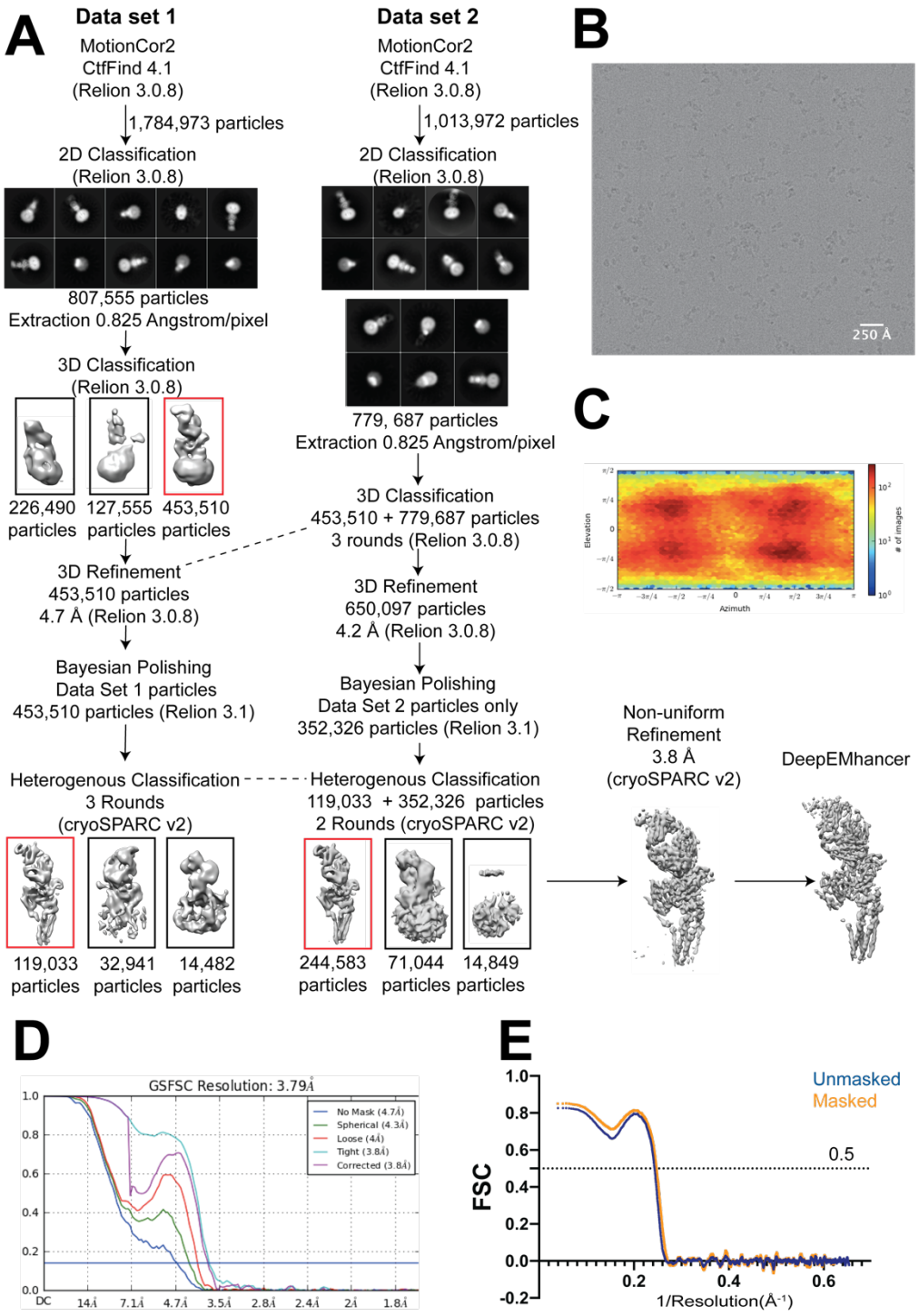

Fig. S2.

