Science Advances

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Supplementary Materials for

Commensal epitopes drive differentiation of colonic T$_{regs}$

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**This PDF file includes:**

Supplementary Figures
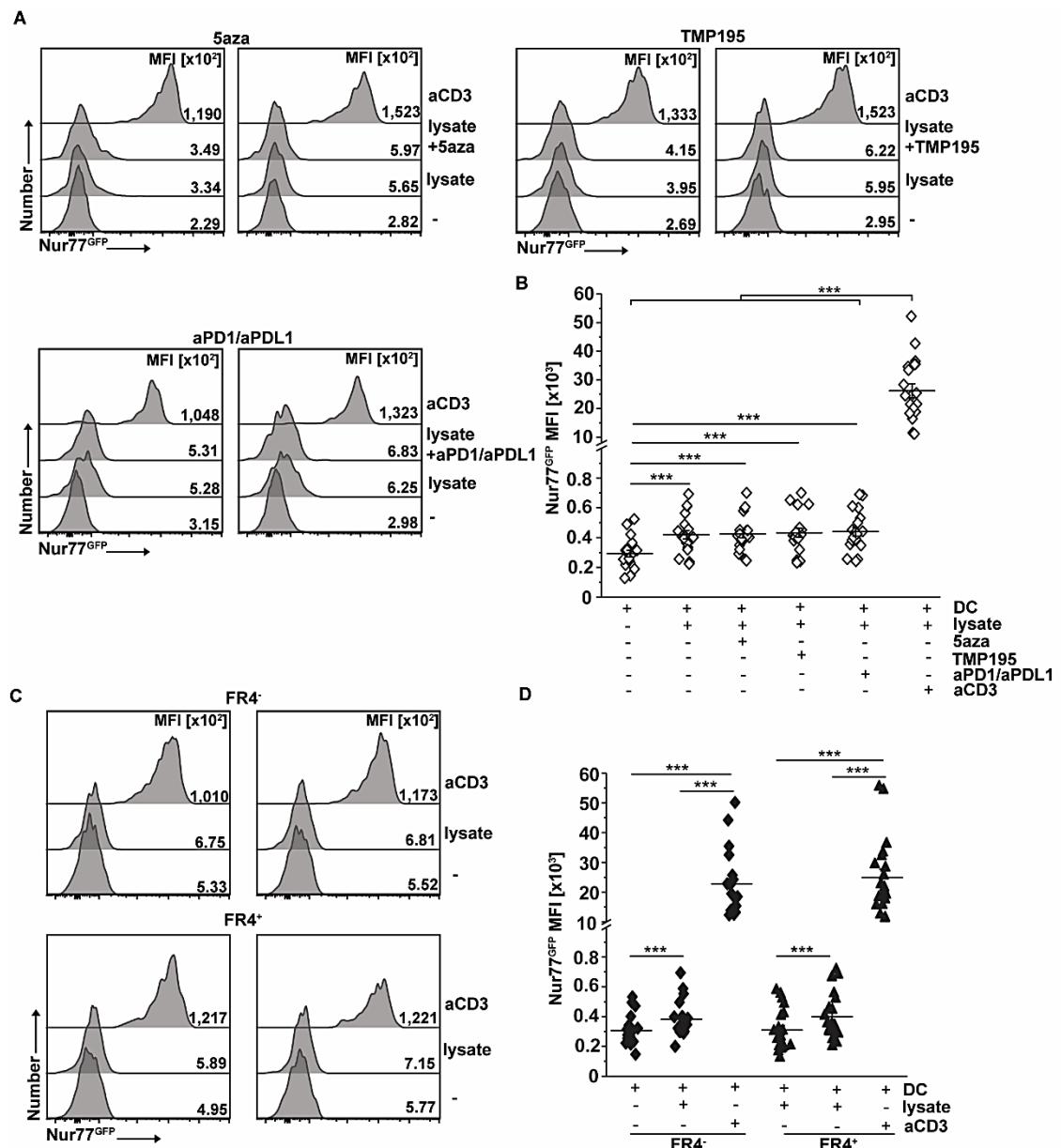
Fig. S1. Tregs-derived hybridomas response to bacterial antigens do not improve after targeted interference with epigenetic methylation/acetylation of histones and PD1/PDL1 signaling pathway. (A) Activation of representative Treg-derived hybridoma with bacterial peptide remains unchanged in the presence or absence of 5aza or TMP195, and blockade of PD1/PDL1 interactions by MoAbs. Representative histograms of 2 out of 20 tested hybridomas are shown and summarized in (B) (each symbol represents separate hybridoma). (C) Hybridomas prepared from anergic CD4+Foxp3-CD44+CD73+FR4+ (FR4+) or naïve/activated non-anergic CD4+Foxp3-FR4- (FR4-) cells similarly responds to bacterial antigens. 2 of 20 tested hybrids are shown and data are summarized in (D). Experiment was repeated three times. Nur77[GFP] MFIs are shown. Paired *t* test, ***p<0.001.
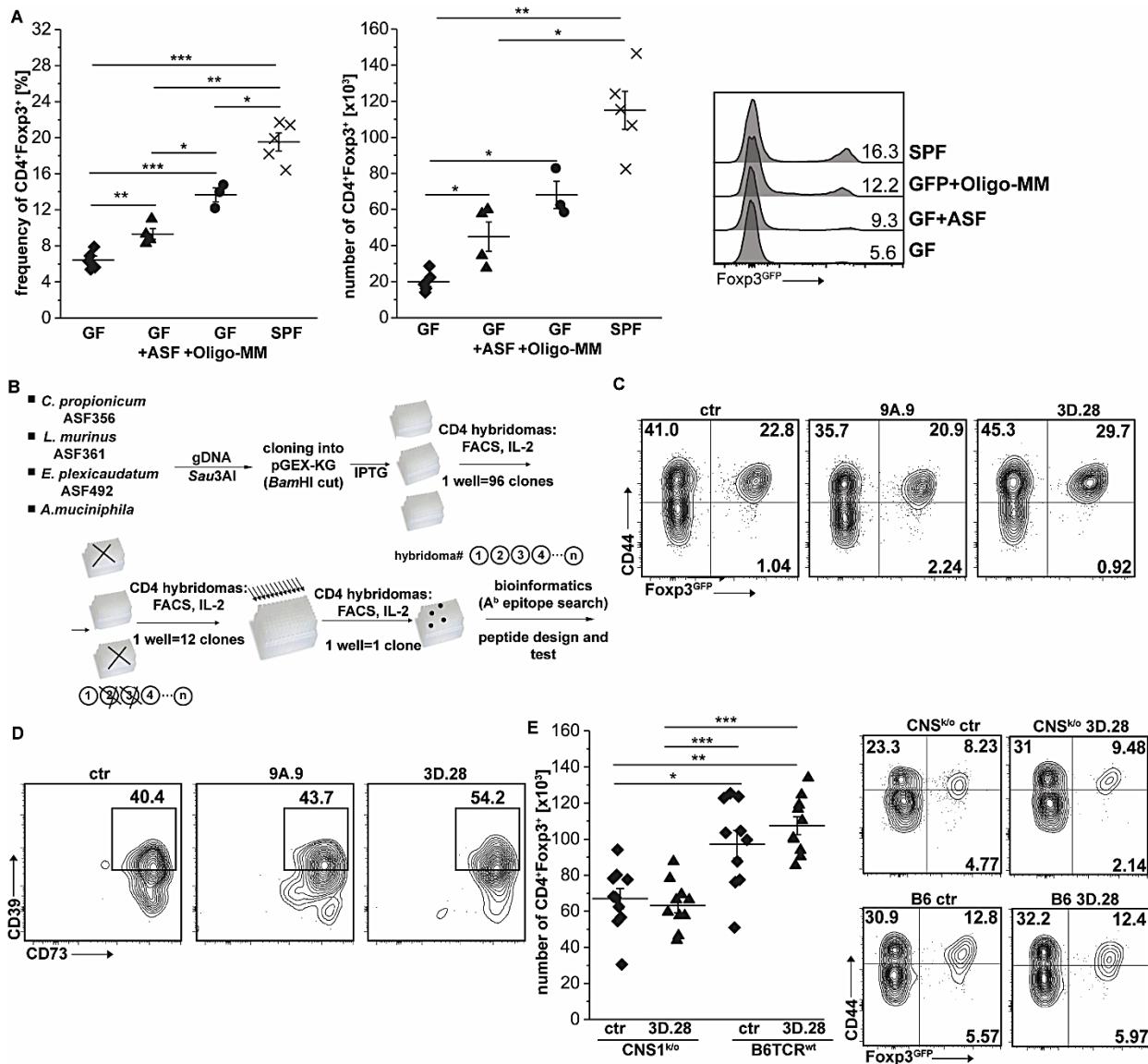
