

Fig. S1. Raw data from T_{reg}-specific demethylated region (TSDR) demethylation assay. The data from Fig. 1D are shown as the difference between cycle threshold (Δ CT) values of demethylated TSDR and *GAPDH*. TSDR demethylation assay was performed in sorted effector T cell 1 (T_{eff1}), effector T cell 2 (T_{eff2}), and regulatory T cell (T_{reg}) populations. Data are presented as mean \pm standard deviation (SD). Statistical significance was evaluated using the Mann-Whitney test; ****P < 0.0001.

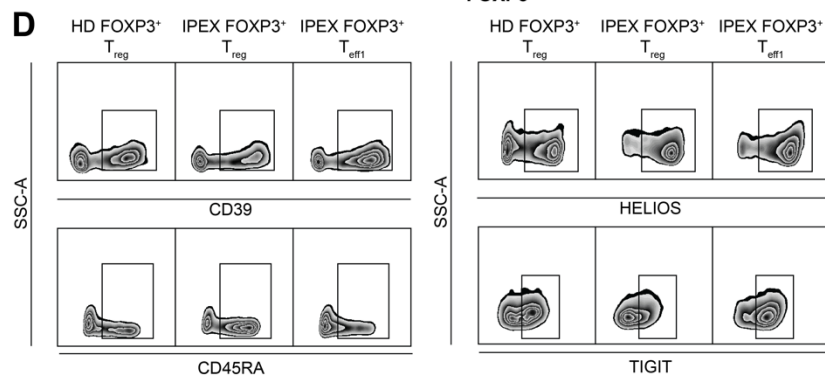
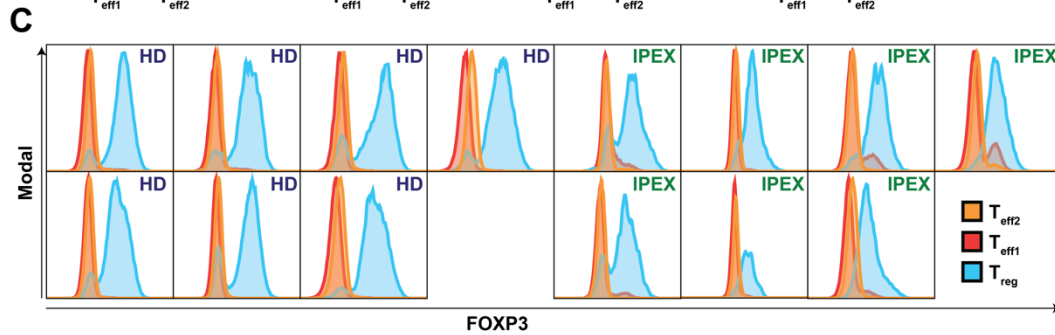
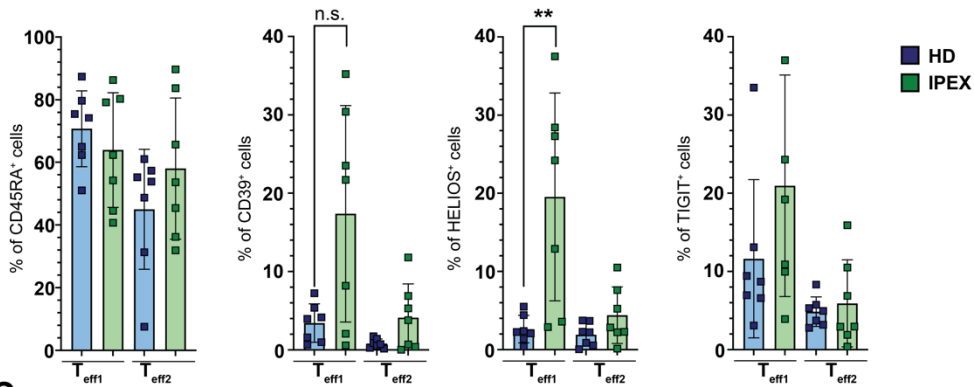
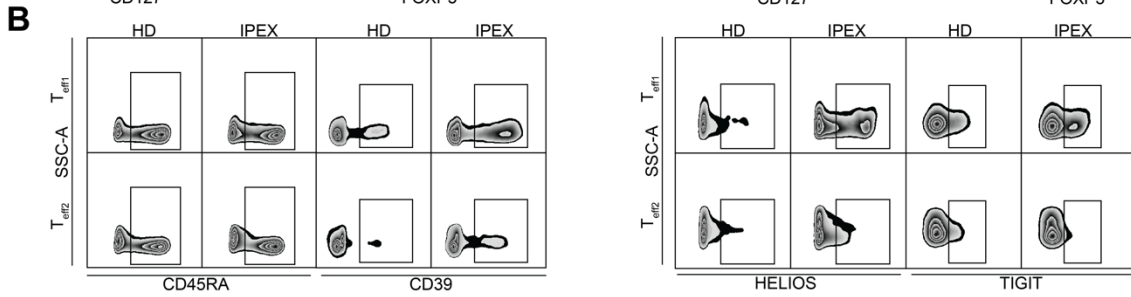
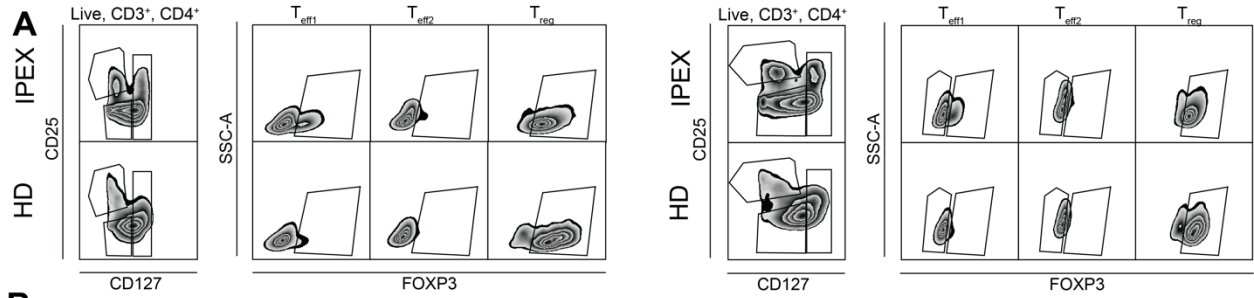
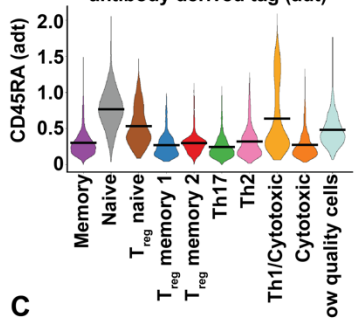
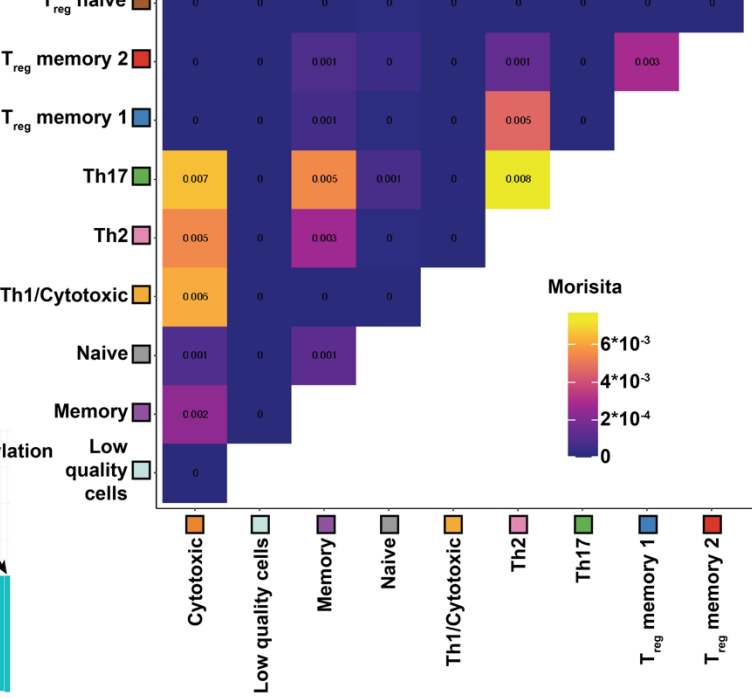


