OMTM, Volume *32*

Supplemental information

High-affinity chimeric antigen receptor signaling induces an inflammatory program in human regulatory T cells

Russell W. Cochrane, Rob A. Robino, Bryan Granger, Eva Allen, Silvia Vaena, Martin J. Romeo, Aguirre A. de Cubas, Stefano Berto, and Leonardo M.R. Ferreira

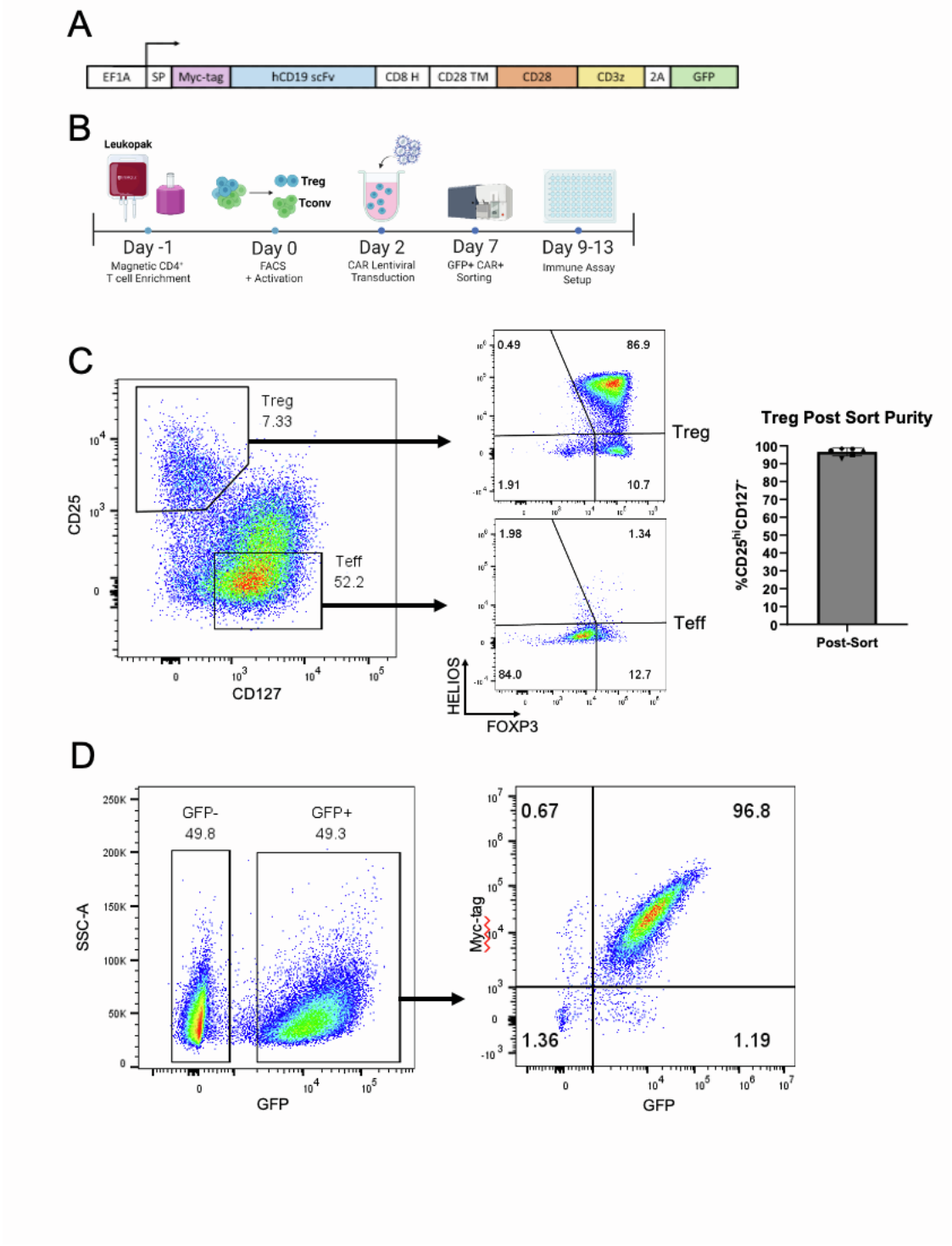

A
EF1A
SP
Myc-tag
hCD19 scFv
CD8 H
CD28 TM
CD28
CD3z
2A
GFP
B
Leukopak
Treg
Tconv
Day -1
Magnetic CD4+
T cell Enrichment
Day 0
FACS
+ Activation
Day 2
CAR Lentiviral
Transduction
Day 7
GFP+ CAR+
Sorting
Day 9-13
Immune Assay
Setup
C
Treg
7.33
Teff
52.2
CD25
CD127
0.49
86.9
1.91
10.7
Treg
1.98
1.34
Teff
84.0
12.7
HELIOS
FOXP3
Treg Post Sort Purity
%CD25hiCD127-
Post-Sort
D
GFP-
49.8
GFP+
49.3
SSC-A
GFP
0.67
96.8
1.36
1.19
Myc-tag
GFP