Fig. S2. Antigens derived from limited bacterial consortia stimulate colonic Tregs. (A) Colonization of GF TCR[mini] mice with ASF or Oligo-MM mini-consortia increase colonic Treg frequency (left) and these cells number (right). Tregs from SPF TCR[mini] mice are also shown for comparison. 3-5 mice of each kind were analyzed. Histograms on the right show typical data of CD4^{+}Foxp3^{+} cell frequencies. (B) Strategy for expression genomic DNA libraries from selected bacterial strains incorporated in ASF or Oligo-MM microbiomes (for details see method section). (C) Immunization of TCR[mini]Foxp3[GFP] mice with *E. plexicaudatum* ASF492-derived 3D.28 but not with 9A.9 peptide induces colonic Tregs. Dot plots show representative FACS analysis of gated colonic CD4^{+} cells. (D) Representative plots of CD39 and CD73 upregulation on Tregs in response to indicated peptide antigen. (E) 3D.28 peptide derived from *E. plexicaudatum* ASF492 induces pTregs in TCR[mini] mice but not in in TCR[mini] CNS1[k/o] mice or B6 animals expressing wild-type TCR repertoire. Each symbol depicts an individual animal. Experiment was repeated twice with a total of n=7 (ctr, cholera toxin only) and n=10 (toxin with 2C.1 peptide) mice. (E) 2C.1 peptide of *A. muciniphila* origin induces pTregs in TCR[mini] mice and slightly enhances (p=0.09536) colonic Treg numbers in B6 animals expressing wild-type TCR repertoire. Four to five mice of each kind were tested in each of two experiments. Symbols represent individual mice. Paired *t* test, *p<0.05, **p<0.01, ***p<0.001. GF-Germ Free, ctr-mice receiving cholera toxin only.
