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Supplementary Information for
Generating Tumor-selective Conditionally Active Biologic Anti-CTLA4 Antibodies Via Protein-associated Chemical Switches

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This PDF file includes:

Detailed Materials and Methods

Reagents.

Human Her2 and Human Nectin4 were produced by BioAtla. Human CD73, Human CTLA4, Human EpCAM, and Human PD1 hexa-histidine tagged extracellular domains (ECD) were purchased from Sinobiological. Human CellExp™ CD3 epsilon & CD3 delta was purchased from BioVision. Anti-Flag (HRP) was purchased from Abcam. Goat anti-human IgG was purchased from Thermofisher. Anti-Human IgG HRP was purchased from Promega. IpA and CAB anti-CTLA4 variants were produced by Evitria SA (Schlieren, Switzerland), using the sequence information provided. CHO-S cells were purchased from Thermofisher. Staphylococcal enterotoxin B (SEB) was purchased from Toxin Technology Inc. Human Normal Peripheral Blood Mononuclear Cells (PBMC) were purchased from Precision for Medicine. Bovine serum albumin was purchased from VWR. Carbonate-bicarbonate coating solution, D-Glucose, Magnesium chloride.$6H_2O$, Krebs-Ringer solution, lactic acid, sodium phosphate dibasic (anhydrous), sodium phosphate monobasic (anhydrous), sodium bicarbonate, tween 20 were purchased from Sigma. Potassium chloride, sodium chloride, sodium phosphate dibasic, potassium phosphate dibasic were purchased from Sinopharm Chemical Reagent. Sodium sulfide nonahydrate was purchased from ACROS. HCl was purchased from VWR and Beijing Reagent. PBS, RPMI1640, Sodium Bicarbonate solution (7.5% solution) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Thermofisher. 16% paraformaldehyde (PFA) was purchased from Electron Microscopy Sciences. The IL2 ELISA Kit was purchased from R&D Systems. ELISA plates were purchased from Thermofisher. SealPlate adhesive microplate seals were purchased from Thomas Scientific or E&K Scientific. Orion™ Star™ A111 pH Benchtop Meter was purchased from VWR. The Molecular Devices SpectraMax i3X Plate reader was used for all ELISA readouts. The NovoCyte Flow Cytometer from ACEA Biosciences and Thermo Scientific™ MaxQ™ 2000 shaker from Thermofisher were used for all pH FACs applications.

Antibody engineering and screening. Antibody variant libraries of Ipilimumab were built using BioAtla's proprietary Comprehensive Protein Evolution (CPE^{TM}) and Comprehensive Protein Synthesis (CPS^{TM}) processes (Supplementary Reference 1). Using BioAtla's proprietary Comprehensive Integrated Antibody Optimization (CIAOTM) process (Supplementary Reference 2), these antibody variants were expressed in CHO-

S cells and the antibodies which contain the Flag tag (DYKDDDDK) at the carboxyl terminus of the heavy chains were secreted into the supernatant. Quantitation ELISA was performed to determine the concentration of antibodies in the supernatant. The same concentration of antibodies in the supernatant was used for the high-throughput, parallel pH affinity ELISA screening process.

Briefly, 100µL of 1µg/mL of human CTLA4 hexa-histidine tagged extracellular domain (ECD) was immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with either pH6.0 ELISA assay solution (Krebs-Ringer solution with 1.25g/L bicarbonate, 10g/L BSA and 1.5g/L lactic acid, pH6.0) or pH7.4 ELISA assay solution (Krebs-Ringer solution with 1.25g/L bicarbonate, 10g/L BSA and 0.09g/L lactic acid, pH7.4) at room temperature for one hour then washed with the corresponding pH ELISA assay solution. The supernatant was diluted in the corresponding pH ELISA assay solution to a final antibody concentration of 25ng/mL and added to the previously blocked and washed wells. ELISA plates containing the supernatant were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH ELISA assay solution. 100µL of anti-FLAG HRP secondary antibody was added to each well at a concentration of 500ng/mL diluted in the corresponding pH ELISA assay solution. Subsequently, the plates were sealed and incubated at room temperature for one hour with shaking. Following incubation, the plates were washed three times with the corresponding pH ELISA assay solution. 50µL of TMB Peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50µL of 0.1N HCl. The OD at 450nm was collected using a Microplate Spectrophotometer. Based on the OD450nm values, antibody variants which exhibited higher binding activities to human CTLA4 ECD in pH6.0 ELISA assay conditions compared to pH7.4 ELISA assay conditions were selected for further confirmation.

Confirmation pH Affinity ELISA. Selected anti-CTLA4 variants (clones 87CAB1, 87CAB2 and 87CAB3) which have higher binding activities to human CTLA4 ECD at pH6.0 compared to pH7.4 were produced by Evitria with the Flag tag at the carboxyl terminus. In addition, an IpA with the Flag tag was produced at Evitria to be used as the bench-mark antibody. Serially diluted antibodies were bound to human CTLA4 extracellular domain immobilized in the wells and detected as described in *Antibody engineering and screening* except that the amount of bound variants was quantified using anti-human IgG antibody conjugated to HRP, which then reacted with TMB

colorimetric substrate to generate a detectable chromatic product. The OD450nm in each well is proportional to the variants bound in the different pH ELISA assay conditions. EC50 values at pH6.0 and at pH7.4 for binding to human CTLA4 were determined using the nonlinear fit model (variable slope, four parameters) of GraphPad Prism version 7.03.