Fig. S2. Flow cytometry gating strategy for cells isolated from patients with IPEX and HDs analyzed in Fig. 1E and H; FOXP3 expression in patients analyzed in Fig. 1G, and extended phenotypes of T_{eff1} and T_{eff2} populations. (A) Representative plots illustrating the flow cytometry gating strategy of two antibody panels (left and right). The data quantifications and further sub-gating strategies are shown in Fig. 1E and H, fig. S2B and D. SSC-A, side scatter area. **(B)** Extended phenotype analyses of the T_{eff1} and T_{eff2} populations. Flow cytometry gating strategy is shown in the top panel, and quantification is shown in the bottom panel. The T_{eff1} and T_{eff2} were gated as shown in (A). Statistical significance was evaluated using the Mann-Whitney test; $**P < 0.01$. Data are presented as mean \pm standard deviation (SD). **(C)** FOXP3 expression in patients analyzed in Fig. 1G. Flow cytometry plots showing FOXP3 expression in T_{eff1} (red), T_{eff2} (orange), and T_{reg} (blue) populations from seven patients with IPEX and seven HDs. **(D)** Representative plots illustrating the flow cytometry gating strategy used in Fig. 1H. The T_{eff1} and T_{eff2} were gated as shown in (A).

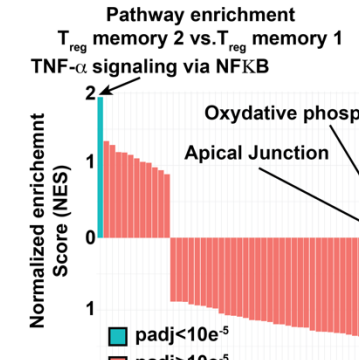
A CD45RA expression in clusters antibody derived tag (adt)



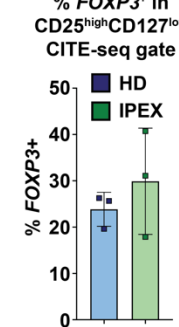
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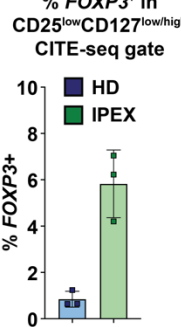
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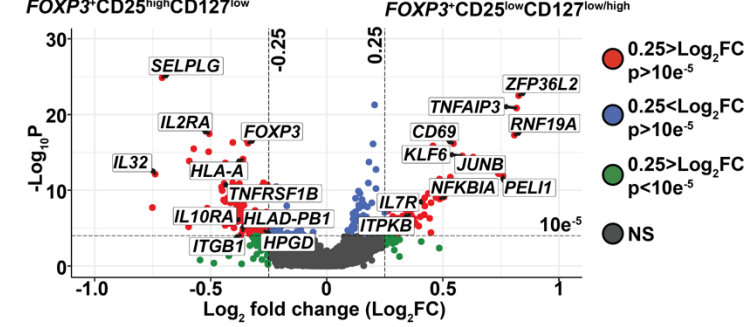
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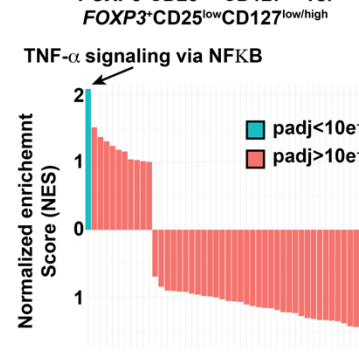
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F