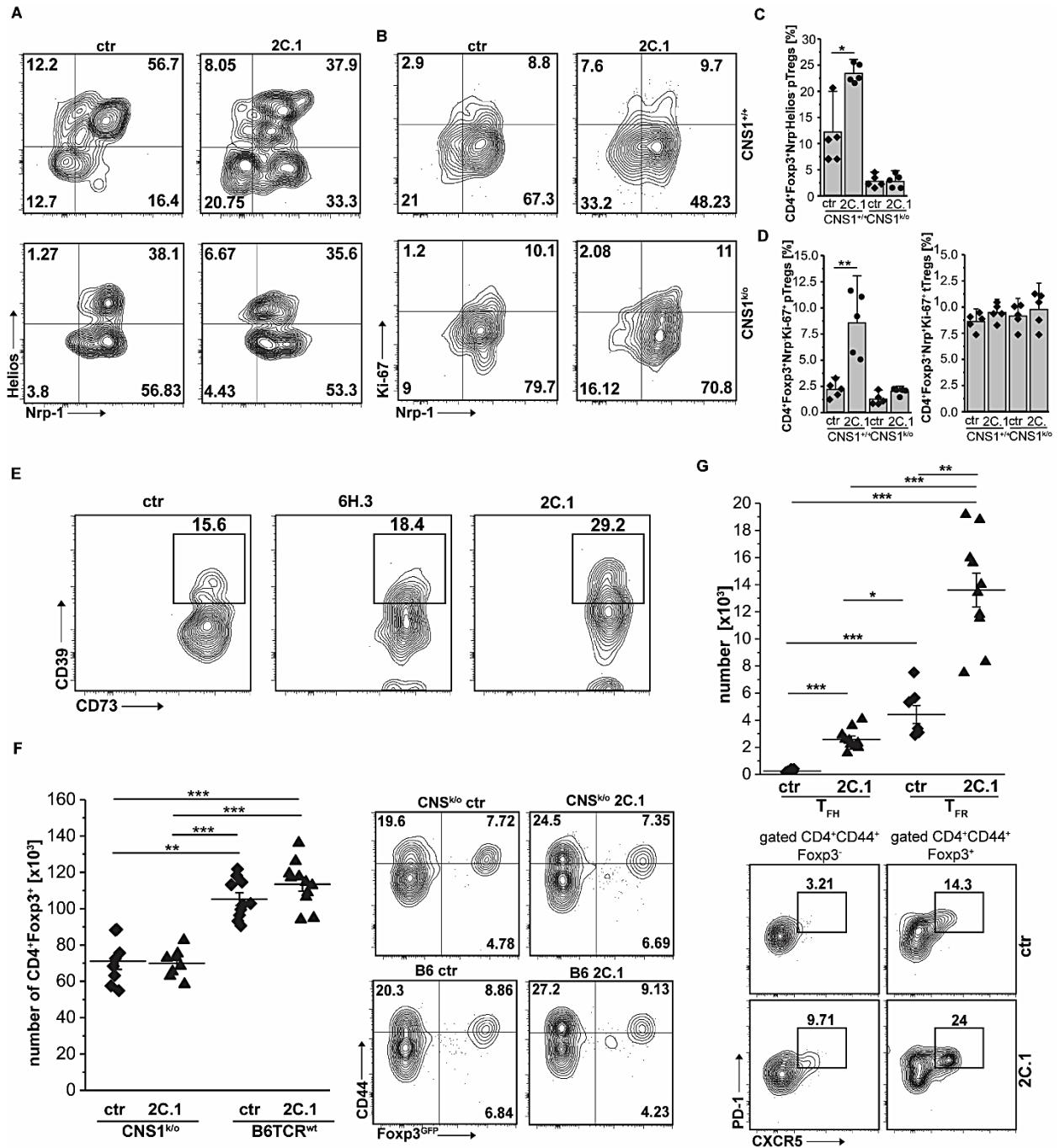
Fig. S3. *A. muciniphila*-derived peptide 2C.1 induces colonic Tregs. (A) Expression of Nrp-1 and Helios measured *in situ* indicate pTreg induction in response to 2C.1 peptide in CNS1^{+/+} but not CNS1[k/o] mice. Typical FACS data are shown and summarized in (C). (B) Proliferation of pTregs in response to 2C.1 peptide measured by Ki-67 upregulation. Typical data are shown and summarized in (D). (E) 2C.1 peptide induces higher expression of CD39 and CD73 on colonic Tregs. Representative FACS dot plots are shown. (F) 2C.1 peptide induces pTregs in TCR[mini] mice but not animals expressing wt TCRs (B6). Summary (left) and typical FACS data (right) are shown. (G) 2C.1 peptide induces PD-1^{+}CXCR5^{+} follicular helper and follicular regulatory cells. Co-expression of PD-1 and CXCR5 was assessed by flow cytometry. Numbers of colonic helper (T_{FH}) and regulatory (T_{FR}) follicular cells and representative dot plots are depicted. Each symbol represents individual mouse. Statistical significances were calculated with paired Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001. ctr-mice receiving cholera toxin only.
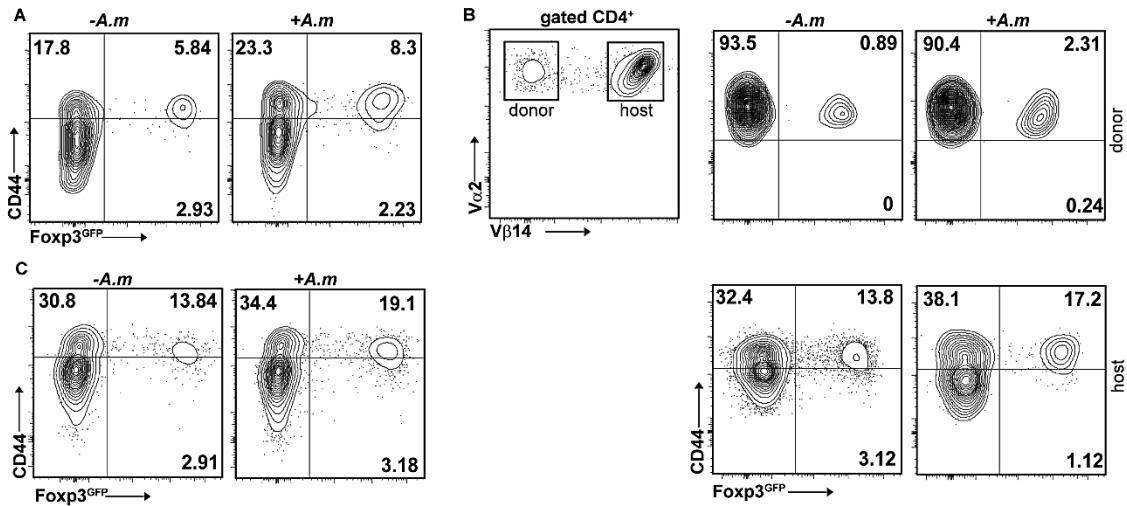