Confirmation pH Range ELISA. The activity of the selected anti-CTLA4 variants were tested in a range of pH ELISA assay conditions (pH6.0 to pH7.4), mimicking tumor microenvironment pH (pH6.0 to pH6.7) and the normal physiological pH (pH7.4). Briefly, 100μL of 1μg/mL of human CTLA4 ECD was immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with various pH ELISA assay solutions (see *Antibody engineering and screening* section, pH6.0, 6.2, 6.5, 6.7, 7.0 and 7.4) at room temperature for one hour then washed with the corresponding pH ELISA assay solution. IpA and anti-CTLA4 variants were diluted in the corresponding pH ELISA assay solution to a final antibody concentration of 25ng/mL and then added to the wells which were previously blocked and washed. ELISA plates containing the diluted antibodies were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH ELISA assay solution. 100μL of anti-human IgG HRP secondary antibody was added to each well at a dilution of 1:2500 in the corresponding pH ELISA assay solution. Subsequently, the plates were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH ELISA assay solution. 50μL of TMB peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50μL of 0.1N HCl. The plates were read at OD 450nm using a microplate spectrophotometer. Average OD values (from two independent experiments with two replicates) at the different pH were plotted against the pH value of the solution using GraphPad Prism curve fitting by the 4-parameter model built into the software. The inflection point of the pH curve (50% binding activity) equals IC50 of the fitting equation. Binding activity at pH6.0 was set to 100%.

pH reversibility Affinity ELISA. Initially, selected anti-CTLA4 variants were incubated at pH6.0 or pH7.4 in ELISA assay solution. Subsequently, the activity was tested in pH7.4 or pH6.0 ELISA assay solution to determine whether the activities of anti-CTLA4 variants are reversible. Briefly, 100μL of 1μg/mL of human CTLA4 ECD was

immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with pH6.0 or pH7.4 ELISA assay solution (see solution composition in *Antibody engineering and screening* section) at room temperature for one hour then washed with the corresponding pH ELISA assay solution.

IpA and CAB anti-CTLA4 variants were tested in four different conditions: 1. Antibodies were first diluted in pH6.0 ELISA assay solution to an antibody concentration of 250ng/mL and incubated for 30 minutes, then further diluted to 25ng/ml in pH6.0 ELISA assay solution and added to the wells which were previously blocked and washed with pH6.0 ELISA assay solution. 2. Antibodies were first diluted in pH7.4 ELISA assay solution to an antibody concentration of 250ng/mL and incubated for 30 minutes, then further diluted to 25ng/ml in pH7.4 ELISA assay solution and added to the wells which were previously blocked and washed with pH7.4 ELISA assay solution. 3. Antibodies were first diluted in pH6.0 ELISA assay solution to an antibody concentration of 250ng/mL and incubated for 30 minutes, then further diluted to 25ng/ml in pH7.4 ELISA assay solution and added to the wells which were previously blocked and washed with pH7.4 ELISA assay solution. 4. Antibodies were first diluted in pH7.4 ELISA assay solution to an antibody concentration of 250ng/mL and incubated for 30 minutes, then further diluted to 25ng/ml in pH6.0 ELISA assay solution and added to the wells which were previously blocked and washed with pH6.0 ELISA assay solution. ELISA plates containing the diluted antibodies from these four test conditions were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH ELISA assay solution. 100μL of anti-human IgG HRP secondary antibody was added to each well at a dilution of 1:2500 in the corresponding pH ELISA assay solution. Subsequently, the plates were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH ELISA assay solution. 50 μL of TMB peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50 μL of 0.1N HCl. The plates were read at OD 450nm using a microplate spectrophotometer. Data from two independent experiments with two replicates were normalized to test condition 1.

pH Affinity ELISA to Identify Protein Associated Chemicals Switch (PaCS). The activity of clone 87CAB3 was measured in a variety of pH ELISA assay conditions in the absence of one component per experiment (10mM glucose, 1mM magnesium chloride,

4.5mM potassium chloride, 119mM sodium chloride, 0.7 mM sodium phosphate dibasic, 1.1mM sodium phosphate monobasic, 15mM bicarbonate, 16mM lactic acid (for pH6.0) or 1mM lactic acid (for pH7.4))

Briefly, 100μL of 1μg/mL of human CTLA4 ECD was immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with various pH ELISA assay conditions (For complete solution composition, see *Antibody engineering and screening* section. Each test condition had one component removed from the complete solution as indicated in Fig.2 and the pH was adjusted to either 6.0 or 7.4) at room temperature for one hour then washed with the corresponding pH ELISA assay solution. IpA and clone 87CAB3 were diluted in the corresponding pH ELISA assay solution to a final antibody concentration of 25ng/mL and then added to the wells which were previously blocked and washed. ELISA plates containing the diluted antibodies were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH ELISA assay solution. 100μL of anti-human IgG HRP secondary antibody, diluted in the corresponding ELISA assay solution to 1:2500, was added to each well and the plates were incubated at room temperature for one hour. The plates were then washed three times with the corresponding pH ELISA assay solution. 50μL of TMB peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50μL of 0.1N HCl. The plates were read at OD 450nm using a microplate spectrophotometer. The normalized activity values from two independent experiments with duplicate reactions are shown.

Effect of PaCS Solution Titration on the Activity of Clone 87CAB3. The activity of clone 87CAB3 was measured in various PaCS assay conditions at either pH6.0 or pH7.4. Briefly, 100μL of 1μg/mL of human CTLA4 extracellular domain (ECD) was immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with various PaCS assay solutions at room temperature for one hour then washed with the corresponding PaCS assay solution. The PaCS assay solutions consist of PBS buffer (1mM KH_2PO_4, 154mM NaCl, 5.6mM Na_2HPO_4) supplemented with increasing concentration of the following chemicals in each solution: sodium bicarbonate (Fig.2C, 0 to 37.8mM), sodium chloride (Fig.2D, 0 to 308mM) or sodium sulfide (Fig.2D, 0 to 2mM), at pH6.0 or pH7.4. IpA and clone 87CAB3 were diluted in the corresponding PaCS assay solution to a final antibody concentration of

25ng/mL and then added to the previously blocked and washed wells. ELISA plates containing the diluted antibodies were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding PaCS assay solution. 100μL of anti-human IgG HRP secondary antibody, diluted in the corresponding PaCS assay solution to 1:2500, was added to each well and the plates were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding PaCS assay solution. 50μL of TMB peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50μl of 0.1N HCl. The plates were read at OD 450nm using a microplate spectrophotometer. Data was normalized to pH6.0, 0mM (sodium bicarbonate, sodium sulfide) or pH 6.0, 154mM (sodium chloride) using two independent experiments with duplicate reactions.