Figure S1. Human CAR Treg generation. (A) Chimeric antigen receptor (CAR) construct used in this study. (B) Workflow to isolate human CD4+ regulatory T cells (Tregs) and effector T cells (Teff), introduce a CAR, expand, and sort CAR-expressing cells for immune assays. (C) Representative dot plots of Treg sorting strategy with $CD25^{hi}CD127^{low}$ Tregs and $CD25^{low}CD127^{hi}$ Teff on the left and Treg post sort phenotype assessment with $FOXP3^{+}HELIOS^{+}$ Tregs and $FOXP3^{-}HELIOS^{-}$ Teff cells on the right. Post-sort purity of $CD25^{hi}CD127^{low}$ Tregs across multiple donors (n=6). (D) Representative dot plots of Treg transduction efficiency with CD19CAR-2A-GFP lentivirus, based on GFP expression on the left and CAR surface expression (Myc-tag) and reporter gene expression (GFP) after sorting GFP+ cells on the right.

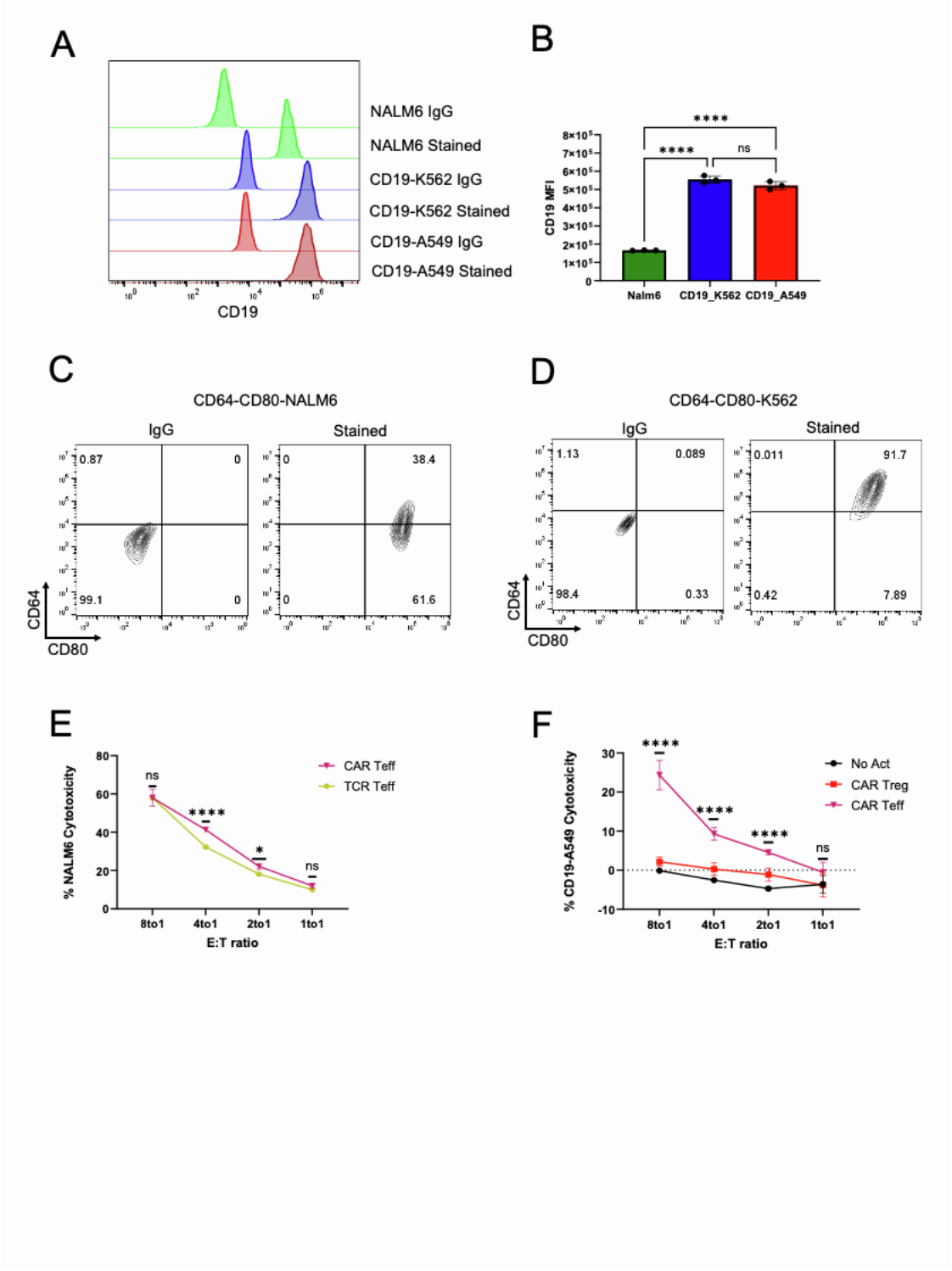

A
NALM6 IgG
NALM6 Stained
CD19-K562 IgG
CD19-K562 Stained
CD19-A549 IgG
CD19-A549 Stained
CD19
B
CD19 MFI
****
****
ns
Nalm6
CD19_K562
CD19_A549
C
CD64-CD80-NALM6
IgG
Stained
0.87
0
99.1
0
0
38.4
0
61.6
CD64
CD80
D
CD64-CD80-K562
IgG
Stained
1.13
0.089
98.4
0.33
0.011
91.7
0.42
7.89
CD64
CD80
E
% NALM6 Cytotoxicity
CAR Teff
TCR Teff
ns
****
*
ns
8to1
4to1
2to1
1to1
E:T ratio
F
% CD19-A549 Cytotoxicity
No Act
CAR Treg
CAR Teff
****
****
****
ns
8to1
4to1
2to1
1to1
E:T ratio

Figure S2. Characterization of target cell lines and cytotoxicity towards them. (A) Histograms of CD19 surface expression on CD19-K562, NALM6, and CD19-A549 target cells. (B) Mean fluorescence intensity (MFI) of CD19 surface expression on CD19-K562, NALM6, and CD19-A549 target cells. (C) Contour plot of CD80 and CD64 surface expression on CD64-CD80-NALM6 cells. (D) Contour plot of CD80 and CD64 surface expression on CD64-CD80-K562 cells. (E) Effector T (Teff) cell cytotoxicity towards NALM6 cells at different effector to target (E:T) ratios. (F) Treg and Teff cytotoxicity towards CD19-A549 cells at different E:T ratios. Values represent technical replicates of representative experiment. Bars represent mean ± standard deviation (SD). To determine statistical significance, one way ANOVA with Tukey's multiple comparison correction was used in Figure S2B, and two-way ANOVA test with Tukey's multiple comparison correction was used in Figure S2E and S2F. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