Fig. S4. *A. muciniphila* **expands colonic tTregs and induces pTregs**. (A) Typical FACS dot plots of colonic CD4^{+} cells from GF mice colonized or not with *A. muciniphila*. (B) Induction of pTregs and tTreg expansion. *A. muciniphila*-deficient CNS1[k/o]TCR[mini] (Vα2^{+}Vβ14^{+}) mice received an adoptive transfer of naive CD4^{+} cells from Vα2 (Vα2^{+}Vβ14[wt]) mice. (left) Strategy of gating for donor and host CD4^{+} cells based on Vα2 and Vβ14. (right) Typical dot plots showing donor and host Treg frequencies in indicated mice. (C) Colonization of CNS1[k/o] TCR[mini] mice with *A. muciniphila* increases colonic tTregs. Representative FACS data are shown. *A.m.-A. muciniphila.*
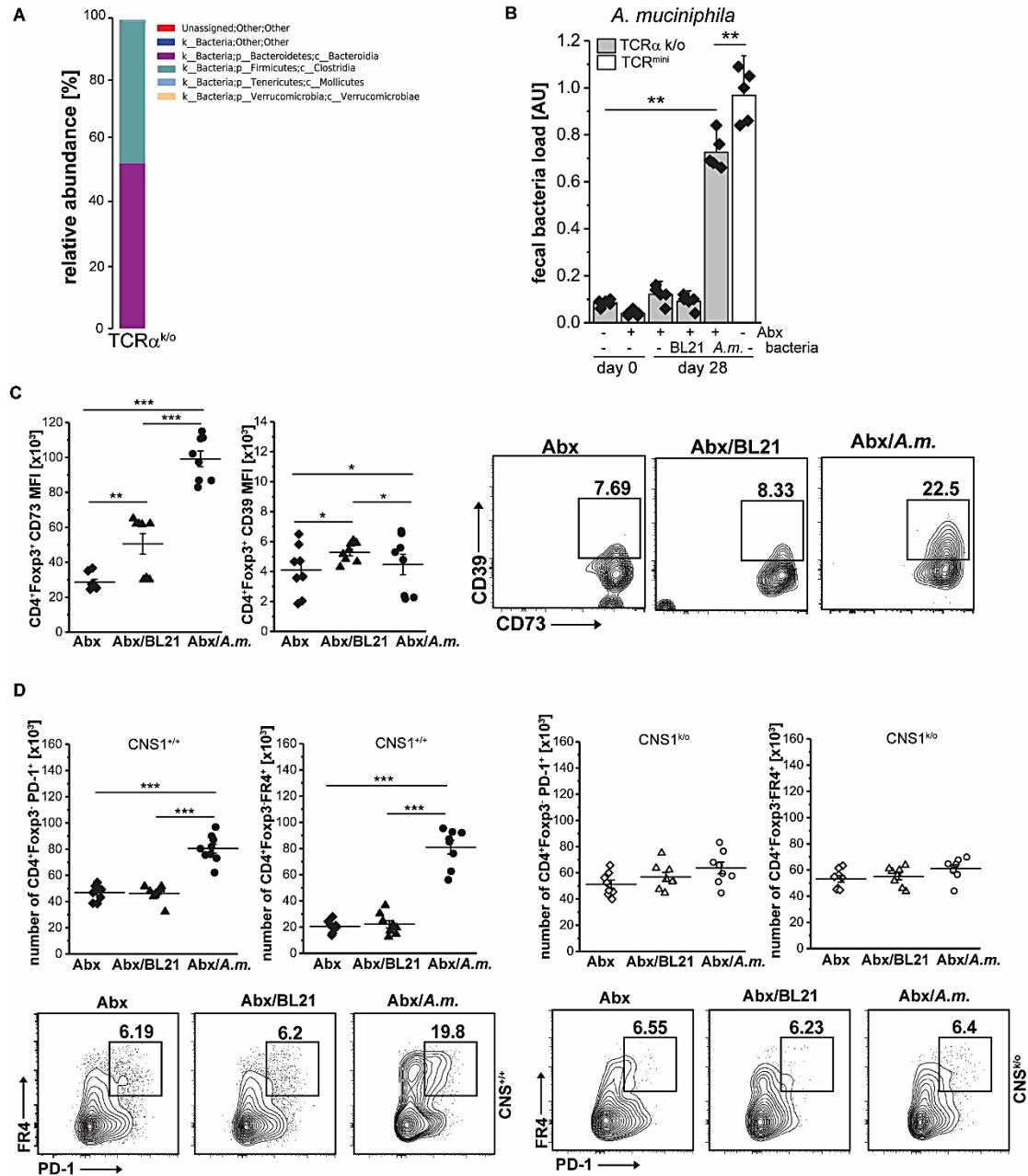
**Fig. S5.** ***A. muciniphila* induces protective colonic pTregs in adaptive transfer model of colitis.** (A) *A. muciniphila* (*Verrucomicrobia* genus) is underrepresented in TCRα$^{k/o}$ mice. 16S rRNA analysis of fecal bacteria is shown. (B) Bacterial load in TCRα$^{k/o}$ mice pretreated with antibiotic cocktail (Abx) and colonized with *A. muciniphila* (*A.m.*) or *E. coli* BL21 (BL21). At day 0 and 28 fecal samples were collected and *A. muciniphila* was detected by RT-qPCR. Samples from TCRα$^{k/o}$ and TCR^{mini} mice served as controls. Bars indicate relative expression [AU] calculated from five mice of each group. Reads were first normalized to UniF340/UniR514 and then to TCR^{mini} *A. muciniphila* signal. Average +/-SD are shown. (C, D) Colonization with *A. muciniphila* induces CD73 and CD39 expression on colonic Tregs (C) and anergic phenotype (FR4$^+$, PD1$^+$) on colonic CD4$^+$-Foxp3$^-$ cells (D). Summaries and representative FACS dot plots are shown. Paired *t* test, *p<0.05, **p<0.01, ***p<0.001.
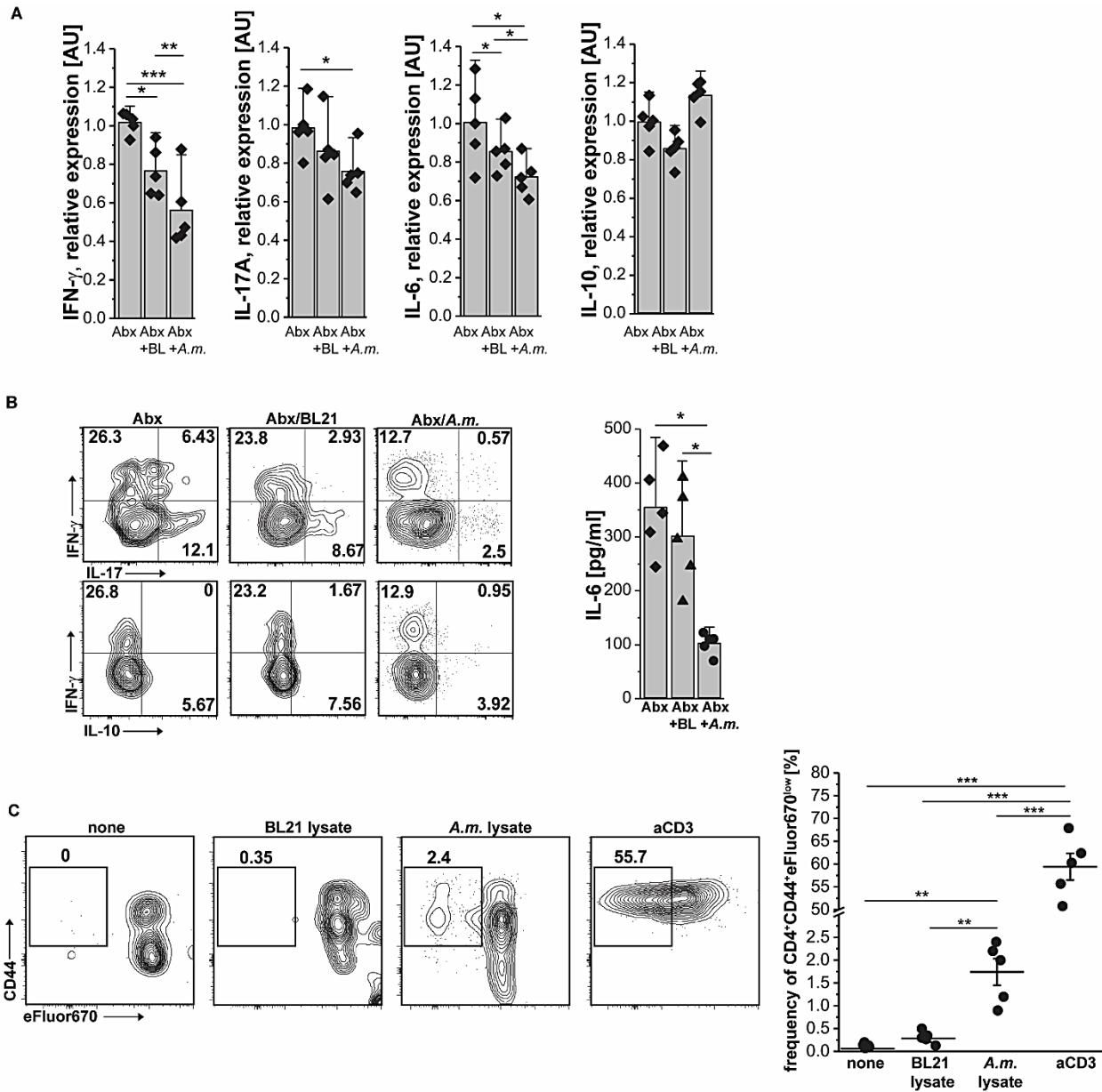
Fig. S6. *A. muciniphila*-induced pTregs prevent colitis in adaptive transfer model. (A, B) Expression of cytokines was measured by RT-qPCR (A) and intracellular staining or ELISA (B) in controls and experimental mice that received *E. coli* BL21 or *A. muciniphila.* Each symbol indicates a sample from an individual mouse.   (C) Colonic CD4⁺ cells from recipients of *A. muciniphila* were tested were tested for response to this bacterium. Cells were loaded with eFluor670 dye and stimulated with a bacterial lysate from *E. coli* BL21DE3 or *A. muciniphila*. Cells without added antigen (none) or samples stimulated with aCD3 MoAb served as additional controls. Dilution of eFluor670 in gated CD4⁺ cells in one experiment of two is shown (summarized on right). Paired *t* test, *p<0.05, **p<0.01, ***p<0.001. BL-*E. coli* BL21DE3, *A.m.-A. muciniphila*.
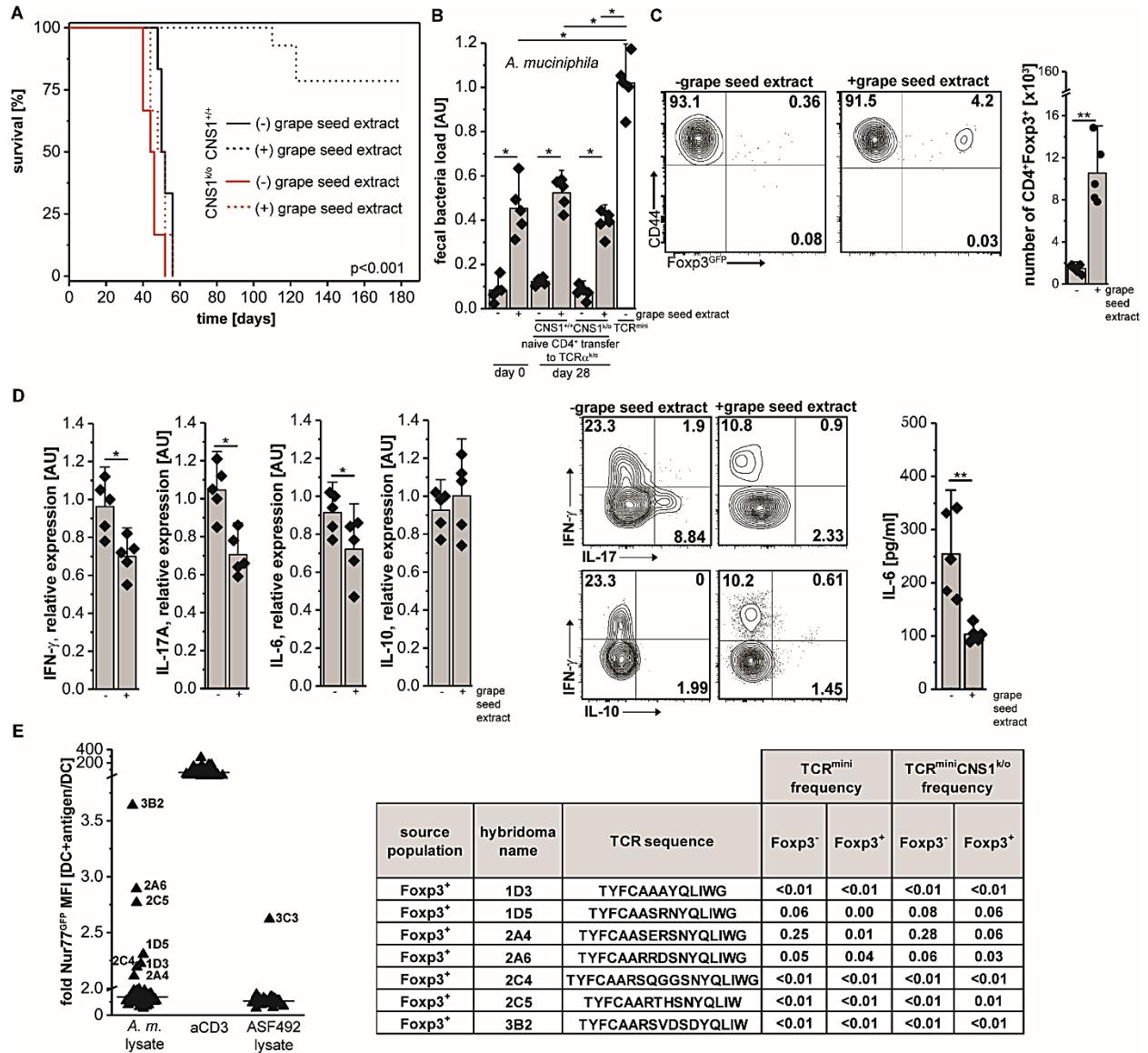