Effect of PaCS Solution on the Activity of anti-CTLA4 variants. The activities of selected anti-CTLA4 variants were measured in various PaCS assay conditions at either pH6.0 or pH7.4. Briefly, 100μL of 1μg/mL of human CTLA4 extracellular domain (ECD) was immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with various PaCS assay solutions at room temperature for one hour then washed with the corresponding PaCS assay solution. The PaCS assay solutions consist of PBS buffer (1mM KH_2PO_4, 154mM NaCl, 5.6mM Na_2HPO_4) containing the following chemicals: bicarbonate (Fig.2F, 30mM), sodium chloride (Fig.2G, 154mM) or sodium sulfide (Fig.2H, 0.1mM), at pH6.0 or pH7.4. IpA and anti-CTLA4 variants were diluted in the corresponding PaCS assay solution to a final antibody concentration of 25ng/mL and then added to the previously blocked and washed wells. ELISA plates containing the diluted antibodies were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding PaCS assay solution. 100μL of anti-human IgG HRP secondary antibody, diluted in the corresponding PaCS assay solution to 1:2500, was added to each well and the plates were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding PaCS assay solution. 50μL of TMB peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50μL of 0.1N HCl. The plates were read at OD 450nm using a microplate spectrophotometer. Data was normalized to

pH6.0, 0mM (sodium bicarbonate, sodium sulfide) or pH 6.0, 154mM (sodium chloride) using two independent experiments with duplicate reactions.

Sodium Sulfide Reversibility Affinity ELISA. Initially, clone 87CAB3 and IpA were incubated at pH6.0 or pH7.4 in PaCS assay solution (1mM KH_2PO_4, 154mM NaCl, 5.6mM Na_2HPO_4) containing various concentrations of sodium sulfide. Subsequently, the activity was tested in pH7.4 or pH6.0 PaCS assay solution with or without sodium sulfide to determine whether the reduced activities of clone 87CAB3 and IpA in the presence of sodium sulfide are reversible.

Briefly, 100μL of 1μg/mL of human CTLA4 ECD was immobilized on 96 well ELISA plates in carbonate-bicarbonate coating solution overnight at 4°C. Plates were blocked with PaCS assay solution containing either 0, 0.1, 0.5, 1 or 2mM sodium sulfide at room temperature for one hour then washed with the corresponding PaCS assay solution. Clone 87CAB3 and IpA were diluted in pH6.0 or pH 7.4 PaCS assay buffers containing various concentration of sodium sulfide to 1,000ng/mL and incubated at room temperature for 30 minutes. The antibodies were further diluted to 2ng/mL in pH6.0 or pH7.4 PaCS assay buffers with or without sodium sulfide incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding assay solution. 100μL of anti-human IgG HRP secondary antibody was added to each well at a dilution of 1:2500 in the corresponding pH PaCS assay solution. Subsequently, the plates were sealed and incubated at room temperature for one hour with shaking. The plates were then washed three times with the corresponding pH PaCS assay solution. 50μL of TMB peroxidase substrate solution was added to each well and the reactions were stopped after 3 minutes with 50μL of 0.1N HCl. The plates were read at OD 450nm using a microplate spectrophotometer. Data from two independent experiments with two replicates were normalized to pH6.0, 0mM sodium sulfide data.

Effect of PaCS Conditions on the Activity of CABs Against Various Cancer or T Cell Surface Antigens. The activities of selected CABs (generated using BioAtla's PaCS process) against various cancer or T cell surface antigens (EpCAM, Her2, Nectin-4, CD73, and CD3) were tested in various PaCS assay conditions at either pH6.0 or pH7.4 using the same assay conditions as described in the above section (*The Effect of PaCS Conditions on the Activity of anti-CTLA4 variants)* except that the corresponding antigens were used to coat the ELISA plates.

pH Flow Cytometry. Binding affinity of anti-CTLA4 antibody variant clone 87CAB3 to human CTLA4 expressing CHO stable cells was measured by flow cytometry under pH6.0 and pH7.4 conditions, in the absence or presence of bicarbonate. IpA was used as the benchmark control. Briefly, the stable cells in log phase were detached and seeded in 96-well plates on the day of assay. Antibodies serially diluted in pH flow cytometry assay solution (PBS with 1% BSA) with or without 30 mM bicarbonate were added to the cells and incubated for one hour at 4 °C in the dark with shaking. The mixture containing cells and antibodies was spun down then washed with the corresponding pH flow cytometry assay solution before the secondary antibody (goat anti-human IgG, Invitrogen) labelled with the fluorophore FITC was added to each well and incubated for 45min at 4°C in the dark. Cells were then washed, paraformaldehyde-fixed and scanned with NovoCyte flow cytometer (ACEA Biosciences). EC50 values of antibodies at pH6.0 and pH7.4 for binding activities to human CTLA4 cells were determined using mean fluorescence intensity and calculated with the nonlinear fit model (variable slope, four parameters) of GraphPad Prism version 7.03.