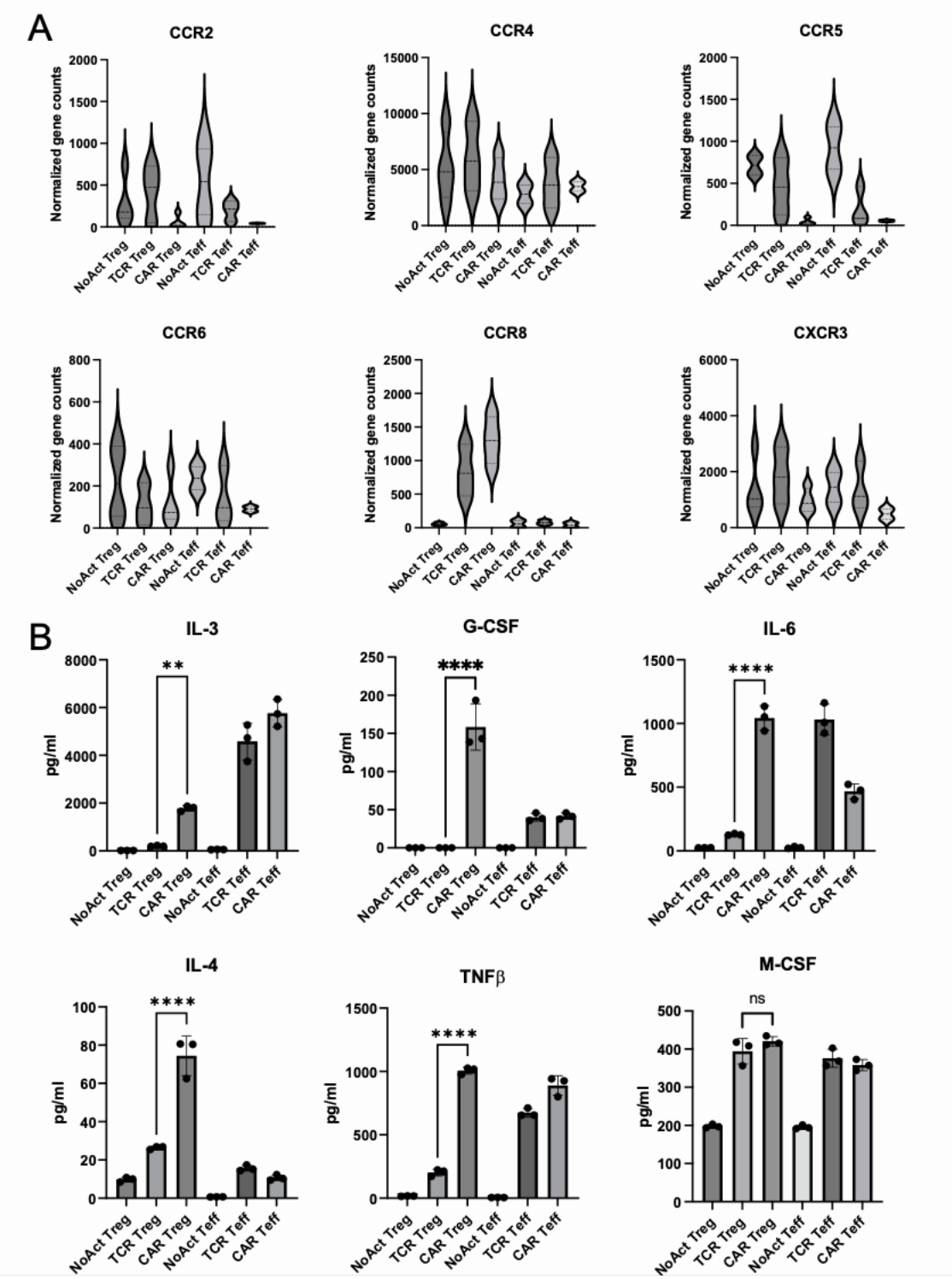
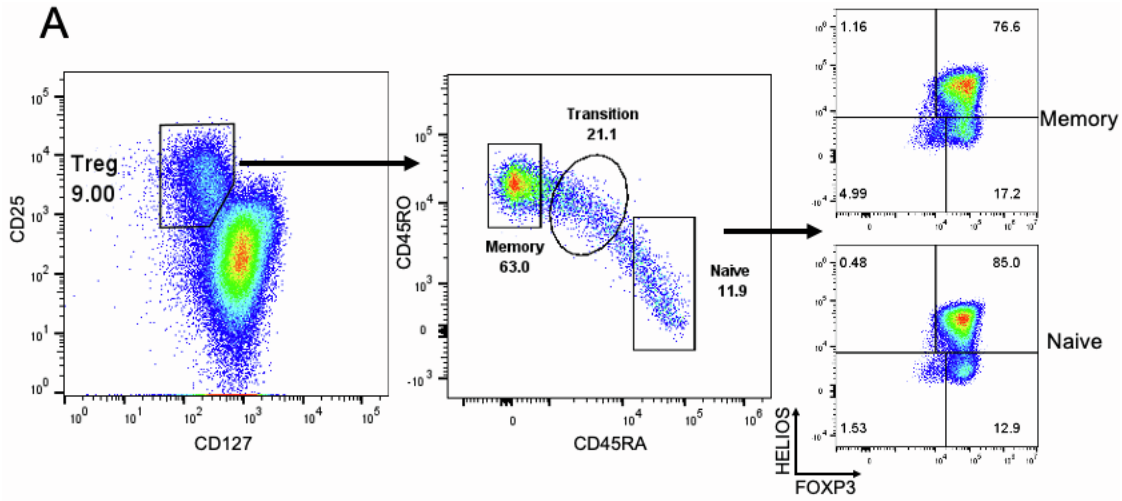
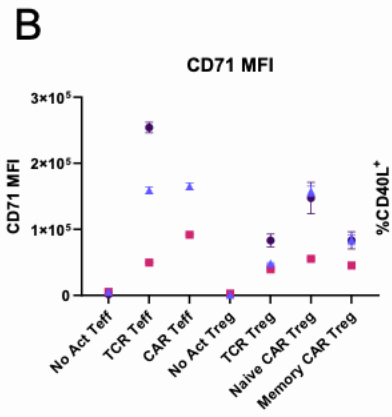
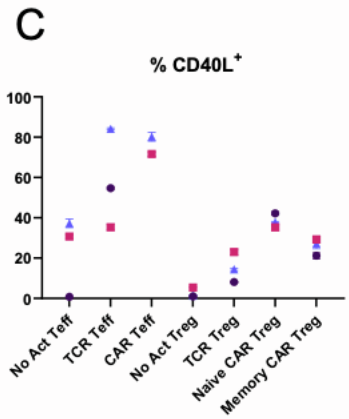
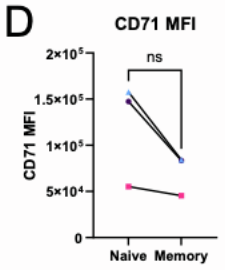

A
CCR2
CCR4
CCR5
CCR6
CCR8
CXCR3
Normalized gene counts
NoAct Treg
TCR Treg
CAR Treg
NoAct Teff
TCR Teff
CAR Teff
B
IL-3
G-CSF
IL-6
IL-4
TNFβ
M-CSF
pg/ml
**
****
ns