G



H

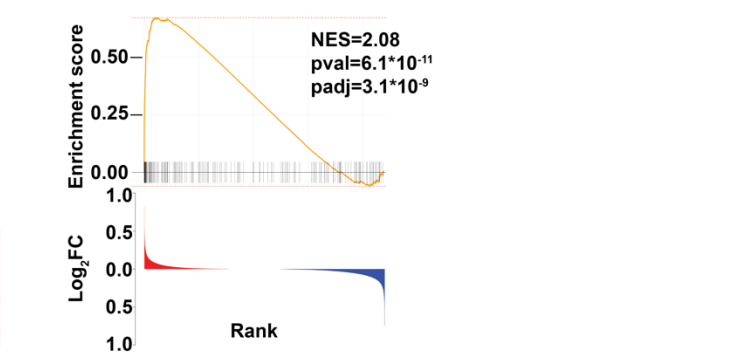


Fig. S3. Single-cell multi-omic RNA, protein, and T cell receptor (TCR) profiling supplementary data for CD4⁺ T cells isolated from patients with IPEX and HDs. (A) Antibody derived tag (ADT)-based analyses of CD45RA expression in T cell populations identified as shown in Fig. 2. (B) Analysis of TCR repertoire overlap between the T cell populations using Morisita overlap. (C) Results of fast gene set enrichment analysis (FGSEA) in T_{reg} memory 2 over T_{reg} memory 1 populations. (D) Percentage of *FOXP3*⁺ (mRNA) cells among CD25^{high}CD127^{low} [ADT] cells in HDs and patients with IPEX. (E) Percentage of *FOXP3*⁺ (mRNA) cells among CD25^{low}CD127^{low/high} [ADT] cells in HDs and patients with IPEX. (F) Differential expression analyses between *FOXP3*⁺ CD25^{high}CD127^{low} and *FOXP3*⁺ CD25^{low}CD127^{low/high} cells from patients with IPEX. The relevant genes are indicated by a label. P values were calculated using the Wilcoxon rank sum test. The genes with positive fold change are upregulated in *FOXP3*⁺ CD25^{high}CD127^{low} cells compared to *FOXP3*⁺ CD25^{low}CD127^{low/high} cells. (G) Results of FGSEA in *FOXP3*⁺ CD25^{high}CD127^{low} over *FOXP3*⁺ CD25^{low}CD127^{low/high} cells from patients with IPEX. (H) Detail of the tumor necrosis factor (TNF)- α signaling from FGSEA analysis from (G). Genes were ranked based on fold changes, the top panel shows the enrichment score, and the bottom panel shows the fold change of the genes. Data are presented as mean \pm standard deviation (SD).