In Vitro Assay Using Human Lymphocytes. Frozen peripheral blood mononuclear cells (PBMCs) from normal de-identified healthy donors were seeded at 1 x 10^5 cells/well and stimulated with 2.5μg/mL Staphylococcal enterotoxin B (SEB). 10, 1 and 0.1μg/mL of clone 87CAB3, IpA and an isotype control antibody (against B12) were added to the SEB-stimulated PBMC in pH6.2 or pH7.4 assay media (RPMI-1640 media, pH6.2 or pH7.4). IL-2 secretion was measured by IL2 ELISA (R&D System, Quantikine IL2 ELISA) on day 3.

SPR Analysis. Binding kinetics of anti-CTLA4 antibodies were measured by surface plasmon resonance on a SPR2/4 instrument (Sierra Sensors, Hamburg, Germany) and flat amine sensor chips. The SPR sensor contains four flow cells (FC1-FC4), each of which can be addressed individually or in groups. CTLA4 ECD was immobilized in FC2 and FC4, while no protein was immobilized in FC1 and FC3 (control surface). All injections were done at a flow rate of 25μL/min and 25°C. The sensor surface was activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (200mM/50mM) for 480 seconds. Human CTLA4 ECD (25μg/mL in 10mM NaAc, pH5.0) was injected for 480s and the surface was inactivated by injecting 1M ethanolamine-HCl for 480s. The control surface was activated and deactivated using the same conditions, but without injecting protein. PBST buffer (PBS

pH7.4 with 0.05% TWEEN20) was used as running buffer for the surface preparation. The running solution was switched to PBST with 30 mM sodium bicarbonate with the pH adjusted as indicated in the figures before the analyte injections. The instrument was equilibrated with the running solution for one hour before the first analyte injection. 100µL analyte diluted in the corresponding running solution (34.25nM, 13.70nM, 6.85nM, 3.42nM, 1.37nM, and 0.0nM) was injected overflow cells 1 and 2 or overflow cells 3 and 4. Off-rate was measured for 360s. The chip surface was regenerated after each cycle of interaction analysis by injecting 6µL of 10mM glycine (pH 2.0). Flow cell 1 (or 3) without immobilized protein was used as control surface for reference subtraction. In addition, data with buffer only as analyte (0nM analyte) was subtracted from each run. Double subtracted data was fitted with the provided analysis software Analyzer R2 (Sierra Sensors) using a 1:1 binding model. A molecular weight of 146kDa was used to calculate the molar concentrations of the analytes.

Animal Model Assessment of Efficacy and Safety. Tumor xenograft models were performed in human CTLA4 knock-in mice (WuXi Apptec) using the murine syngeneic colon adenocarcinoma cell line MC-38 as previously described (54). Safety and immunotoxicity was analyzed in naïve non-human primates (cynomolgus macaques) by combination treatment with NiA (20mg/kg) and anti-CTLA4 antibody (IpA, 87CAB2 and 87CAB3 at 15mg/kg) given once weekly for 4 weeks. The purpose of this study was to evaluate the toxicity and determine the toxicokinetics for each test article, IpA, 87CAB3 and 87CAB2 administered via intravenous injection (IV) once every week (for a total of four doses) immediately (within 5 minutes) following IV administration of anti-PD1 antibody NiA to female cynomolgus monkeys for 4 weeks. Assessment of toxicity was based on mortality, clinical observations, body weights, quantitative food consumption, and both clinical and anatomic pathology. Blood samples were collected for toxicokinetic evaluation, biomarker analysis, and immunophenotyping. All animals survived to their scheduled euthanasia. Kruskal-Wallis analysis (95% CI $P<0.05$) followed by Benjamini and Yekutieli multiple comparisons were used to determine statistical significance between groups. Tumor: %CD8 of T cells: $H(34)=9.99$, $p < 0.05$; CD8/Treg ratio: $H(3)=7.26$. $p < 0.05$; Peripheral blood:CD4eff in CD4 T cells: $H(3)= 8.69$, $p < 0.05$, Spleen: CD4eff in CD4 T cells: no statistical significant difference found between the groups.