Cryo-EM image processing workflow. (A) Two-dimensional class averages of the CD19-CD81-F_{ab} particles and image processing workflow. Particles were extracted with a box size of 344. (B) A representative raw cryo-EM image. (C) Angular distribution of the CD19-CD81-F_{ab} particles in the final round of 3D refinement in CryoSPARC. (D) Fourier shell correlation (FSC) used to determine overall resolution of the map. The blue line marks the resolution corresponding to an FSC value of 0.143.(E) Map to model FSC curve.

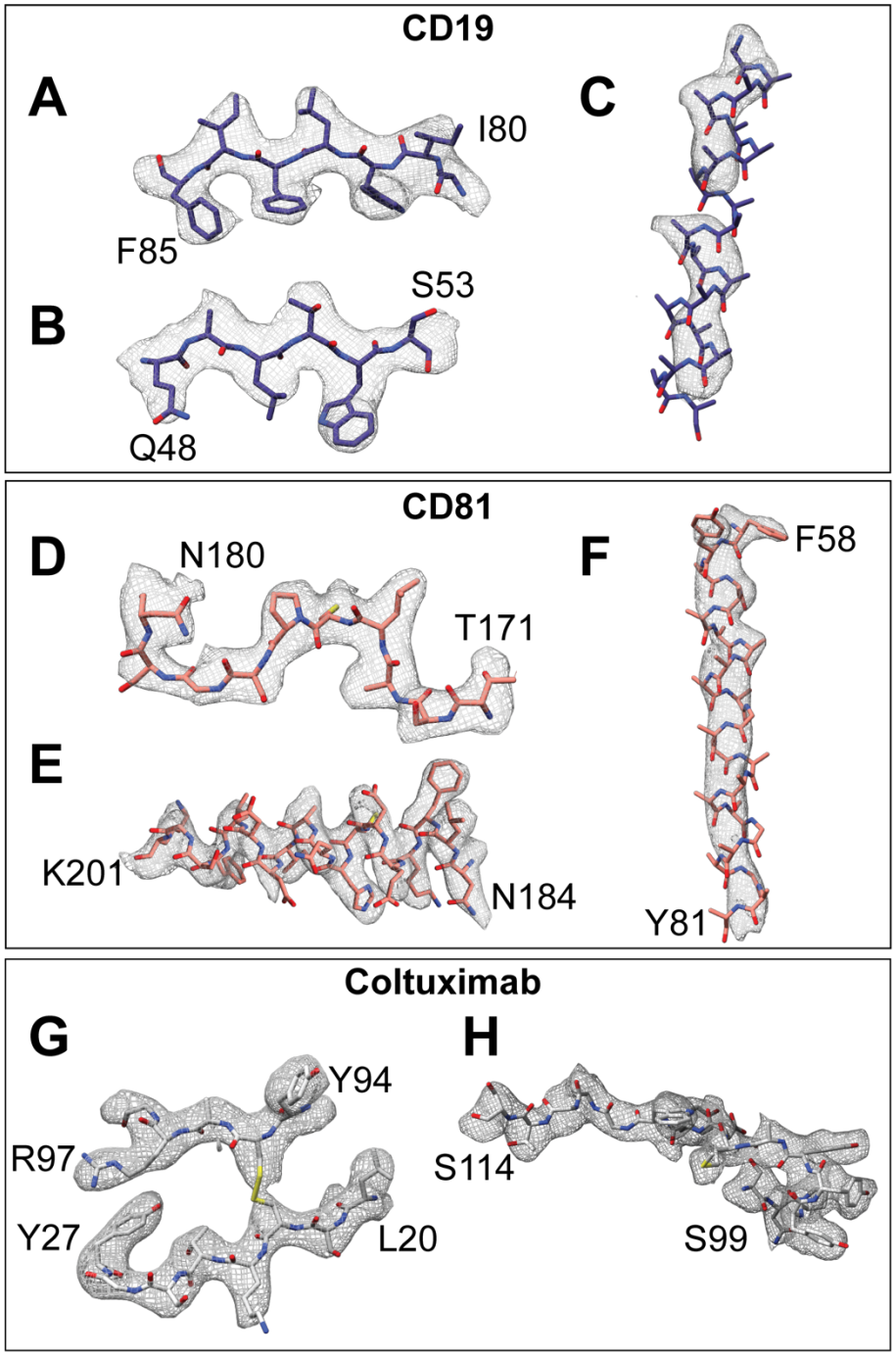

Fig. S3.

Fit of the atomic model into the cryo-EM map. The model (shown as sticks) is show with the corresponding cryo-EM density for: (A) a beta strand