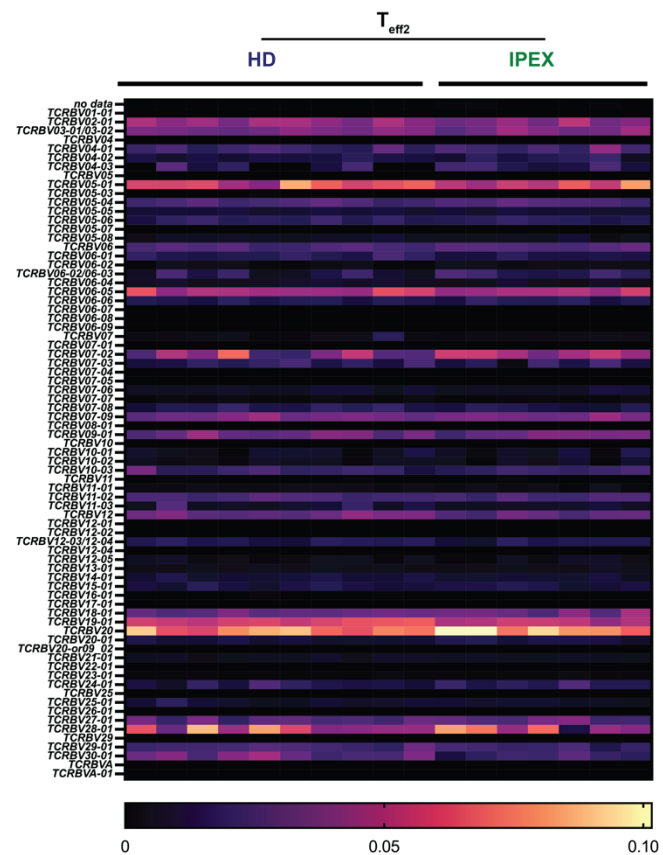
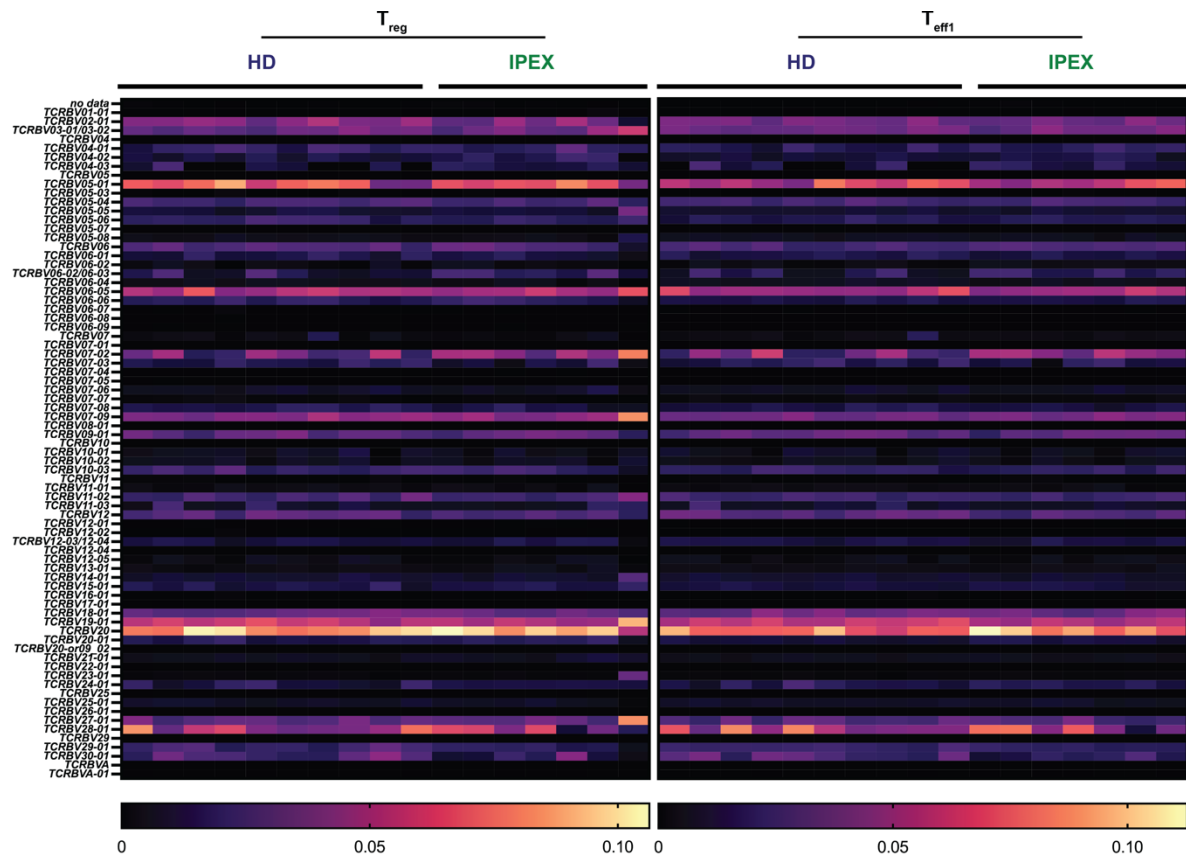


Fig. S4. *TCRB* gene usage in T_{regs} , T_{eff1s} , and T_{eff2s} isolated from patients with IPEX and from HDs.