Fig. S1A: Sodium Bicarbonate

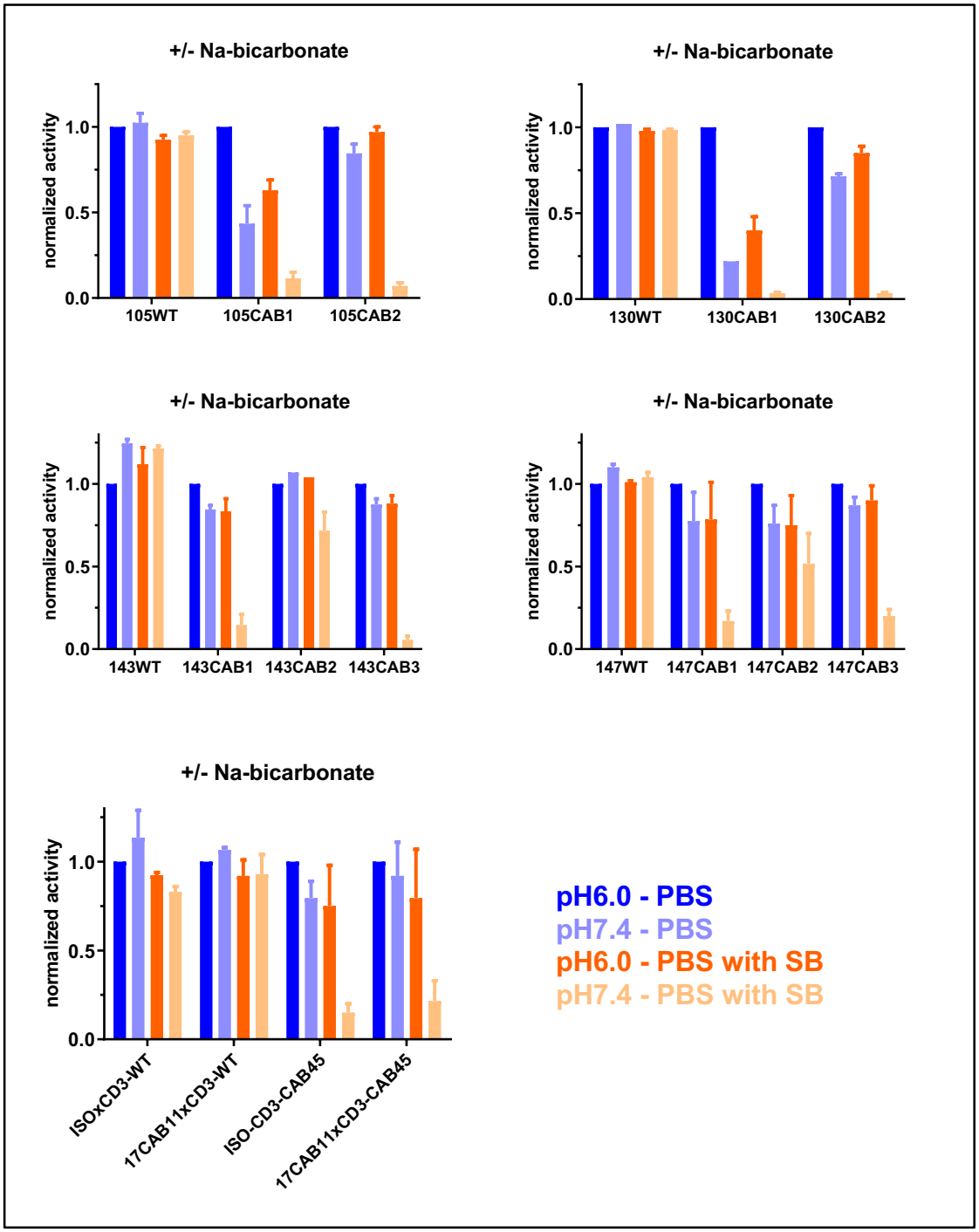


Fig. S1B: Sodium Chloride

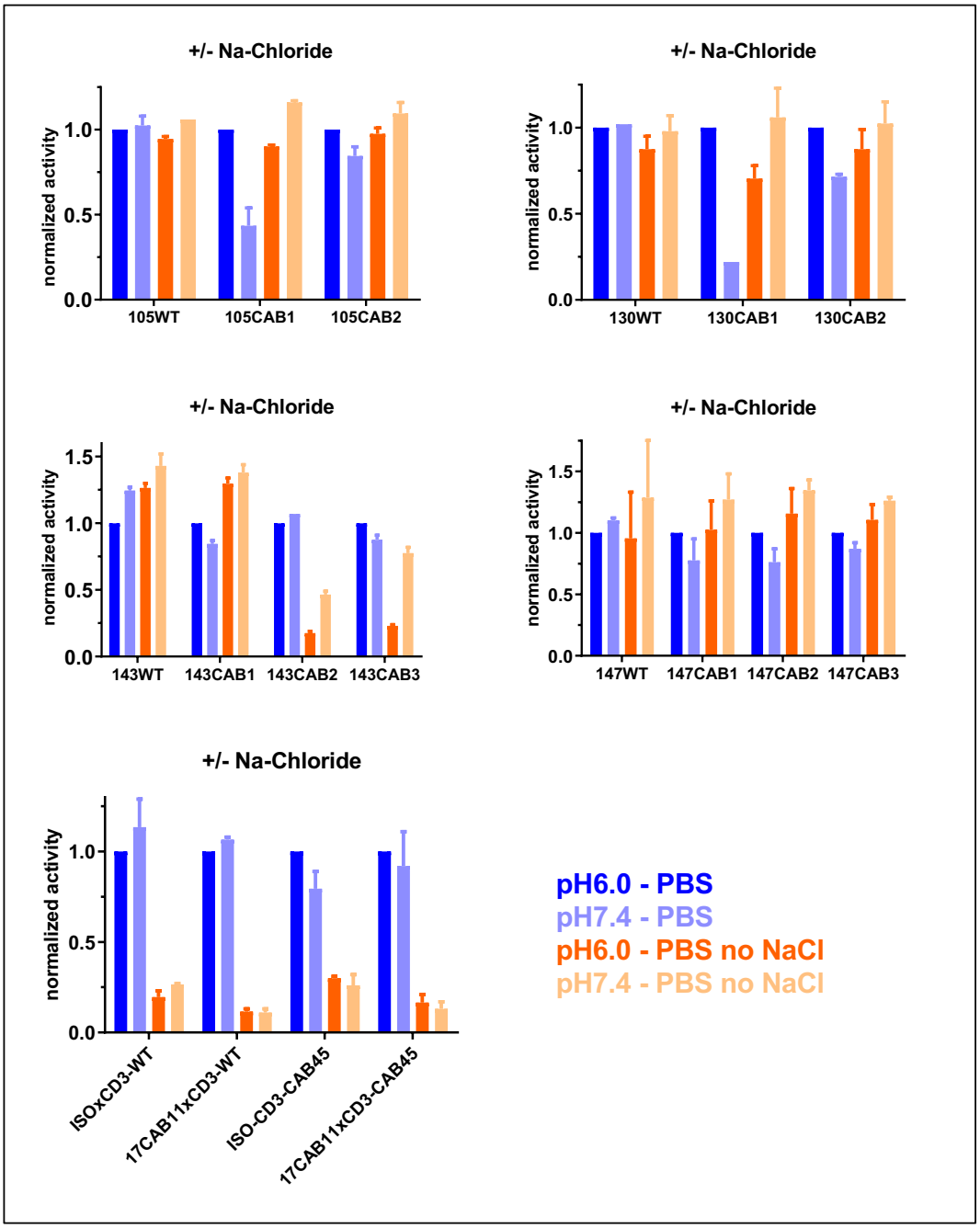


Fig. S1C: Sodium Sulfide

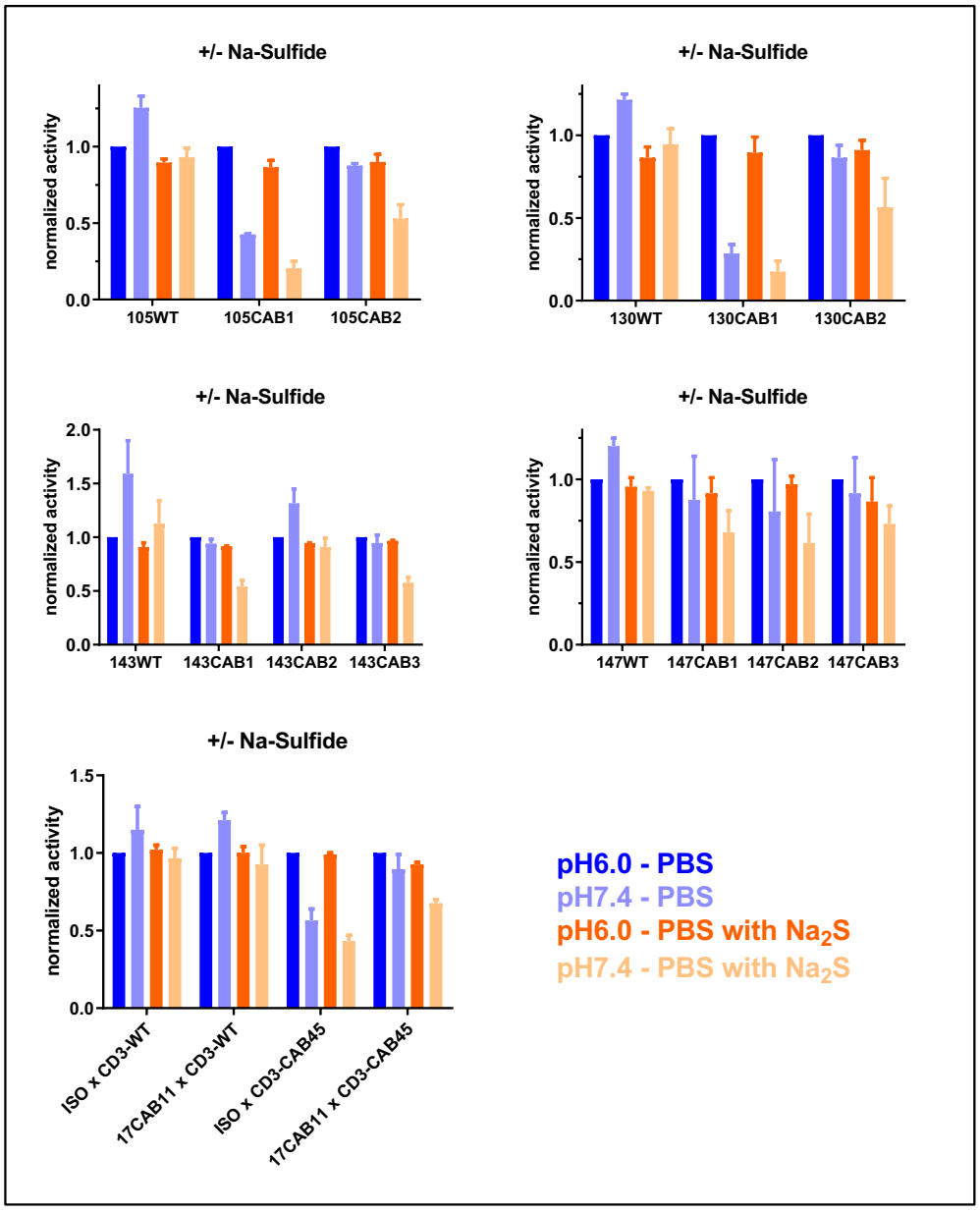


Figure S1: CAB Antibodies against other Targets
Effect of Sodium bicarbonate, Sodium chloride, and Sodium sulfide on pH selectivity of CAB antibodies against other targets**.** Binding activities of CABs to various targets (90: B7-H3, 105: EpCAM, 130: Her2, 143: Nectin4, 147: CD73, 150: EpCAM x CD3 bispecific) at pH6.0 (dark panels) and at pH7.4 (light panels) in the absence (blue) or presence (orange) of A. 20mM sodium bicarbonate, B. 154mM sodium chloride and C. 0.1mM sodium sulfide. Binding activities were determined using pH affinity ELISA. Y-axis: OD 450nm. X-axis: sample ID. Data from duplicate for each clone were normalized

to pH6.0. Data were normalized to pH6.0. Normalized data from two independent experiments with duplicate reactions are shown.

