nature protocols

https://doi.org/10.1038/s41596-023-00930-8

Protocol

Check for updates

Production and use of antigen tetramers to study antigen-specific B cells

In the format provided by the authors and unedited

Supplementary Table 1: Mouse Flow Cytometry Reagents

Target	Conjugate	Clone	Company	Cat.	Dilution	Stain
B220	BV785	RA3-6B2	BioLegend	103246	100	Extracellular
B220	AF700	RA3-6B2	BioLegend	103232	100	Extracellular
CD138	PE/Dazzle 594	281-2	BioLegend	142528	100	Extracellular
CD138	BV605	281-2	BioLegend	142516	100	Extracellular
CD138	APC	281-2	BioLegend	142506	100	Extracellular
CD16/32	Purified	93	BioLegend	101302	25	FcBlocking
CD19	PerCPCy5.5	6D5	BioLegend	115534	100	Extracellular
CD3	BV711	17A2	BioLegend	100241	100	Extracellular
CD3	APC-eF780	17A2	eBioscience	47-0032-82	200	Extracellular
CD38	PE-Cy7	90	BioLegend	102718	100	Extracellular
CD45.1	BV605	A20	BioLegend	110738	100	Extracellular
CD45.2	BV421	104	BioLegend	109832	100	Extracellular
CD79b (IgB)	FITC	HM79-12	BioLegend	132805	100	Extracellular
F4/80	BV711	BM8	BioLegend	123147	100	Extracellular
F4/80	APC-eF780	BM8	ThermoFisher	MF48027	200	Extracellular
Fixable Viability Dye	eFluor 780		ThermoFisher	65-0865-18	600	Extracellular
Fixable Viability Dye	Aqua		ThermoFisher	L34957	600	Extracellular
GL7	PerCPCy5.5	GL7	BioLegend	144610	200-300	Extracellular
GL7	AF647	GL7	BioLegend	144606	100	Extracellular
GL7	AF647	GL7	BD Biosciences	561529	100	Extracellular
Gr-1	APC-eF780	RB6-8C5	eBioscience	47-5931-82	200	Extracellular
H+L	Pacific Blue	Polyclonal	ThermoFisher	P31581	1000	Intracellular
IgA	Biotin	RMA-1	BioLegend	407004	100	Intracellular
IgD	BV605	11-26c.2a	BioLegend	405727	100	Extracellular
IgD	BV510	11-26c.2a	BioLegend	405723	100	Extracellular
IgD	BV650	11-26c.2a	BioLegend	405721	100	Extracellular
IgE	FITC	RME-1	BioLeged	406906	500	Intracellular
IgE	Purified	RME1-1	BioLegend	406902	20	FcBlocking
IgG1	BV421	RMG1-1	BioLegend	406615	50	Extracellular
IgG1	BV421	RMG1-1	BioLegend	406615	500	Intracellular
IgG1	Purified	RMG1-1	BioLegend	406602	20	FcBlocking
IgG1	APC	RMG1-1	BioLegend	406610	50	Extracellular
IgG1	APC	RMG1-1	BioLegend	406610	200-500	Intracellular
IgG2b	PE	RMG2b-1	BioLegend	406708	100	Extracellular
IgG2b	PE	RMG2b-1	BioLegend	406708	200	Intracellular
IgG2c	FITC	Polyclonal	Southern Biotech	1078-02(SB)	100	Intracellular
IgG3	BV650	R40-82	BD OptiBuild	744136	100	Extracellular
IgM	BV786	II/41	BD OptiBuild	743328	50	Extracellular
OVA	FITC		Molecular Probes	O23020	100	Extracellular
Streptavidin	BV605		BioLegend	405229	400	Intracellular

Supplementary Table 2: Human Flow Cytometry Reagents

Target	Conjugate	Clone	Company	Cat.	Dilution	Stain
IgG2	FITC	HP6002	Southern Biotech	9070-02	20	Extracellular
IgG1	PE	12E4.23.20	Miltenyi Biotec	130-119-946	20	Extracellular
CD38	PE-Cy7	HB-7	BioLegend	356608	100	Extracellular
CD19	PerCPCy5.5	SJ25C1	BioLegend	363016	20	Extracellular
IgD	BV421	IA6-2	BioLegend	348226	20	Extracellular
Fixable Viability Dye	Aqua		ThermoFisher	L34957	600	Extracellular