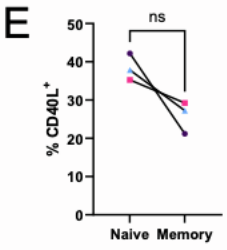
Figure S3. Chemokine and Cytokine Expression of Tregs and Teff cells. (A) Chemokine receptor gene expression levels in No Act Tregs, TCR Tregs, CAR Tregs, No Act Teff, TCR Teff, and CAR Teff 24 hours post activation. Violins represent mean ± standard deviation (SD) of RNA-seq gene counts across blood donors. (B) Cytokine secretion levels by No Act Tregs, TCR Tregs, CAR Tregs, No Act Teff, TCR Teff, and CAR Teff 48 hours post activation. Values represent technical replicates of representative experiment. Bars represent mean ± SD. One-way ANOVA test with Tukey's multiple comparison correction was used to assess statistical significance. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

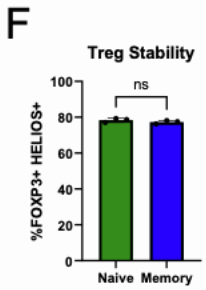

A
Treg
9.00
CD25
CD127
Transition
21.1
Memory
63.0
Naive
11.9
CD45RO
CD45RA
1.16
76.6
Memory
4.99
17.2
0.48
85.0
Naive
1.53
12.9
HELIOS
FOXP3

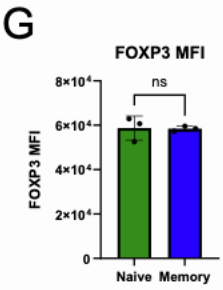

B
CD71 MFI
CD71 MFI
No Act Teff
TCR Teff
CAR Teff
No Act Treg
TCR Treg
Naive CAR Treg
Memory CAR Treg

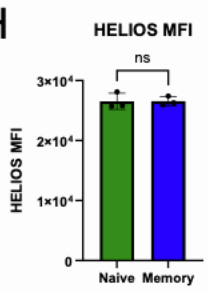

C
% CD40L+
%CD40L+
No Act Teff
TCR Teff
CAR Teff
No Act Treg
TCR Treg
Naive CAR Treg
Memory CAR Treg


D
CD71 MFI
ns
CD71 MFI
Naive
Memory


E
ns
% CD40L+
Naive
Memory


F
Treg Stability
ns
%FOXP3+ HELIOS+
Naive
Memory


G
FOXP3 MFI
ns
FOXP3 MFI
Naive
Memory


H
HELIOS MFI
ns
HELIOS MFI
Naive
Memory

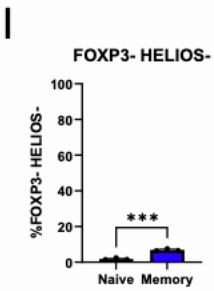

I
FOXP3- HELIOS-
***
%FOXP3- HELIOS-
Naive
Memory

Figure S4. CAR activation in naïve or memory Tregs results in phenotypically similar Tregs. (A) Representative dot plots of sorting strategy to obtain $CD25^{hi}CD127^{low}$ Tregs of either $CD45RO^{+}CD45RA^{-}$ (memory), $CD45RO^{+}CD45RA^{+}$ (transition), and $CD45RO^{+}CD45RA^{-}$ (naïve) phenotype on the left. Naïve and memory Treg post sort phenotype (FOXP3 and HELIOS expression) assessment on the right. (B) Summary data across donors (n=3) of CD71 surface expression 48h after Treg and effector T (Teff) cell activation. (C) Summary data across donors (n=3) of CD40L surface expression 48h after Treg and Teff cell activation. (D) CD71 surface expression (mean fluorescence intensity – MFI) 48h after CAR naïve and memory Treg activation. (E) CD40L surface expression ($\%CD40L^{+}$ cells) 48h after CAR naïve and memory Treg activation. (F) Percentage of $FOXP3^{+}HELIOS^{+}$ cells in CAR naïve and memory Tregs eight days post CAR activation.(G) FOXP3 MFI in CAR naïve and memory Tregs eight days post CAR activation (H) HELIOS MFI in CAR naïve and memory Tregs eight days post CAR activation. (I) Percentage of $FOXP3^{-}HELIOS^{-}$ cells in CAR naïve and memory Tregs eight days post CAR activation. For Figure S4B and S4C, values represent mean ± standard deviation (SD) of technical triplicates per blood donor (n=3). For Figure S4D and S4E, values represent mean of technical triplicates per blood donor (n=3), with lines collecting the data points from the same donor. Statistical significance was assessed using paired Student's t test. For Figure S4F, S4G, S4H, and S4I, values represent technical replicates of representative experiment. Bars represent mean ± SD. Statistical significance was assessed using unpaired Student's t test. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