Figure S2: PaCS-responsive amino acid changes

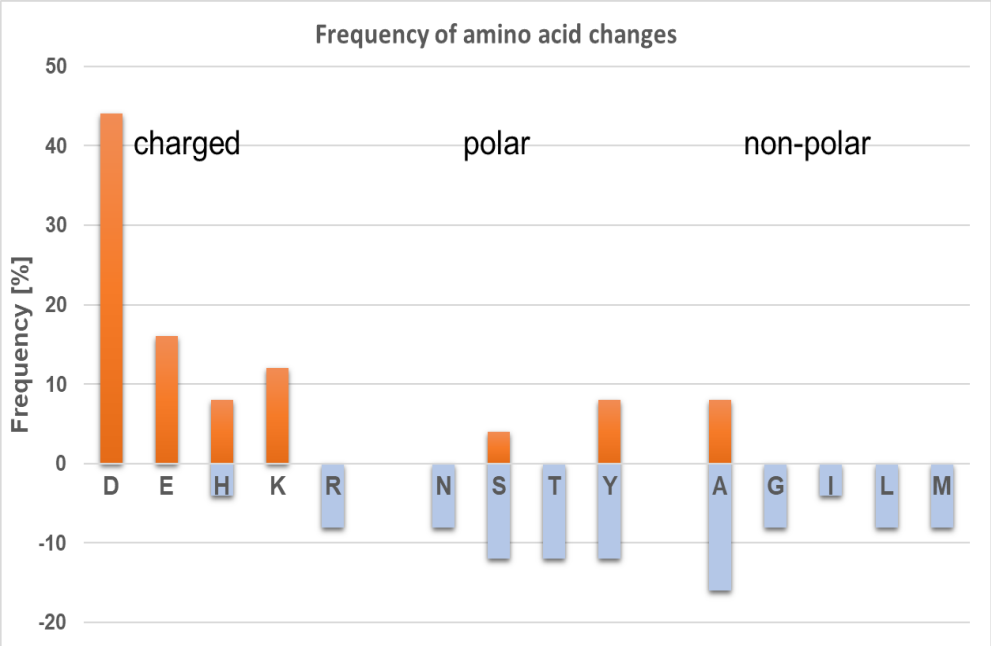
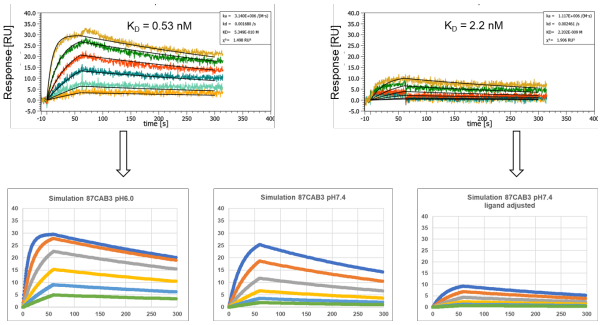


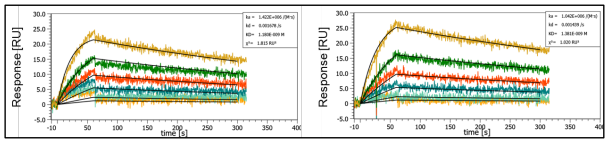
Figure S2: PaCS-responsive amino acid changes. The graph shows the frequency of certain amino acids that are replaced in CAB clones (blue bars) and the frequency of certain amino acids that are introduced (orange bars). This table includes only changes for the CAB molecules shown in this report.

Figure S3: Kinetic analysis of clone 87CAB3 binding to CTLA4 at pH6.0 and pH7.4 performed by SPR.

A



B



C

	pH6.0			pH7.4		
	Ka [M·s]	Kd[s^{-1}]	KD[M]	Ka [M·s]	Kd[s^{-1}]	KD[M]
87CAB3	3.14E+06	1.68E-03	5.35E-10	1.12E+06	2.46E-03	2.20E-09
IpA	1.42E+06	1.68E-03	1.18E-09	1.04E+06	1.44E-03	1.38E-09

Figure S3: Kinetic analysis of clone 87CAB3 binding to CTLA4 at pH6.0 and pH7.4 performed by SPR. Kinetic analysis was performed using a SPR2/4 instrument. The binding signals were generated using a multi-cycle approach with CTLA4 immobilized on the sensor surface and varying concentrations of clone 87CAB3 (or Ipilimumab analogue) as indicated below. Representative sensorgrams are shown. Experiments were performed at pH6.0 or pH7.4 with PBST running buffer with 30mM sodium bicarbonate

Fig. S3A: Binding kinetics of anti-CTLA4 clone 87CAB3 was measured by SPR at pH6.0 (top left) and pH7.4 (top right) in the presence of 30mM sodium bicarbonate. Shown are the SPR traces of one representative experiment at each pH. The affinity drops from 0.53nM at pH6.0 to 2.2nM at pH7.4. In addition, the SPR signal drops from 30RU to 10RU.

The bottom panels show simulated SPR diagrams using the Ka and Kd values from the experiments shown above. Left: simulation of run at pH6.0; middle: simulation of run at pH7.4; right simulation of run at pH7.4 with adjusted ligand density.

Fig. S3B: Binding kinetics of anti-CTLA4 clone Ipilimumab analogue was measured by SPR at pH6.0 (left) and pH7.4 (right) in the presence of 30 sodium bicarbonate.

Binding curves in each sensorgram correspond to 34.24nM, 13.70nM, 6.85nM, 3.42nM, and 1.37nM analyte, top to bottom. Signals are double subtracted (reference cell and buffer injection).

Fig. S3C: Measured affinities of clone 87CAB3 and Ipilimumab analogue.

Figure S4: Binding activity of clone 87CAB3 to human CTLA4 at pH 6.0 and pH 7.4 with or without sodium bicarbonate determined by Flow Cytometry