Fig. S7. Grape seed extract expands *A. muciniphila* which induces pTregs and protects host mice from colitis in adoptive transfer model. (A) Selected TCRα[k/o] mice were fed with grape seed extract after which they received adoptive transfer of naïve CD4^{+}CD45RB^{high} cells (1x10^{6}) from either CNS1^{+/+} or CNS1[k/o] TCR[mini] mice (n=6 CNS1^{+/+}, n=14 CNS1^{+/+} +grape seed extract, n=6 CNS1^{-/-}, n=6 CNS1^{-/-} +grape seed extract). Kaplan-Meier survival curves are shown. (B) Grape seed extract induces *A. muciniphila*. RT-qPCR analysis of fecal bacterial DNA from indicated groups is shown from 5 individual mice. (C) Expansion of *A. muciniphila* by grape seed extract results in colonic pTreg induction. Representative FACS dot plots and data summary are shown. (D) Induction of pTregs decreases proinflammatory cytokines. qPCR data (left) and intracellular staining or ELISA (right) are shown. (E) Colonic pTregs fom mice that received grape seed extract were converted into hybridomas and tested against *A. muciniphila* or ASF492 lysates. Responding hybridomas were marked, their TCRs were sequenced and their frequencies of *in situ* CD4^{+}Foxp3^{-} or Foxp3^{+} colonic cells were calculated (table). aCD3 was used as a control. Experiment was repeated twice. Each symbol represents individual mouse. For survival analysis, log-rank test was performed, otherwise, paired *t*-tests were applied, *p<0.05, **p<0.01, ***p<0.001. BL-*E. coli* BL21DE3, *A.m.-Akkermansia muciniphila*.