CD27	BV605	O323	BioLegend	302830	20	Extracellular
IgM	BV650	MHM-88	BioLegend	314526	20	Extracellular
CD3	BV711	OKT3	BioLegend	317328	100	Extracellular
CD14	BV711	M5E2	BioLegend	301838	100	Extracellular
CD16	BV711	3G8	BioLegend	302044	100	Extracellular

Supplementary Table 3: Sample Mouse Flow Cytometry Panel for APC Tetramer

Target	Conjugate	Clone	Company	Cat.	Dilution	Stain
IgG2c	FITC	Polyclonal	Southern Biotech	1078-02(SB)	100	Intracellular
IgG2b	PE	RMG2b-1	BioLegend	406708	200	Intracellular
GL7	PerCPCy5.5	GL7	BioLegend	144610	200	Extracellular
CD38	PE-Cy7	90	BioLegend	102718	100	Extracellular
IgG1	BV421	RMG1-1	BioLegend	406615	500	Intracellular
Viability	Aqua		ThermoFisher	L34957	600	Extracellular
CD138	BV605	281-2	BioLegend	142516	100	Extracellular
IgD	BV650	11-26c.2a	BioLegend	405721	100	Extracellular
CD3	BV711	17A2	BioLegend	100241	100	Extracellular
F4/80	BV711	BM8	BioLegend	123147	100	Extracellular
IgM	BV786	II/41	BD OptiBuild	743328	50	Extracellular
Antigen Tetramer	APC					
B220	AF700	RA3-6B2	BioLegend	103232	100	Extracellular
Control Tetramer	APC-DL755					

Supplementary Text

Tracing of antigen-specific B cells using tetramer enrichment.

We incubated cells from the spleen and mesenteric lymph nodes of unimmunized CD45.1 mice with OVA-PE tetramers and enriched using anti-PE microbeads. Enriched cells from one donor mouse were adoptively transferred into one recipient CD45.2 B cell deficient (μMT) mouse prior to immunization with OVA/Alum (**Extended Data Fig. 2A**). At day 9, a large population of OVA-specific B cells were detected in OVA-enriched fractions from the spleen and mesenteric lymph nodes of immunized mice but were absent in samples from unimmunized mice (**Extended Data Fig. 2B**). Immunized mice had detectable levels of circulating OVA-specific IgG1 and IgE, demonstrating that transferred enriched cells can generate secreted antibodies of multiple isotypes (**Extended Data Fig. 2C**). >98% of OVA PE tetramer-binding cells were CD45.1-expressing, indicating that the B cell response was derived from the transferred cells (**Extended Data Fig. 2D**). Robust populations of donor-derived antigen-specific GC B cells ($B220^+$ $GL7^+$ $CD38^-$) and ASCs ($B220^-$ $CD138^+$) could be detected in recipient mice, while a much smaller population of memory B cells ($B220^+$ $GL7^+$ $CD38^-$) was detected, though this is expected at an early timepoint such as 9 days post-immunization (**Extended Data Fig. 2E-F**). Upon transfer, antigen enriched B cells behave similarly to endogenous B cells in terms of differentiation and antibody secretion, and therefore these cells can be used for tracing experiments.

Explanation and interpretation of blots to assess biotinylation ratio

This assay works by pre-incubating biotinylated antigens with known concentrations of SA, thereby generating large multimers that will not run down the gel in the same way that the free antigen would. Further, since SA-AF680 is used for detection on the nitrocellulose membrane, the pre-incubation with SA-PE blocks available SA-binding sites. Unbiotinylated antigens will run down the gel but cannot be detected using SA-AF680 and therefore are not considered. It is only when the concentration of SA-PE can no longer capture all of the biotinylated antigen that free antigen will be able to run down the gel and be detected using SA-AF680. Since we know that SA will bind 4 biotinylated antigens, and we know the concentration of SA that was no longer able to capture all the biotinylated antigen, we can mathematically determine the concentration of biotinylated antigen.