Fig. S4A: pH Flow Cytometry

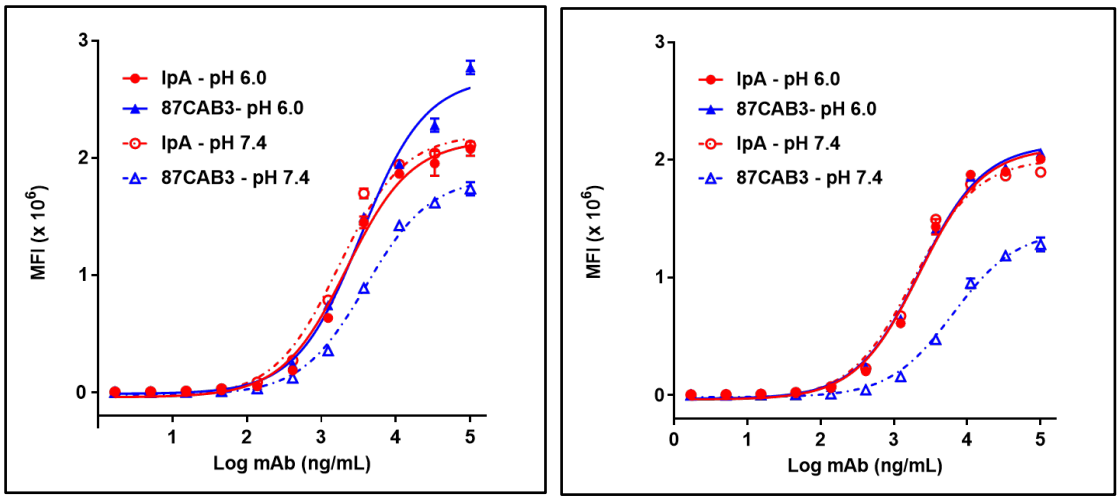


Fig. S4B: EC50 Values determined by pH Flow Cytometry

Clone ID	Sodium Bicarbonate	EC50 (ng/mL) pH 6.0	EC50 (ng/mL) pH 7.4
87CAB3	-	2,415 +/- 873	2,622 +/- 1,163
87CAB3	+	1,703 +/- 693	5,024 +/- 1,922
IpA	-	1,748 +/- 393	1,423 +/- 271
IpA	+	1,722 +/-693	1,474 +/- 535

Figure S4: Binding activity of clone 87CAB3 to human CTLA4 at pH 6.0 and pH 7.4 with or without sodium bicarbonate determined by Flow Cytometry

Fig.S4A: Binding activities of clone 87CAB3 (blue) and the control, Ipilimumab analogue (IpA) (red) to human CTLA4 at pH6.0 (solid lines) and at pH 7.4 (dotted lines) in the absence (left) or presence (right) of 30mM sodium bicarbonate. Binding activity was measured by flow cytometry using CHO cells expressing human CTLA4 on the cell surface. The mean fluorescence intensity (MFI) values from a representative experiment with two duplicate reactions are shown.
Y-axis: MFI values x 10^6; X-Axis: antibody concentration (log ng/mL)

Fig.S4B: EC50 values of clone 87CAB3 and the control, Ipilimumab analogue (IpA) binding to human CTLA4 at pH6.0 and at pH7.4 were calculated using the nonlinear fit (variable slop, four parameters) model built into GraphPad Prism software version 7.03. Data from two independent experiments were used to calculate EC50 values.

Figure S5: Clone 87CAB3 potentiates IL-2 Secretion in SEB-stimulated human PBMCs

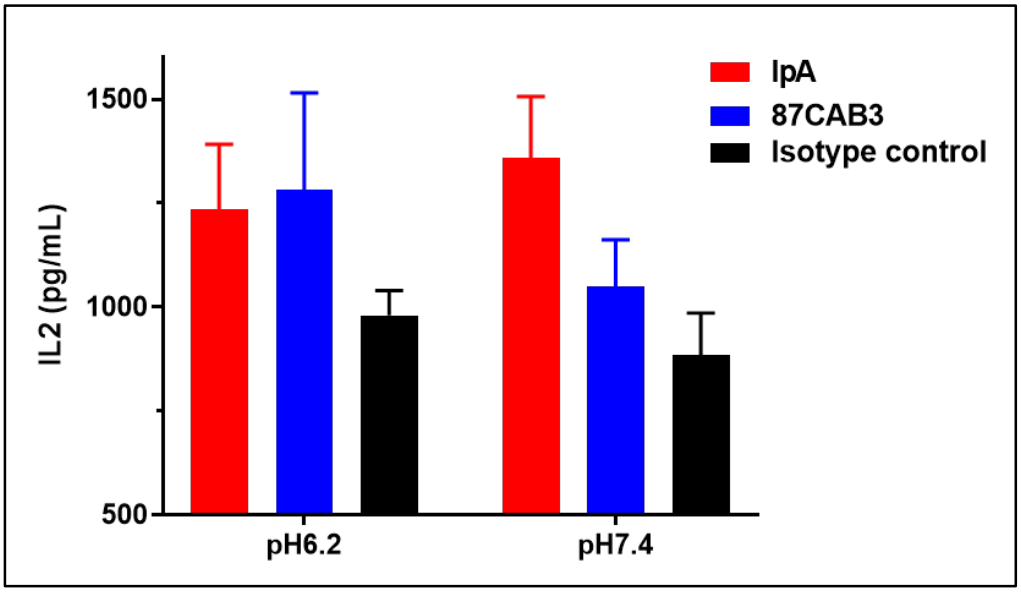


Figure S5: Clone 87CAB3 potentiates IL-2 Secretion in SEB-stimulated human PBMC at acidic pH**.** Frozen PBMC from healthy normal de-identified donors were seeded at $1x10^5$ cells/well and stimulated with 2.5µg/mL SEB. 10µg/mL of clone 087CAB3 (blue), and Ipilimumab analogue (IpA, red) were added to the SEB-stimulated PBMC in pH6.2 or pH7.4 assay media. B12 antibody (isotype control, black) was used as the negative control. IL-2 secretion was measured by ELISA on day 3. Y-axis: IL-2 concentration (pg/mL); X-axis: antibody concentration (µg/mL). The IL-2 concentrations from a representative experiment with two duplicate reactions are shown.

SI References

1. J.M. Short, Methods Of Protein Evolution. US patent US 10,106,576 (2018)
2. J.M. Short, Simultaneous, Integrated Selection And Evolution Of Antibody/protein Performance And Expression In Production Hosts. US patent US 8,859,467 (2014)