within the CD19 ectodomain that binds to CD81 (B) an additional beta strand in the CD19 ectodomain (C) the CD19 transmembrane domain (D) a region of the CD81 ectodomain that binds to CD19 (E) an alpha helix in the CD81 ectodomain (F) an alpha helix (TM2) within the CD81 transmembrane domain (G) a beta sheet in heavy chain of Coltuximab (H) a portion of CDR3 from the heavy chain of Coltuximab. Images were prepared in Chimera using a 1.9 Å carve radius.

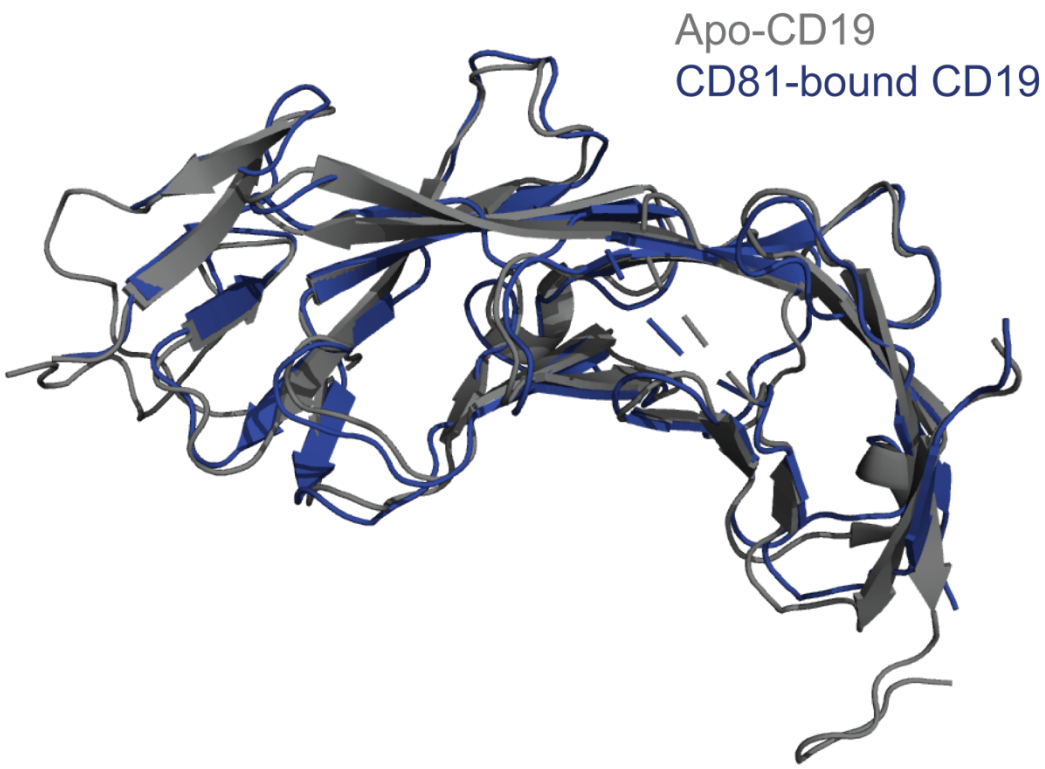

Fig. S4.
Comparison of CD19 structures in uncomplexed and complexed states. Ribbon diagrams showing superposition of the structure of uncomplexed CD19 (PDB:6AL5; gray) on the structure of CD81-complexed CD19 (blue).