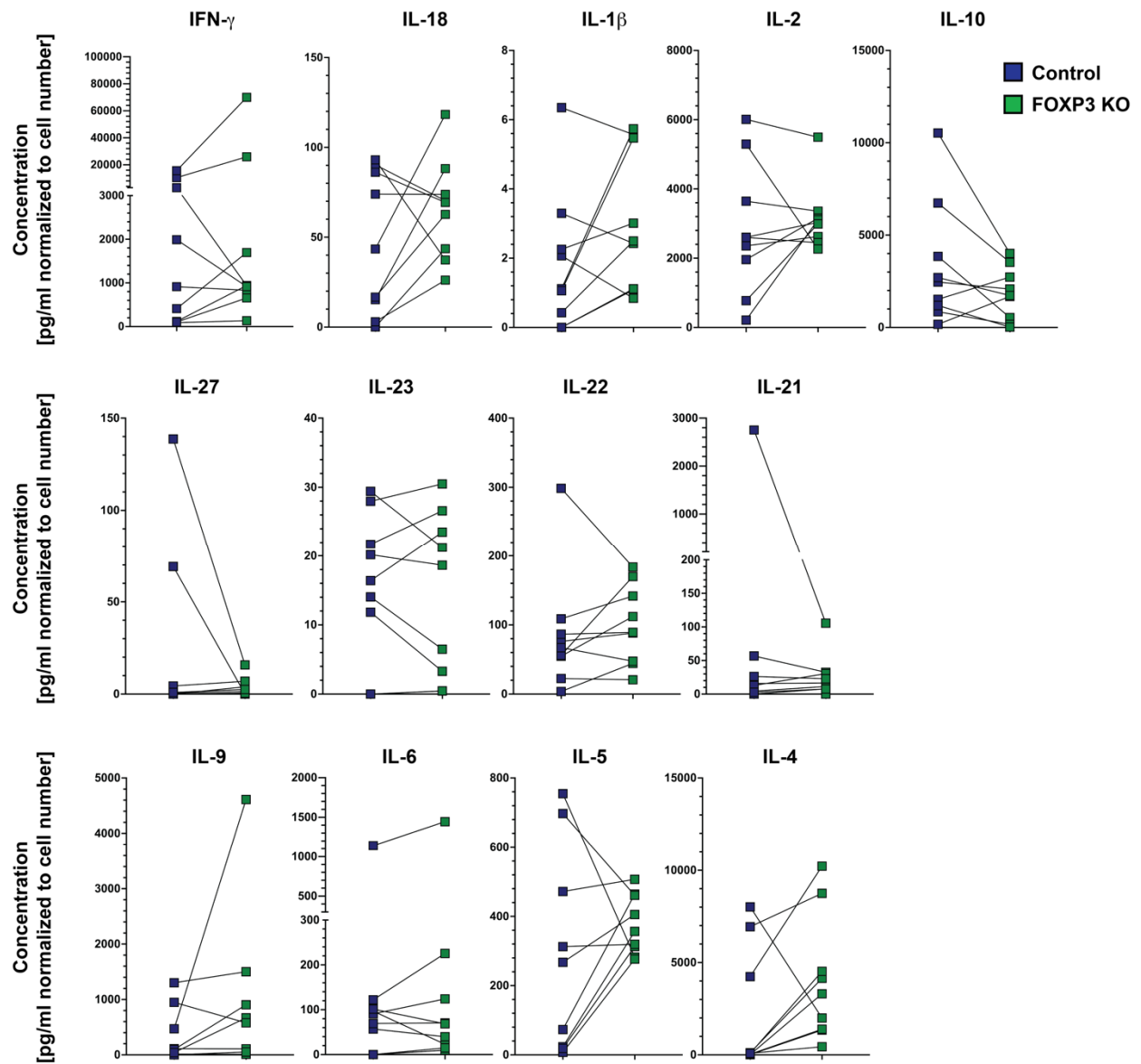


Fig. S5. Cytokine production by FOXP3 knockout (KO) and control T_{regs} using Luminex in addition to those shown in Fig. 7D. IFN, interferon; IL, interleukin.

Fig. S6. Comparable expansion and proinflammatory cytokine production in ScrambleGuideRNA/Cas9 and AAV6 treated T_{regs} (scramble T_{regs}) as in control T_{regs} (nucleofected) in response to TCR stimulation in vitro. (A) Representative flow cytometry plot (left), and quantification (right) of FOXP3 expression in scramble T_{regs} and control T_{regs} from 8 HDs. FSC-A, forward scatter area. **(B)** T_{regs} expansion upon CD3/CD28 stimulation. Cells were counted 3 days post anti-CD3 and anti-CD28 stimulation. The data are plotted as a fold increase of the cell number from day 0. Lines connect control and scramble T_{regs} from the same donor. **(C)** Frequency of TSDR-demethylated cells in control and scramble T_{regs}. **(D)** Analyses of cytokine production from supernatants of control and scramble T_{regs} using a Luminex-based multiplexed assay. GM-CSF, granulocyte-macrophage colony-stimulating factor. The data were analyzed using the Wilcoxon matched-pairs test (A to D); *p<0.05, **P<0.01. Data are presented as ± standard error of the mean (SEM).

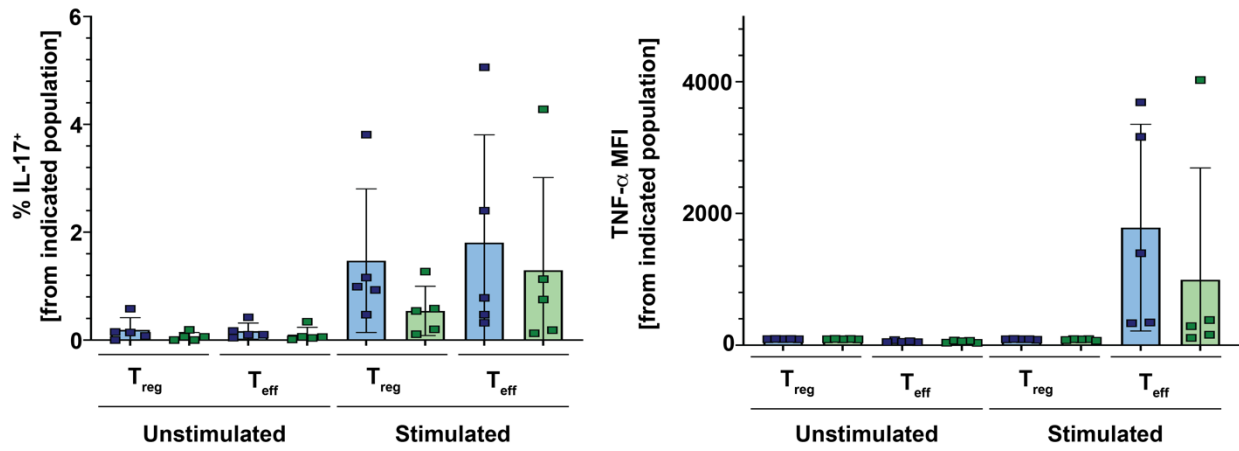


Fig. S7. Cytokine production by Tregs and Tregs from patients with IPEX and HDs. The data show non-significant comparisons from Fig. 7E and F. Data are presented as mean \pm SD.