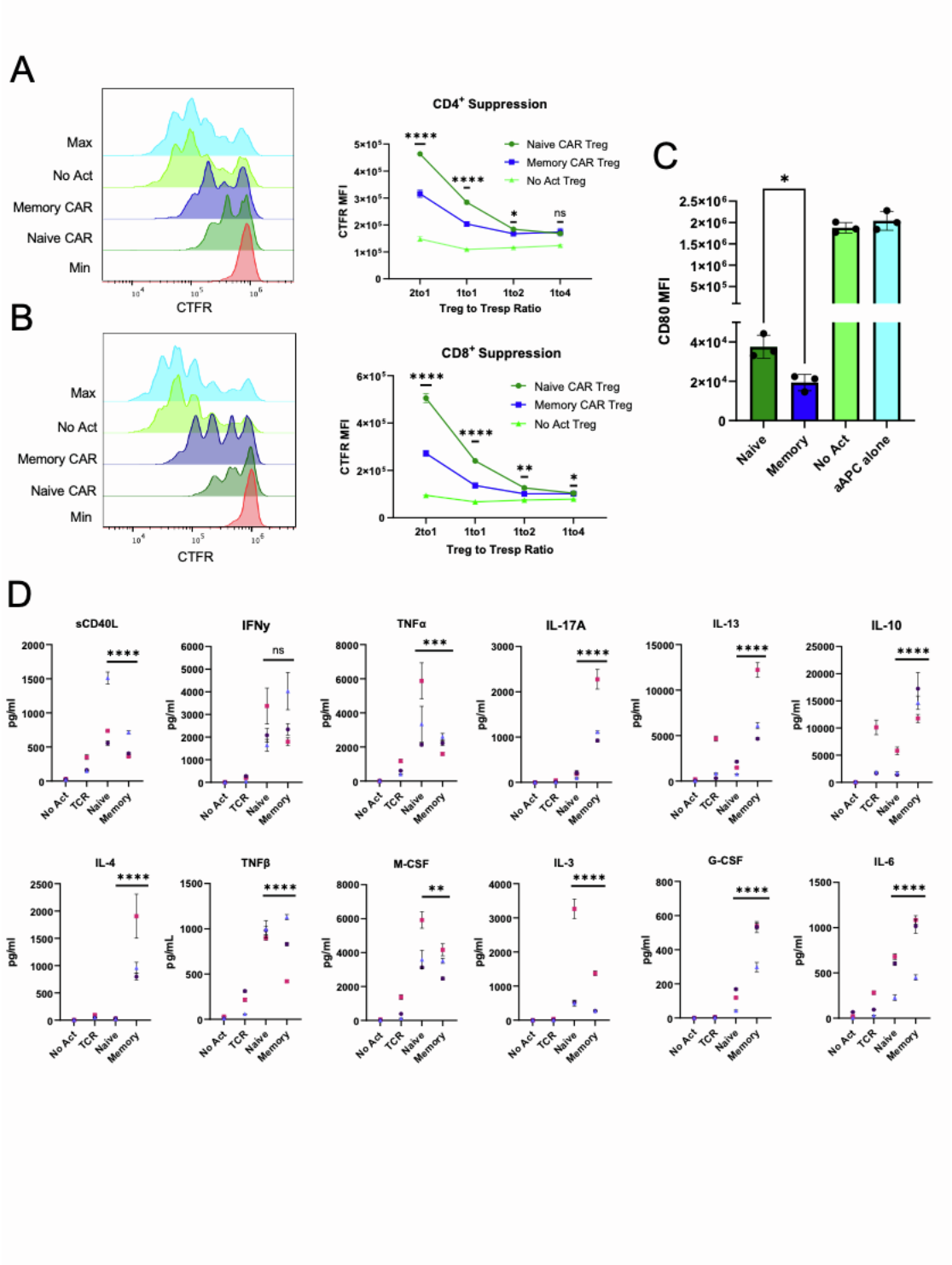

A
Max
No Act
Memory CAR
Naive CAR
Min
CTFR
CD4+ Suppression
Naive CAR Treg
Memory CAR Treg
No Act Treg
CTFR MFI
Treg to Tresp Ratio
2to1
1to1
1to2
1to4
B
CD8+ Suppression
C
CD80 MFI
Naive
Memory
No Act
aAPC alone
D
sCD40L
IFNγ
TNFα
IL-17A
IL-13
IL-10
IL-4
TNFβ
M-CSF
IL-3
G-CSF
IL-6
pg/ml
No Act
TCR
Naive
Memory