Blots for assessing biotinylation do not need to be of analytical quality. We often see multiple bands for proteins, especially those that are purified from natural products rather than produced recombinantly. Biotin will be added to polypeptides of any length if there is an available lysine residue. Most often, all bands on the blot will re-emerge at the same concentration of biotin. If a band of any intensity is present in lane 3, that indicates that a 1:2 ratio of biotinylated antigen to available biotin binding sites on SA was not sufficient to incorporate all of the antigen. This is only possible if there is more than 1 biotinylation per protein or if the concentration of either the SA-PE or biotinylated protein was inaccurately measured (**Extended Data Fig. 3A**). An appropriately biotinylated antigen will have no detectable band in lane 3, and a band that is similar to the band in lane 2 (no SA-PE) should re-emerge at some concentration of SA (**Extended Data Fig. 3B**). Weak bands may appear at a higher concentration of SA-PE, as can be seen at the 4:1 concentration in **Extended Data Fig. 3B**, but it is prudent to select a lower SA-PE concentration where the band fully re-emerges as this will slightly under-estimate the amount of biotinylated

antigen. This will result in a slight excess of biotinylated antigen being incubated with SA-fluorophore when constructing antigen, ensuring proper loading of the tetramer. The blot in **Extended Data Fig. 3C** intentionally included a 32:1 ratio that is not recommended in the protocol to demonstrate that an antigen may be biotinylated at a ratio below 16:1. These biotinylated antigens can be used to generate tetramers, but it is important to purify the tetramer from unincorporated antigen as non-biotinylated antigen will be present in excess and could interfere with tetramer staining.

Supplementary Methods

Mice

Mice were housed either at the Central Animal Facility at McMaster University, the University of Minnesota or at the Fred Hutchinson Cancer Center in specific pathogen free conditions. All procedures were approved either by McMaster University's animal research ethics board or in accordance with University of Minnesota or Fred Hutchinson Cancer Center Institutional Animal Care and Use Committee approval and National Institutes of Health guidelines. C57Bl/6, CD45.1, and μMT mice[57] were purchased from Charles River or Jackson Laboratories. The following strains were bred at McMaster University: IgG1-deficient mice[58] were provided by Dr. Amy Kenter (University of Illinois at Chicago), Verigem mice[51] were provided by Dr. Christopher D.C. Allen (University of California San Francisco). Mice were kept on a 12-hour light/dark cycle and given food and water *ad libidum*. MD4 $Rag1^{-/-}$ mice were bred from C57BL/6-Tg(IghelMD4)4Ccg/J and B6.129S7-*Rag1*^{tm1Mom}/J mice obtained from The Jackson Laboratory.

Participants

S2P-tetramer enrichment: PBMCs were purified from blood donated by adult volunteers prior to the COVID-19 pandemic as a part of the Fred Hutchinson Seattle Area Control study, with approval from the Institutional Review board of the Fred Hutchinson Cancer Center or the University of Washington. All participants gave informed consent.

RBD-tetramer enrichment: a 26-year-old sex male participate >1-year post-immunization with COVID-19 mRNA vaccine donated blood for a study approved under the Hamilton Integrated Research Ethics Board (#3417). PBMCs were purified by density gradient centrifugation and cryopreserved in liquid nitrogen.

Flow sorting, antibody production, and biolayer interferometry

S2P-enriched cells were stained in 50 μL of FACS buffer containing 6.24 μg/mL anti-IgM FITC (G20–127, BD Biosciences), 2.5 μL anti-CD19 BUV395 (SJ25C1, BD Biosciences), 1 μL anti-CD3 BV711 (UCHT1, BD Biosciences), 1 μL anti-CD14 BV711 (M0P-9, BD Biosciences), 1 μL anti-CD16 BV711 (3G8, BD Biosciences), 2.5 μL anti-CD20 BUV737 (2H7, BD Biosciences),

2.5 µL anti-IgD BV605 (IA6–2, BD Biosciences), 3.125 µg/mL anti-CD27 PE-Cy7 (LG.7F9, eBioscience), and 0.5 µL Ghost Dye Violet 510 (Tonbo Biosciences) for 25 minutes on ice. After the incubation, ~4 mL of FACS buffer was added and the samples were centrifuged at 300 × g for five minutes at 4°C. Supernatants were discarded and cells resuspended in FACS buffer and antigen tetramer$^+$ control tetramer$^-$ B cells were individually sorted using a 5-laser (355nm, 405nm, 488nm, 561nm, 640nm) FACS Aria (BD Biosciences) into empty 96-well low profile PCR plates (Labcon), which were sealed with PCR microplate sealing foil (Eppendorf), and flash frozen prior to storage at -80°C.