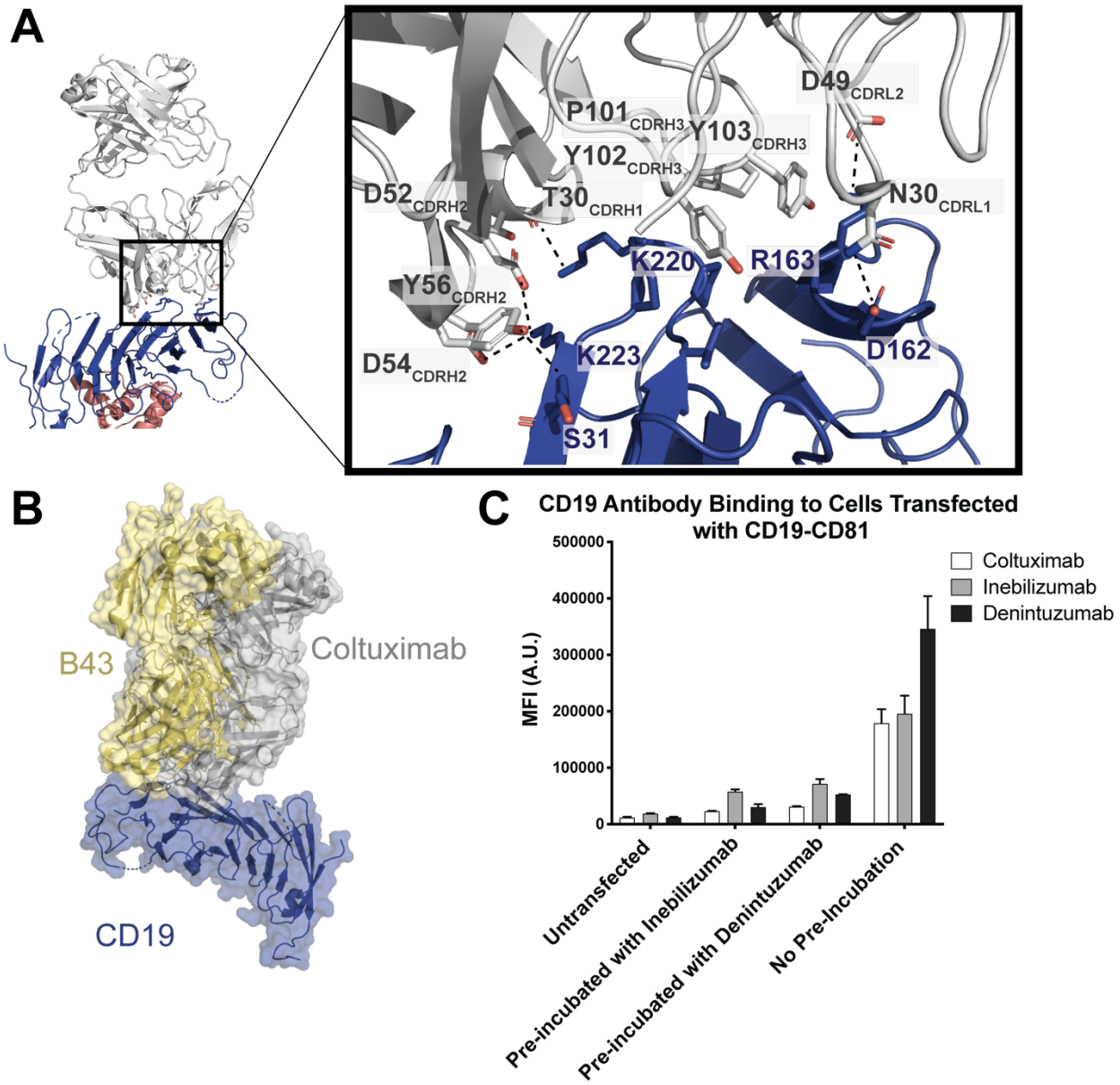

Fig. S5.
Coltuximab epitope and competition with other anti-CD19 antibodies. (A) CD19-Coltuximab binding interface. Hydrogen bonding interactions are indicated with dotted lines. (B) Superposition of the CD19-B43 Fab co-crystal structure (PDB: 6AL5) with the Coltuximab-CD19 interface in the CD81-CD19-Coltuximab structure, highlighting the overlap of the binding interface. (C) CD19 antibody binning experiment. Pre-incubation of cells expressing the CD19-CD81 fusion protein with unlabeled inebilizumab or denintuzumab was performed prior to staining of the cells with fluorescently labeled inebilizumab, denintuzumab, or coltuximab. Pre-incubation with either antibody blocks binding of all three antibodies to CD19. Error bars represent mean ± SEM of two independent experiments.