Table S1: Key resources

Monoclonal antibodies

Antibody	Clone	Conjugate	concentration used in the study [µg/ml]	Supplier	Catalog number	Lot number
CD3	145-2C11	unconjugated	variable (0.0001-5)	BioLegend	100331	B270450
CD4	GK1.5	BV510	0.8	BD	743155	B25813
CD4	GK1.5	APC	0.2	BioLegend	100412	B258297
CD4	RM4-5	biotin	0.4	BD	553045	86055
CD11c	N418	PE	0.8	eBioscience	12-0114-86	E01080-1630
CD16/32	2.4G2	unconjugated	supernatant (1:10 dilution)	ATCC	HB-197	n/a
CD25	PC61	PECy7	0.4	BioLegend	102016	B136654
CD28	37.51	unconjugated	1 or 5	Invitogen	16-0281-86	1934012
CD39	5F2	biotin	0.8	BioLegend	135703	n/a
CD44	IM7	eFluor450	0.4	eBioscience	48-0441-80	4314446
CD44	IM7	PE	0.8	BioLegend	103008	B140386
CD62L	MEL14	APCCy7	0.4	BioLegend	104428	B15773
CD71	RI7217	PECy7	0.4	BioLegend	113812	B259648
CD73	TY/11.8	PE	0.4	BioLegend	127206	B199645
CD80	16-10A1	FITC	0.4	BD	553768	48602
CXCR5	2G8	biotin	0.2	BD	551960	81949
FR4	12A5	APC/Fire750	0.8	BioLegend	125014	B248137
Helios	22F6	PE	1.0	BD	137216	B141151
Ki-67	16A8	PE	0.8	BioLegend	652403	
Nrp-1	3DS304M	SB600	0.8	eBioscience	501122305	2031416
PD1	J43	SB702	0.4	eBioscience	67-9985-80	1981574
PD1	RMP1-14	unconjugated	10	eBioscience	16-9982-81	E07013-1630
PDL1	10F.9G2	PECy7	0.4	BioLegend	124314	n/a
PDL1	MIH5	unconjugated	10	eBioscience	16-5982-81	4281874
streptavidin	streptavidin	PECy5	0.2	BioLegend	405205	B141105
streptavidin	streptavidin	PECy7	0.4	BioLegend	405206	B207311
streptavidin	streptavidin	APCCy7	0.4	BioLegend	405208	B215107
IFN-γ	XMG1.2	APC	2.0	BioLegend	505810	E028187
IL-17A	TC11-18H10.1	PE	1.0	BioLegend	506903	B118589
IL-10	JES5-16E3	PE	2.0	BioLegend	505007	B122294
Vα2	B20.1	PE	0.4	BD	553289	B245806
Vα2	B20.1	PerCP-Cy5.5	0.8	BD	560529	B219567

Vβ14	14-2	biotin	0.4	BD	553257	5176771
TCRβ	H57-597	APCCy7	0.4	BioLegend	109220	B230161
Y3P	Y3P	biotin	0.8	ATCC	HB-183	in house