Antibody heavy and light chain sequences were amplified from frozen PCR plates containing individually sorted B cells as described previously[59]. PCR products were sequenced by Genewiz, and sequences were analyzed using IMGT/V-Quest to identify V, D, and J gene segments. Paired heavy chain VDJ and light chain VJ sequences were cloned into pTT3-derived expression vectors containing the human IgG1, Igκ, or Igλ constant regions using In-Fusion cloning (Clontech), and monoclonal antibodies were produced and purified as previously described[60].

BLI assays were performed on the Octet Red instrument (ForteBio) at RT with shaking at 500 rpm. Anti-human IgG Fc capture biosensors (ForteBio) were loaded with 10 µg/mL of purified antibody in kinetics buffer (0.01% bovine serum albumin, 0.02% Tween 20, and 0.005% NaN3 in 1xDPBS, pH 7.4) for 2.5 minutes. After loading, a baseline was recorded for 1 minute in kinetics buffer. The sensors were then immersed in 62.5 nM of S2P in kinetics buffer for 5 minutes to assess association, followed by immersion in kinetics buffer for 5 minutes to assess dissociation. A 1:1 binding model using ForteBio data analysis software was used for curve fitting.

Antigens, immunization, and sensitization

OVA-tetramers were constructed using >98% pure OVA (Sigma, A5503). RBD produced recombinantly in Expi293 cells by Drs. Matthew Miller and Mark Larché (McMaster University) and generously gifted. Cor a 8 was produced recombinantly in *Pichia pastoris*. Media obtained after induction was dialyzed against 0.1 M ammonium acetate and lyophilized overnight. After resuspension in 0.1 M ammonium acetate, the solution was fractioned by size exclusion chromatography on a Superdex HR 75 16/26 column (Pharmacia Biotech), eluting with 0.1 M ammonium acetate (1 mL/min). Fractions enriched in Cor a 8 were pooled, lyophilized, resuspended in 0.1% trifluoroacetic acid and separated by reversed phase high-performance liquid chromatography on a Nucleosil 300-C4 column (Sugelabor), eluting with a linear gradient of acetonitrile (15% during 10 min, 15%-85% during 120 min, and 85% during 10 min). Peaks containing Cor a 8 (elution time ≈ 60 min) were identified by SDS-PAGE, Western blot and mass spectrometry. Alt a 1 was produced recombinantly in *Pichia pastoris* as previously described[61]. B-lactoglobulin was purchased from Sigma (L3908). GST was produced and purified as described previously[62]. Natural Ara h 1 was a generous gift from ALK Abello A/S.

Figure legends indicate which model was used for each experiment. OVA/CT: mice were gavaged once per week for four weeks with 1 mg OVA (Sigma; A5378) and 5 µg cholera toxin (CT; List Labs; 100B). OVA/Alum: 250 µg of OVA (Sigma; A5378) and 2 mg of Alhydrogel (Invivogen; VAC-ALU-250) were given by intraperitoneal injection. OVA/MPLA: 250 µg of OVA (Sigma;

A5378) and 20 µg of MPLA-SM VacciGrade (Invivogen; vac-mpla). CFA Experiments: 1.4 nmol of OVA, SA or PE in 25 µL of PBS emulsified with 25 µL CFA injected subcutaneously in the base of the tail. GST/CFA: Experiments: 5 nmol of GST in 25 µL of PBS emulsified with 25 µL CFA injected subcutaneously in the base of the tail. Cor a 8/Alum: 250 µg of Cor a 8 and 2 mg of Alhydrogel (Invivogen; VAC-ALU-250) were given by intraperitoneal injection. Alt a 1/Alum: 250 µg of Alt a 1 and 2 mg of Alhydrogel (Invivogen; VAC-ALU-250) were given by intraperitoneal injection. BLG/Alum: 250 µg of BLG (Sigma, L3908) and 2 mg of Alhydrogel (Invivogen; VAC-ALU-250) were given by intraperitoneal injection. RBD/Alum: 20 µg RBD and 2 mg of Alhydrogel (Invivogen; VAC-ALU-250) were given by intraperitoneal injection. Ara h 1/Alum: 20 µg Ara h 1 and 2 mg of Alhydrogel (Invivogen; VAC-ALU-250) were given by intraperitoneal injection.

Tissue collection and processing

Mice were anesthetized using isofluorane and euthanized in accordance with McMaster University or Fred Hutchinson Cancer Center animal research ethics approvals. Spleen and mesenteric lymph node samples were collected into Hanks balanced salt solution (HBSS; Gibco). Spleens were crushed through a 40 µm strainer (Corning Inc., 352340) using the plunger of a 3 ml syringe into HBSS. Mesenteric lymph node samples were crushed between frosted glass slides into HBSS. Cells were centrifuged at 300 x g for 10 minutes at 4°C and resuspended in FACS buffer.