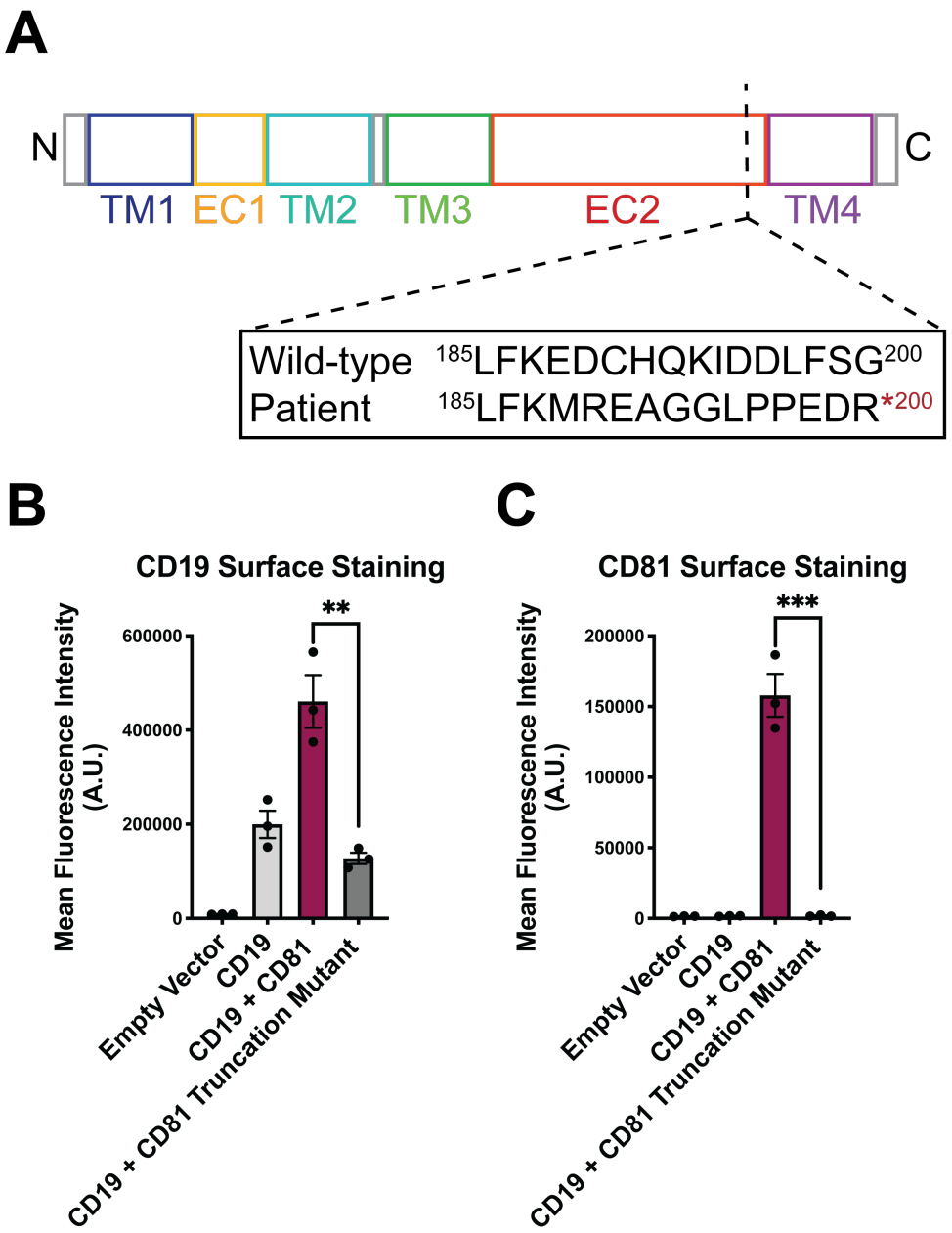

Fig. S6.
Effect of a CD81 immunodeficiency disease mutation on CD19 surface delivery. (A) Schematic representation of the CD81 domain organization, highlighting the amino sequence and the site of the premature stop codon resulting from a frameshift mutation within CD81 found in a patient with type VI immunodeficiency. (B) Effect of the CD81 truncation mutation on CD19 export. Surface CD19 was detected by flow cytometry using an Alexa 488-coupled anti-CD19 antibody. (C) Surface staining of the CD81 truncation mutant compared to wild type CD81, confirming that the truncation mutant is not detected on the cell surface. Statistical analysis was performed in GraphPad Prism using an unpaired t test. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