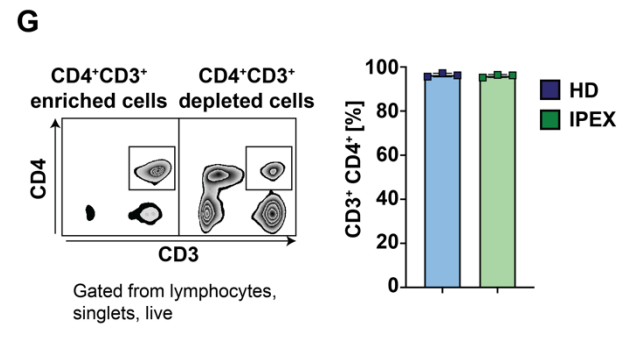
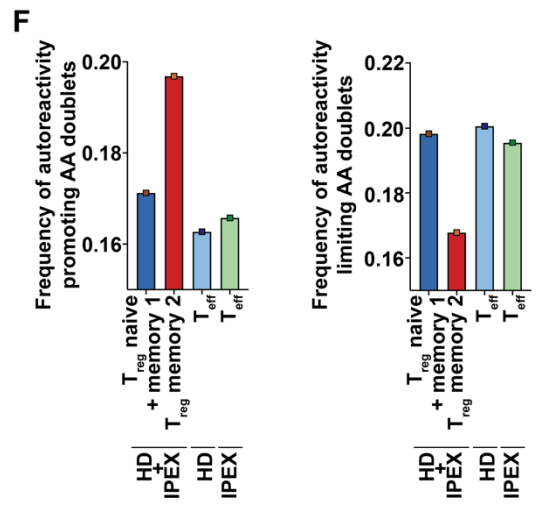
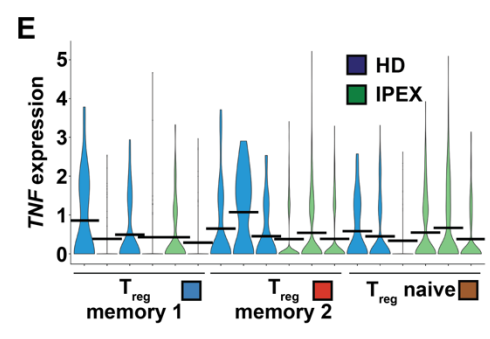
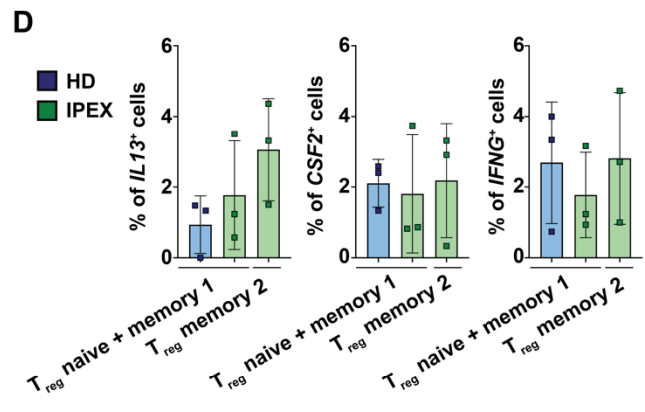
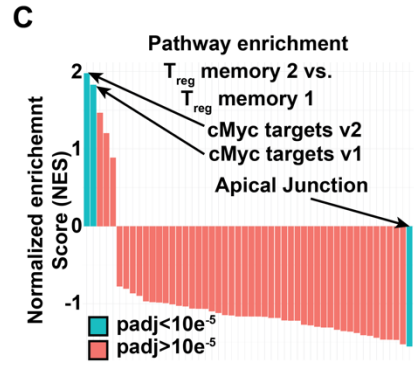
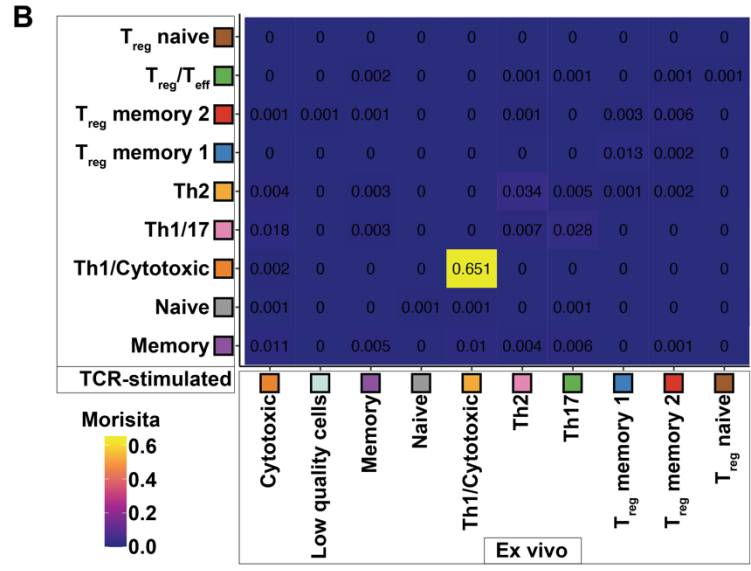
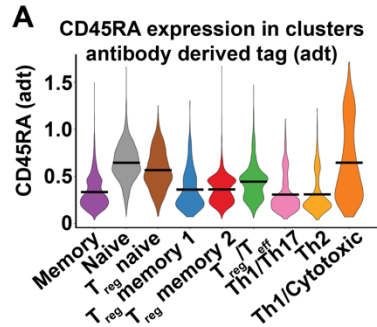