Small intestines were flushed with ~10 ml PBS and collected into PBS. Intestines were opened by a longitudinal cut, mucus was gently removed by gliding the side of straight forceps along the intestine, and then cut into 1 cm thick pieces. Tissues were incubated in a 37°C shaker at 200 rpm for 10 minutes in 1mM DL-dithiothreitol (MilliporeSigma, Cat: 43816). Tissues were vortexed, passed through a metal sink strainer, collected into 5 mM EDTA (ThermoFisher Scientific, Cat: E468-500), 10 mM HEPES, 10% heat inactivated FBS (ThermoFisher Scientific, Cat: 10437028)-PBS, and incubated in a 37°C shaker at 200 rpm for 10 minutes. The tissues were washed and incubated in EDTA-HEPES-FBS-PBS a total of 3 times. The tissues were then transferred into 0.5 mg/ml Collagenase A (F. Hoffmann-La Roche Ltd., Cat: 10103586001) and 100 µg/ml DNAse I (F. Hoffmann-La Roche Ltd., Cat: 10104159001) and incubated for 60 minutes in a 37°C shaker at 200 rpm. Digested tissues were crushed through a 70 µm filter (Corning Inc., Cat: 352350) using the plunger of a 3 ml syringe. Immune cells were enriched by resuspending cells in 40% Percoll (GE Healthcare, Cat: 17-0891-01) in RPMI (ThermoFisher Scientific, Cat: 31800089), underlaying 70% Percoll in RPMI, and spinning at 1000x g for 30 minutes. Cells were centrifuged at 300 x g for 10 minutes at 4°C and resuspended in FACS buffer.

To collect bone marrow, two hind legs per mouse were collected intact in PBS. The femur and tibia were separated, the ends of the bone were cut, and the bone marrow was flushed with PBS using a 25G 5/8 in. needle (BD, Cat: 305122) attached to a 10 ml syringe (BD, Cat: 309604). The bone marrow was pipetted up and down to break up bone marrow chunks and passed through a 40 µm cell strainer. Cells were centrifuged at 300 x g for 10 minutes at 4°C and resuspended in FACS buffer.

Flow cytometry staining

Mouse: Up to 3 million cells resuspended in FACS buffer were plated in a 96 well U bottom plate (Corning, 353077) for staining. Cells were pelleted and resuspended in 25 µl FACS buffer with anti-CD16/32 Clone 93, Biolegend, 101302) for 15 minutes on ice, for Fc blocking. In experiments where IgE was detected, anti-mouse IgE (Clone RME-1, BioLegend, 406902) was included in the Fc blocking step to block surface IgE as previously described[51]. In experiments where IgG1 was detected intracellularly, anti-mouse IgG1 (Clone RMG1-1, BioLegend, 406602) was included in the Fc blocking step to block the mouse IgG1 antibodies present on anti-PE and anti-APC microbeads. 25 µl of a mix of fluorescent antibodies targeting extracellular markers was added on top of the Fc blocking solution and incubated for 30 minutes on ice, covered from light. Cells were washed twice with 200 µl of FACS. In some experiments, rat IgM anti-GL7 was added in a second 30-minute staining step in 50 µl of FACS buffer to minimize cross reactive labeling by anti-mouse IgM antibodies. For experiments using intracellular staining, cells were resuspended with 100 µl of BD Cytofix/Cytoperm (BD, 554714) for 20 minutes. Cells were washed once with 150 µl BD Perm/Wash (BD, 554714) and once with 200 µl BD Perm/Wash. Cells were then resuspended in a mix of fluorescent antibodies targeting intracellular markers and incubated for 30-45 minutes on ice, covered from light. Cells were washed twice with 200 µl of BD Perm/Wash, then resuspended in 200 µl FACS buffer and run on a BD LSR Fortessa configured with 3 lasers (407, 488, 640).

Human: Up to 3 million cells were resuspended in FACS buffer and plated into a 96 U-bottom plate (Corning, 353077). Cells were then pelleted and resuspended in 25 µl FACS buffer with mouse gamma globulin (Jackson ImmunoResearch, 015-000-002) for 15 minutes on ice to block non-specific Fc staining. Subsequently, 75 µl of an fluorescent antibody mix targeting extracellular markers was added on top and incubated on ice and covered for 30 minutes. Wells were then washed with 150 µl of FACS, pelleted and washed again. Samples were resuspended in a final volume of 200 µl and ran of a BD LSR Fortessa configured with 3 lasers (407, 488, 640).