Figure S5. CAR activation leads to the same levels of IFNγ secretion in naïve and memory Tregs. (A) On the left, representative flow cytometry histograms of proliferation of Cell Trace Far Red (CTFR)-labeled $CD4^+$ T responder (Tresp) cells co-cultured with activated CAR Tregs at a 2: 1 Treg to Tresp ratio. On the right, suppression of proliferation of CTFR-labeled $CD4^+$ Tresp cells by Tregs at various Treg to Tresp ratios (2:1, 1:1, 1:2, and 1:4). (B) On the left, representative flow cytometry histogram of proliferation of CTFR-labeled $CD8^+$ Tresp cells co-cultured with activated CAR Tregs at a 2:1 Treg to Tresp ratio. On the right, suppression of proliferation of CTFR-labeled $CD8^+$ Tresp cells by Tregs at various Treg to Tresp ratios (2:1, 1:1, 1:2, and 1:4). Values represent mean ± standard deviation (SD) of technical replicates of representative experiment, with statistical significance computed by one-way ANOVA with Tukey's multiple comparison correction. (C) Representative histograms of downregulation of CD80 surface expression in CD80-CD64-NALM6 cells (aAPC – artificial antigen presenting cells) by Tregs. Bars represent mean ± SD of technical replicates of representative experiment, with statistical significance assessed by unpaired Student's t test. (D) Levels of cytokines secreted by No Act Tregs, TCR Tregs, CAR naïve Tregs, and CAR memory Tregs 48h post-activation. Values represent mean ± SD of technical triplicates per blood donor (n=3), with statistical significance assessed by one-way ANOVA test with Tukey's multiple comparison correction. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

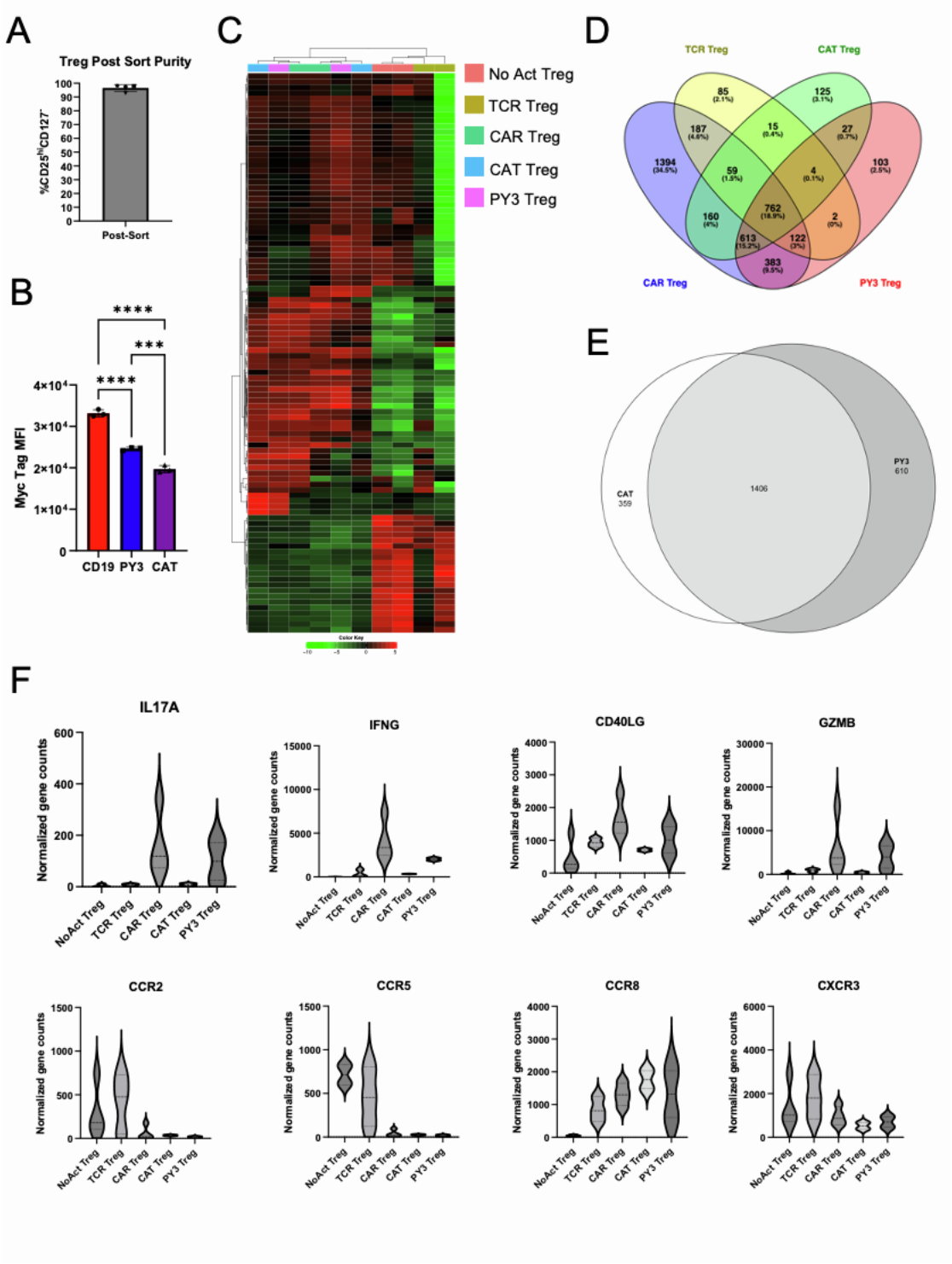

A
Treg Post Sort Purity
%CD25hiCD127-
Post-Sort
B
Myc Tag MFI
CD19 PY3 CAT
C
No Act Treg
TCR Treg
CAR Treg
CAT Treg
PY3 Treg
D
TCR Treg
CAT Treg
CAR Treg
PY3 Treg
E
CAT
359
1406
PY3
610
F
IL17A
IFNG
CD40LG
GZMB
CCR2
CCR5
CCR8
CXCR3
Normalized gene counts
NoAct Treg
TCR Treg
CAR Treg
CAT Treg
PY3 Treg