Fig. S8. Single-cell multi-omics RNA, protein, and TCR profiling supplementary data for TCR-stimulated CD4⁺ T cells from patients with IPEX and HDs. (A) ADT-based analyses of CD45RA expression in T cell populations identified in Fig. 7. (B) Analysis of TCR repertoire overlap between the ex vivo identified T cell populations from Fig. 2 and from TCR stimulated T cell populations identified in Fig. 7 using Morisita overlap. (C) Results of FGSEA analysis in T_{reg} memory 2 over T_{reg} memory 1. (D) Percentage of *IL13*⁺, *CSF2*⁺, and *IFNG*⁺ cells in T_{reg} memory 1 and T_{reg} naïve, and T_{reg} memory 2 populations from samples from HDs and patients with IPEX. (E) Violin plot showing *TNF* expression in T_{reg} naïve, T_{reg} memory 1, and T_{reg} memory 2 populations from samples from HDs and patients with IPEX. The black lines indicate the mean. (F) Frequency of autoreactivity promoting (left) and autoreactivity limiting (right) amino acid (AA) doublets in complementarity determining region 3 beta (CDR3β) in T_{reg} memory 1 and T_{reg} naïve, and T_{reg} memory 2 populations from both HDs and from patients with IPEX, and in T_{effs} separately for HDs and samples from patients with IPEX. The T_{reg}/T_{eff} population was not included among the T_{effs}. (G) The purity of CD3⁺CD4⁺ cells used for the single-cell multiomics profiling in Fig. 2 and Fig. 8 from 3 patients with IPEX and 3 HDs. (*Left panel*) The representative flow cytometry plots of CD4⁺CD3⁺ enriched and depleted peripheral blood mononuclear cells (PBMCs). (*Right panel*) Quantification of the CD4⁺CD3⁺ enriched cell purity. Data are presented as mean ± SD.

Table S1. Donor Characteristics. # Indicates that mutation was not confirmed by sequencing but two of the mother's children have the same mutation. *Indicates protein expression in the affected child, or reference to the description of the affected child. NA, not available or applicable.

Patient/ HD	Sex	Age	Affected gene	Mutation	Protein expression	Previously described individual or mutation	Relation between the subjects in this study
20	Male	1	FOXP3	c.340C>T	Yes	(13)	NA
37	Male	29	FOXP3	c.1150G>A	Yes	(13, 33)	NA
64	Male	3	FOXP3	c.1270_1272 del/insC	No	(13, 33)	NA
77	Male	15	FOXP3	c.1129C>G	Yes	(13, 33)	NA
122	Male	13-14	FOXP3	c.1280C>G	Yes	(13)	NA
136	Male	1	FOXP3	c.200C>T and c.1042 T>G	Yes	(13)	NA
139	Male	2	FOXP3	c.694T>G	Yes	(13)	Brother of 140
140	Male	4	FOXP3	c.694T>G	Yes	(13)	Brother of 139
152	Male	9	FOXP3	c.1222G>A	Yes	(13)	Brother of 154 and 153, Mother is 156, Father is 155
154	Male	2	FOXP3	c.1222G>A	Yes	(13)	Brother of 152 and 153, Mother is 156,

							Father is 155
157	Male	9	FOXP3	c.454+4 A>G	Yes	(17)	NA
161	Male	<1	FOXP3	c.782_807de linsTGAGCC	Yes	new mutation	Father is 162, and mother is 163
184	Male	7	FOXP3	c.1222G>A	Yes	(13)	NA
187	Male	15	FOXP3	c.1036A>G	Yes	(57)	NA
188	Male	3	FOXP3	c1087A>G	Yes	(58)	Mother is 189
195	Male	10	FOXP3	c.1190 G>A	Yes	(59)	NA
196	Male	14	FOXP3	c.994 A>G	Yes	(14)	NA
201	Male	17	FOXP3	c.1222 G>A	Yes	(13)	NA
Carrier Mother 156	Female	NA, adult	FOXP3	p.1222G>A#	Yes*	(13)*	Mother of 152, 153, and 154
Carrier Mother 1	Female	NA, adult	FOXP3	p.A384T	Yes*	(60), (30)*	NA
Carrier Mother 2	Female	NA, adult	FOXP3	p.F373A	Yes*	(60), (30)*	NA
Carrier Mother 3	Female	NA, adult	FOXP3	p.A384T	Yes*	(60), (30)*	NA
Carrier Mother 4	Female	NA, adult	FOXP3	IVS8+4A>G	NA	(39)	NA
Carrier Mother 5	Female	NA, adult	FOXP3	c.2T>C	No*	(60), (30)*	NA
Carrier Mother 6	Female	NA, adult	FOXP3	c.2T>C	No*	(60), (30)*	NA
Carrier mother 163	Female	NA, adult	FOXP3	c.782_807de linsTGAGCC	Yes	NA	Mother of 161
Carrier Mother IPEX01	Female	NA, adult	FOXP3	V408M	Yes	(16)*	NA

Mother 189	Female	34	HD	FOXP3 wild type form	Yes	NA	Mother of 188
AIRE07	Female	8	AIRE	c.967_979del13 and c.769C>T	NA	(8, 61), (9)*	NA
AIRE09	Male	13	AIRE	c.967_979del13 homozygote	NA	(8), (9)*	NA
AIRE11	Female	17	AIRE	c.967_979del13 homozygote	NA	(8), (9)*	NA
63	Female	14	AIRE	AIRE c.967_979del confirmed for one allele	NA	(8)	NA
FAT_AIRE07	Male	NA, adult	NA	NA	NA	(9)*	Father of patient AIRE07
MOT_AIRE07	Female	NA, adult	NA	NA	NA	(9)*	Mother of patient AIRE07
MOT_AIRE11	Female	NA, adult	NA	NA	NA	(9)*	Mother of patient AIRE11
MOT_AIRE12	Female	NA, adult	NA	NA	NA	NA	NA
153	Male	7	HD	WT FOXP3	NA	NA	Healthy brother of 152 and 154, Mother is 156, Father is 155
Pediatric donor 1	NA	10	HD	NA	NA	NA	NA
Pediatric donor 2	NA	11	HD	NA	NA	NA	NA
Pediatric donor 202	Male	14	HD	NA	NA	NA	NA
208	Male	23	HD	NA	NA	NA	NA

155	Male	NA, adult	HD	NA	NA	NA	Father of 152, 153, 154
162	Male	NA, adult	HD	NA	NA	NA	Father of 161
HD01	Male	NA, adult	HD	NA	NA	NA	NA
27 healthy donors Stanford blood bank	Female	NA, adult	HD	NA	NA	NA	NA
5 healthy donors Stanford blood bank	NA	NA, adult	HD	NA	NA	NA	NA
37 healthy donors Stanford blood bank	Male	NA, adult	HD	NA	NA	NA	NA

Table S2. List of antibodies.