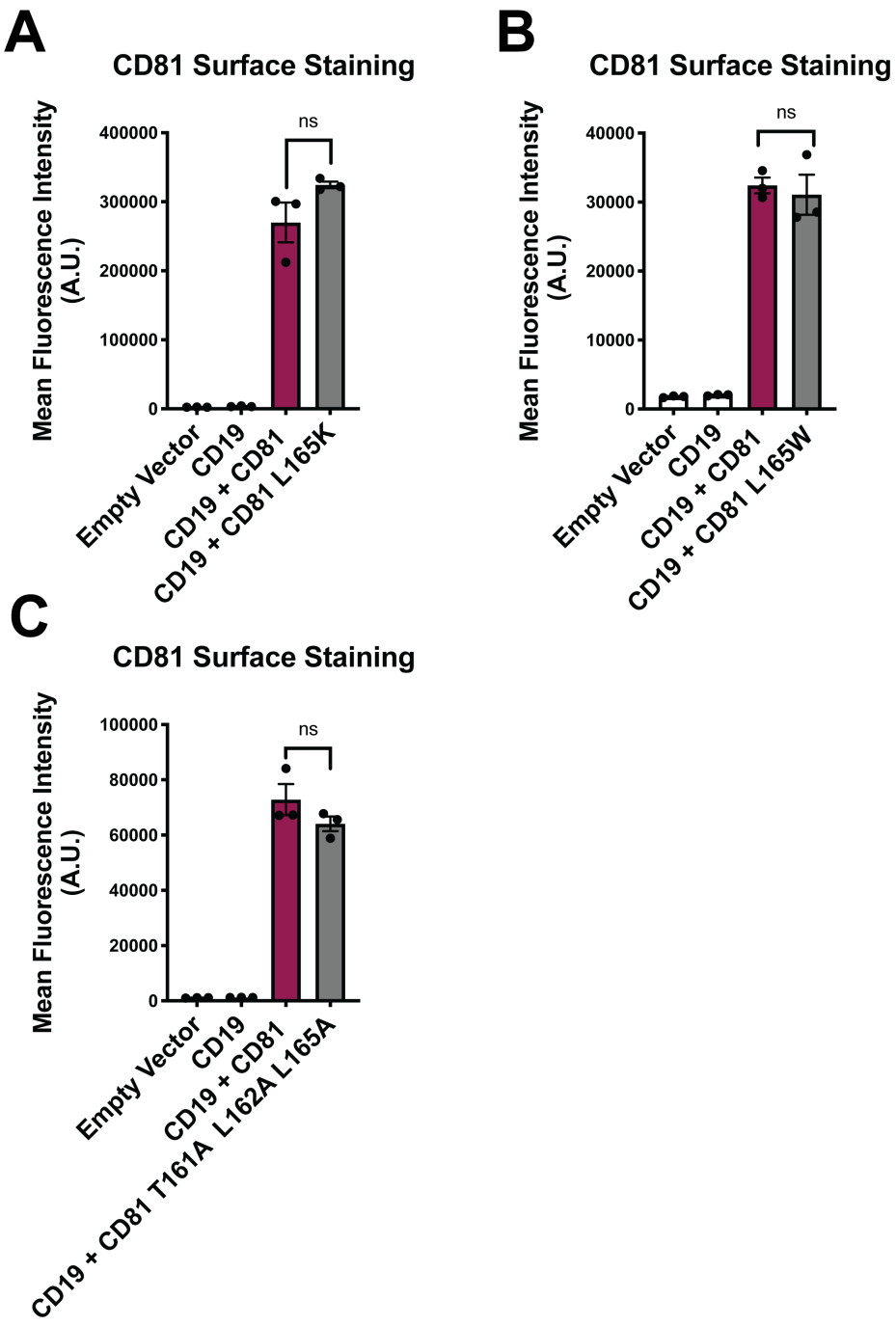

Fig. S7.
Surface staining of CD81 Point Mutants. Surface expression of CD81 L165K (A), L165W (B) and T161A L162A L165A (C). Error bars represent mean ± SEM of three independent experiments. Statistical analysis was performed in GraphPad Prism using an unpaired t test. Statistical analysis was performed in GraphPad Prism using an unpaired t test. Not significant: ns.