Chemicals, reagents, kits

Chemicals, peptides and recombinant proteins	Supplier	Catalog number	Lot number
5 azacytidine	MP	100821	QR12395
660nm Protein Assay Reagent	Fisher	22662	NF173401
Ampicillin	Sigma	A0166-5G	029K05521
*Bam*HI	Fisher	FD0058	377990
BHI broth	Fisher	R452472	R112022
Brefeldin A	BioLegend	420601	N/A
Buffered formalin, 10%	Fisher	3081101G8T	4812-10
Cholera toxin	Sigma	C8052-1MG	107M4001V
CIAP	Fisher	EF0651	416264
Ciprofloxacin	Sigma	17850-25G-F	127M4065V
Collagenase D	Roche	11088882001	34163622
DAPI	Invitrogen	D3571	26992W
DMSO	Corning	25-950-CQC	31217005
DNaseI	Roche	10104159001	35027800
FBS	HyClone	SH30396.03	AD18110280
Nr4a1-GFP BAC	BacPac	GENSAT1-BX1262	n/a
Ghost red 710 viability dye	Tonbo	13-0871-T100	DO871061016133
Grape seed extract	BRI Nutrition	Grape Seed	2017-00785
HBSS	Corning	55-022-PB	20318006
Ionomycin	Sigma	10634-1MG	n/a
IPTG	GoldBio	12481C5	0216.060316A
Lysozyme	Sigma	L4919-500MG	SLBN8071V
MEM	Corning	10-010-CV	2819001
Metronidazole	Sigma	M3761-100G	SLBD5470V
MMLV Reverse Transcriptase	Promega	M170B	24228241
Monensin	BioLegend	420701	N/A
MTT	Sigma	M2128-10G	MKBB9557
Nycodenz	Axell	AN1002423	10198771
PBS	Cellgro	55-031-PC	55031016
pcDNA3	Fisher	V79020	n/a
PEG1450	J.T. Baker	U220-07	K31603
pGEX-KG	ATCC	77103	n/a

PMA	Sigma	P8139-5MG	n/a
PrimeTime Gene Expression Mix	IDT	1055770	406882
ProBlock Gold bacterial protease cocktail	GoldBio	GB-330-1	2001.090916A
Recombinant Murine IL-2	Peprotech	212-12-20UG	608108
Recombinant Murine GM-CSF	Peprotech	315-03-50UG	81455
Recombinant Flt3L	Peprotech	250-31L-10ug	0814352-1
*Sau3*AI	Promega	R619A	124767
Sodium bicarbonate	Sigma	S5761-500GM	047K0072
Streptomycin	Corning	61-088-RM	61088115
SsoAdvanced Universal IT SYBR Green kit	BioRad	172-5017	L006343A
SuperScript III First-Strand Synthesis System	Invitrogen	18080051	813751
T4 ligase	Fisher	EL0014	478874
TMP195	MCE	HY-18361	24939
Vancomycin	Sigma	V2002-5G	058M-4009V
*Xho*I	Promega	R616A	24456004

Assays

Critical Commercial Assays	supplier	catalog number	lot number
eFluor670	eBioscience	65-0840-90	E11832-1632
IL-6 ELISA kit	eBioscience	501128808	N/A
RNeasy Mini Kit	Qiagen	74106	163012131
NucleoFast® 96 PCR Plates	Macherey-Nagel	743100.5	806/001
GenElute bacteria genomic isolation kit	Sigma	NA2100-1KT	SLBQ4038V
GeneJET Plasmid miniprep kit	Fisher	K0503	110096
QIAamp PowerFecal DNA Kit	Qiagen	12830-50	160040265

Primers

organism	primer name	primer sequence (5'->3')	reference
A. muciniphila	AM1	CAGCACGTGA AGGTGGGGAC	48
	AM2	CCTTGCGGTTG GCTTCAGAT	
universal bacteria	UniF340	ACTCCTACGGG AGGCAGCAGT	49
	UniR514	ATTACCGCGGC TGCTGGC	
molecule	primer name	primer sequence (5'->3')	reference
IL-2	fwd	CCTGAGCAGG GAGAATTACA	50
	rev	TCCAGAACATG CCGCAGA	
GFP	fwd	AAGTTCATCTG CACCACCG	51
	rev	TCCTTGAAGAA GATGGTGCG	
β-actin	fwd	GGCTGTATTCC CCTCCATCG	52
	rev	CCAGTTGGTAA CAATGCCATGT	

Assay	TaqMan assay number
IFN-γ	Mm01168134_m1
IL-17	Mm00506606_m1
IL-6	Mm00446190_m1
IL-10	Mm01288386_m1
β-actin	Mm00607939_s1

Software

Software and Algorithms	Source	Identifier
OriginPro v2017	OriginLab	https://www.originlab.com
FlowJo v10	FlowJo LLC	https://www.flowjo.com/
Python-based custom TCR extraction tool	52	
Custom made TCR database	2	
QIIME2		http://qiime.org/

Mice

Experimental Models: Organisms/Strains/Cell lines	Source	Identifier
Mouse: B6.129P2-B2mtm1Unc/J ($\beta 2m^{k/o}$)	Jackson laboratory	2087
Mouse: B6.129S2-Tcratm1Mom/J ($TCR\alpha^{k/o}$)	Jackson laboratory	2116
Mouse: C57BL/6-Tg(Foxp3-GFP)90Pkraj/J ($Foxp3^{GFP}$)	Jackson laboratory	23800
Mouse: B6.129S2-H2dlAb1-Ea/J (A^{b-})	Jackson laboratory	3584
Mouse: TCR^{mini}	17	
Mouse: $CNS1^{k/o}$	19	
Cell line: HT2	17	
Cell line: $BWNur77^{GFP}$	This paper	
Cell line: T cell hybridomas	This paper	

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