Antigen/ reagent	Clone/details	Conjugate, dilution, and catalog number	Company
CD3	OKT3, UCHT1	PE - dilution 1:50, catalog num. 317308; BB700 - dilution 1:50, catalog num. 566818; PerCP-Cy5.5 - dilution 1:50, catalog num. 317336; BUV 496 - dilution 1:50, catalog num. BDB612940	BioLegend, BD Biosciences
CD4	RPA-T4	a700 - dilution 1:25 or 1:50, catalog num. 557922; APC-Cy7 - dilution 1:50, catalog num. 300518; BUV395 - dilution 1:50, catalog num. BDB564724	BioLegend, BD Biosciences
CD14	63D3	PB - dilution 1:50, catalog num. 367122	BioLegend
CD127	A019D5	PE/Cy7 - dilution 1:50, catalog num. 351320; BV421- dilution 1:20, catalog num. 351310; A700 - dilution 1:25, catalog num. 351344	BioLegend
CD25	2A3	APC - dilution 1:25, catalog num. BDB340939; FITC - dilution 1:25 or 1:20, catalog num. BDB347643; BV605 - 1:50, catalog num. BDB562660; B650 - dilution 1:25, catalog num. BDB740634	BD Biosciences
FOXP3+C7	259D, 150D	a488 - dilution 1:20 or 1:25, catalog num. 320212; PE - dilution 1:25 or 1:20, catalog num. 320008; a647 - dilution 1:25 and 1:20, catalog num. 320014 and 320214	BioLegend
NGFR	C40-1457	PE-CF594 - dilution 1:50, catalog num. BDB563452	BD Biosciences
HLA-A2	BB7.2	APC - dilution 1:50, catalog num. 343308	BioLegend
CTLA-4	BNI3, L3D10	BV421 - dilution 1:25, catalog number 369605; APC - dilution 1:20, catalog num. 349908; BV711 - dilution 1:20, catalog num. 369632	BioLegend
Live/Dead Fixable Aqua	Cell Stain Kit	Dilution 1:50, catalog num. L34966	Invitrogen
Hoechst 33258	Staining Dye Solution	Dilution 1:10000, catalog num ab228550	Abcam
LIVE/DEA D Fixable Violet	Cell Stain Kit	Dilution 100x and 50x, catalog num. L34955	Invitrogen

FcR blocking reagent	human	Dilution 1:20 and 1:25, catalog num. 130-059-901	Miltenyi Biotec Inc
CD39	MZ18-23C8	PE - dilution 1:20, catalog num. 130-118-540	Miltenyi Biotec Inc
CD45RA	HI100	BV510 - dilution 1:50, catalog num. 304142	BioLegend
Zombie Red Fixable Viability Kit	Cell Stain Kit	Dilution 1:25, catalog num 423110	BioLegend
TIGIT	MBSA43, VSTM3	PE-Cy7 - dilution 1:20, catalog num. 50-112-9029; BV785 - dilution 1:25, catalog num. 372736	eBioscience, BioLegend
HELIOS	22F6	PE - dilution 1:20, catalog num. 137216; PE/Dazzle 594 - dilution 1:20, catalog num. 137232	BioLegend
IL-13	JES10-5A2	PE-Cy7 - dilution 1:20, catalog num. 501914	BioLegend
TNF- α	MAB11	a488 - dilution 1:20, catalog num. 502915	BioLegend
IL-17A	BL168	BV785 - dilution 1:20, catalog num. 512338	BioLegend
GM-CSF	BVD2-21C11	PE - dilution 1:20, catalog num. 502306	BioLegend
anti-human CD25	BC96	TotalSeq-C0085 - dilution 1:100 (0.5ug per 100ul), catalog num. 302649	BioLegend
anti-human CD45RA	HI100	TotalSeq-C0063 - dilution 1:200 (0.25ug per 100ul), catalog num. 304163	BioLegend
anti-human CD127	A019D5	TotalSeq-C0390 - dilution 1:100 (0.5 ug per 100ul), catalog num. 351356	BioLegend
anti-human Hashtag 2	LNH-94; 2M2	TotalSeq-C0252 - dilution 1:50 (1ug per 100ul) catalog num. 394663	BioLegend
anti-human Hashtag 1	LNH-94; 2M2	TotalSeq-C0251 - dilution 1:50 (1ug per 100ul), catalog num. 394661	BioLegend

Data file S1. List of genes from differential gene expression analysis between ex vivo T_{reg} memory 2 and T_{reg} memory 1 populations.

Data file S2. List of genes from differential gene expression analysis between IPEXs *FOXP3*⁺CD25^{high}CD127^{low} and *FOXP3*⁺CD25^{low}CD127^{low/high} cells.

Data file S3. List of genes from differential gene expression analysis between TCR-stimulated T_{reg} memory 2 and T_{reg} memory 1 populations.

Data file S4. Raw, individual-level data for experiments.