Bead experiments: 50 uL of UltraComp eBeads Plus compensation beads (ThermoFisher Scientific, 01-3333-42) were incubated with 0.125 µg anti-PE (Clone PE001, BioLegend, 408102), anti-APC (Clone APC003, BioLegend, 408002), anti-SA (Clone S10D4, ThermoFisher Scientific, MA1-20010), or anti-RBD clone CV30[63]. 4 mL of FACS buffer was added prior to centrifugation at 400 x g for 5 minutes at 4°C. Supernatants were carefully aspirated, and beads were resuspended with 50 µL FACS buffer. 3 µL of beads were incubated with 0.25 pmol antigen tetramer in a final volume of 50 µL for 25 minutes on ice. 4 mL of FACS buffer were added prior to centrifugation at 400 x g for 5 minutes at 4°C. Supernatants were decanted and samples were resuspended in 200 µL FACS buffer for flow cytometry.

Adoptive transfer

Antigen enriched splenocytes and mesenteric lymph node cells from unimmunized CD45.1 mice were resuspended in 200 µL PBS and transferred by tail vein injection into unimmunized µMT hosts. The next day, mice were immunized intraperitoneally with OVA/Alum.

ELISA

ELISAs were performed used 96-well flat bottom absorptive plates (VWR Cat: 439454). ELISAs were blocked using 5% skim milk in PBS, and unless otherwise specified, reagents were prepared in 1% skim milk in PBS. Washes were done using PBS with 0.05% TWEEN 20 (Sigma, Cat: P-1379) using a Tecan Hydroflex (Tecan Trading AG). Plates were read using a Thermo Scientific Multiskan FC plate reader and analyzed in Excel.

OVA-specific IgG1: Plates were coated with 4 µg/ml of OVA (Sigma, Cat: A5378) in 100 µl of carbonate bicarbonate buffer (Sigma, Cat: C-3041), sealed with an adhesive cover (ThermoFisher, Cat: 3501) and incubated overnight in the fridge. The next day, the plates were blotted entirely and blocked for 2 hours at RT. Plates were washed 3 times and incubated with samples at indicated dilutions overnight in the fridge. The next day, plates were washed 3 times, and incubated with 0.25 µg/ml biotinylated anti-mouse IgG1 (Southern Biotech, Cat: 1070-08) for 2 hours at RT. Plates were washed 3 times and incubated with 1:1000 SA-alkaline phosphatase diluted in 0.3% BSA (Sigma, Cat: A4503) for 1 hour at RT and covered from light. After 3 washes, plates were developed using 4-nitrophenyl phosphate (Sigma, Cat: N-9389) dissolved in 1x diethanolamine substrate buffer (ThermoFisher, Cat: 34064) in dH2O until either signal was detected in no-sample control wells or in the highest sample dilution. Plates were stopped with 25 µl 1N NaOH. Samples were read at 405 nm.

OVA-specific IgE: Plates were coated with 50 µl of 2 µg/ml anti-mouse IgE (R35-72, BD, Cat 553413) in PBS overnight in the fridge. The next day, plates were washed 3 times and blocked for 1 hour at 37°C. Plates were washed 3 times and samples were added at the indicated dilutions and incubated overnight in the fridge. The next day, plates were washed 5 times, incubated with 50 µl of 300 ng/ml OVA, conjugated to digoxigenin following supplier recommendations (ANP Technologies, Cat: 90-1023-1KT) for 90 minutes at RT. Plates were washed 5 times and incubated with 1:5000 anti-digoxigenin POD fragments (Roche, Cat: 11 633 716 001) in 0.3% BSA-PBS for 1 hour at RT, covered from light. Plates were washed 5 times, then detected using 50 µl TMB (Sigma, Cat: T0440) and stopped using 25µl 2N H_2SO_4. Samples were read at 450 nm.

Analysis

Flow cytometry data was analyzed using FlowJo v.10.8.1 (FlowJo LLC). GraphPad Prism 6 (GraphPad Software) was used to generate summary plots and for statistical analysis. Figures were assembled in Adobe Illustrator (Adobe Inc.).