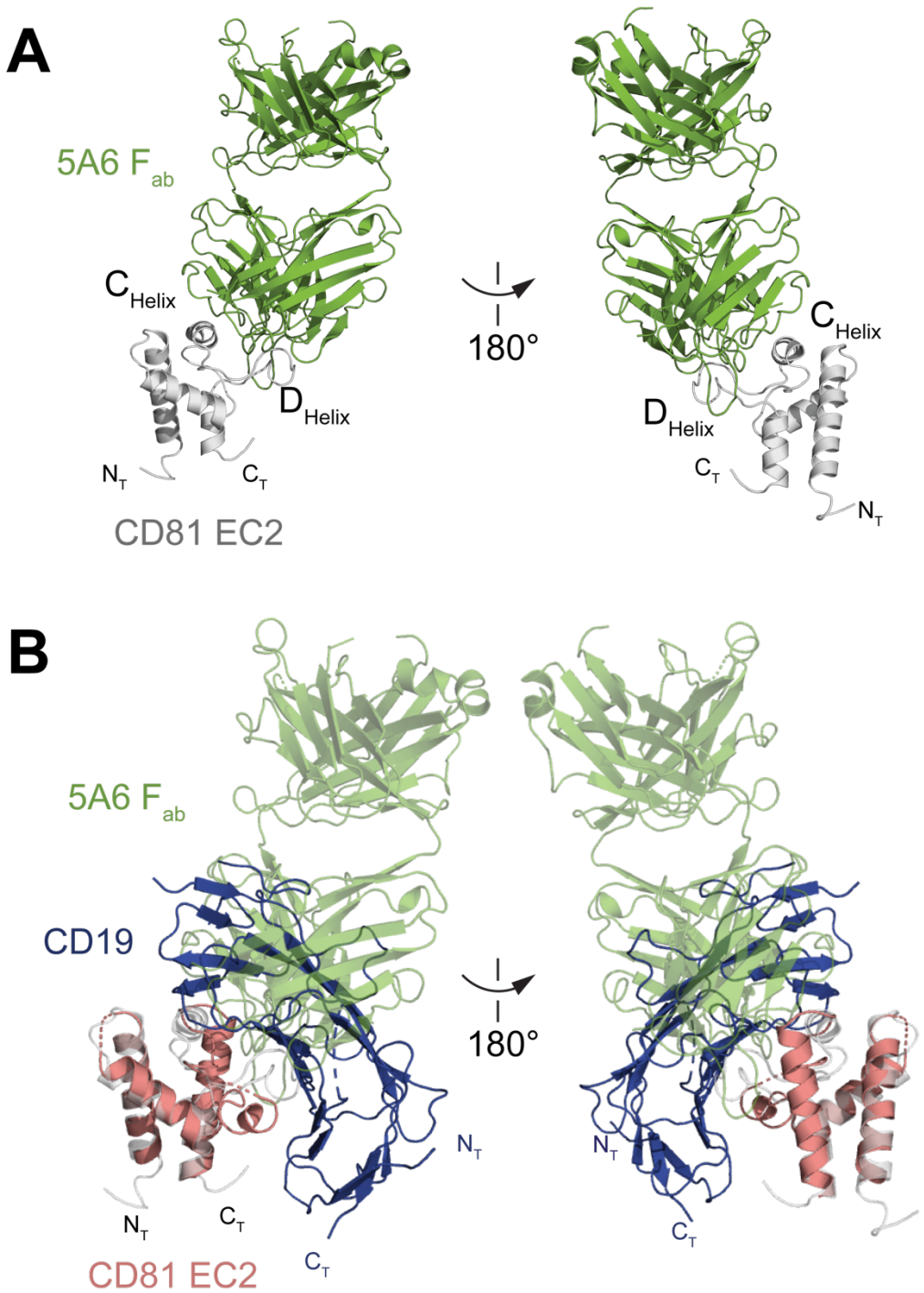

Fig. S8.
Comparison of the CD81-5A6 Fab and CD81-CD19 complexes. (A) Ribbon representation of the structure of 5A6 Fab bound to the EC2 of CD81 (PDB: 6U9S). (B) Superposition of the 5A6-CD81 EC2 complex on the CD19-CD81 structure, highlighting the overlap of the binding sites, and the conformational plasticity of the C and D helices of CD81.

Table S1.
Cryo-EM data collection, refinement, and validation statistics

	CD19-CD81 PDB: 7JIC EMD-22344
Data collection and processing	
Magnification	105,000
Voltage (kV)	300
Electron exposure (e^{-}/Å^2)	~55
Defocus range (μm)	-1.0 to -2.5
Pixel size (Å)	0.825
Symmetry	C1
Initial particle images (no.)	2,798,945
Final particle images (no.)	244,583
Map resolution (Å) FSC threshold	3.8 (0.143)
Refinement	
Initial model used (PDB code)	6AL5; 5TCX; 6ANI
Model resolution (Å) FSC threshold	4.0 (0.5)
Map sharpening *B* factor (Å^2)	-142.4
Model composition Non-hydrogen atoms Protein residues Ligands	 5979 842 0
R.m.s. deviations Bond lengths (Å) Bond angles (°)	 0.002 0.454
Validation MolProbity score Clashscore Poor rotamers (%)	 1.51 4.93 0.00
Ramachandran plot Favored (%) Allowed (%) Disallowed (%)	 96.27 3.73 0.00

Movie S1.
Structural transition of apo-CD81 to CD19-complexed CD81, highlighting the opening of the large extracellular loop (EC2) and inward movement of the transmembrane domain.