Figure S6. CAT Tregs have lower inflammatory gene expression levels than CAR Tregs. (A) Post-sort purity of CD25hiCD127low Tregs across multiple donors (n=4). (B) Surface expression (Myc-tag) of FMC63 CD19 CAR (CAR), CAT-13.1E10 CD19 CAR (CAT), and mutated CD28 signaling domain FMC63 CD19 CAR (PY3). Bars represent mean +/- SD of technical replicates of representative experiment, with statistical significance computed by one-way ANOVA with Tukey's multiple comparison correction. (C) Heatmap clustered by column (sample) and by row (gene) with top 100 most differentially expressed genes between No Act Tregs, TCR Tregs, CAR Tregs, CAT Tregs, and PY3 Tregs. (D) Venn diagram with genes upregulated in TCR Tregs, CAR Tregs, CAT Tregs, and PY3 Tregs in relation to their respective No Act cell types. Number of genes and respective percentage of the total number of genes are indicated in each intersection. (E) Venn diagram with genes upregulated in CAT Tregs and in PY3 Tregs. (F) Inflammatory, cytotoxic, and chemokine receptor gene expression levels in No Act Tregs, TCR Tregs, CAR Tregs, CAT Tregs, and PY3 Tregs. Violins represent mean ± standard deviation (SD) of RNA-seq gene counts from different blood donors.

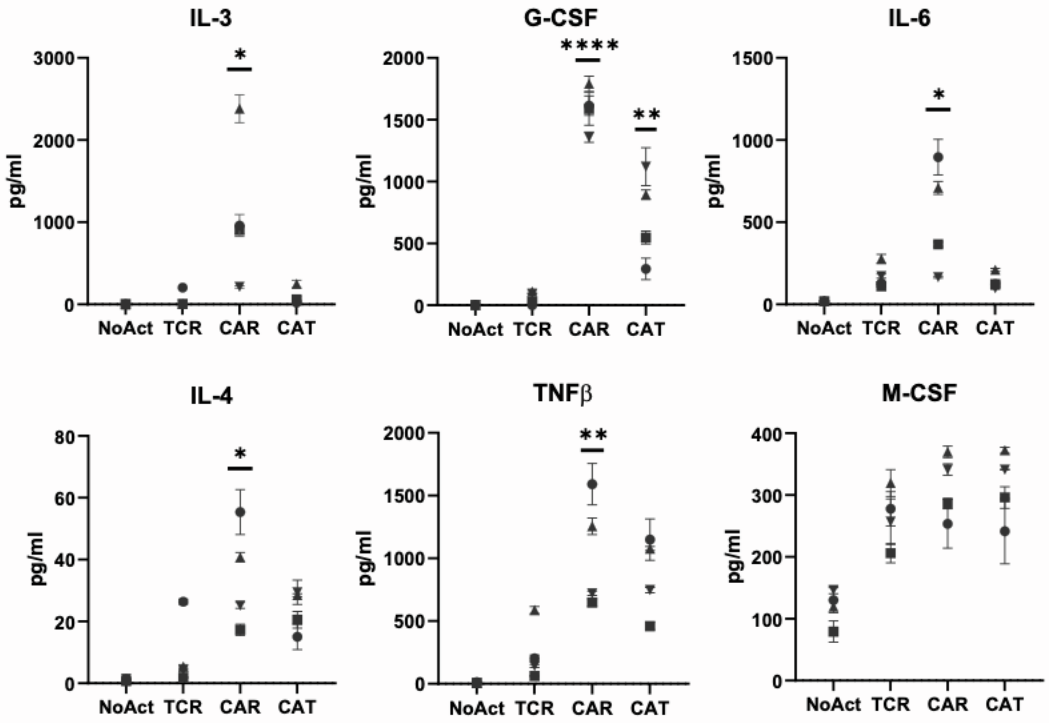

IL-3
G-CSF
IL-6
IL-4
TNFβ
M-CSF
pg/ml
NoAct
TCR
CAR
CAT
*
****
**

Figure S7. Lowering CAR affinity leads to lower cytokine secretion. Cytokine secretion levels by No Act Tregs, TCR Tregs, CAR Tregs, and CAT Tregs 48 hours post activation. Values are the mean ± standard deviation (SD) of technical triplicates per blood donor (n=4), with statistical significance assessed by one-way ANOVA with Tukey's multiple comparison correction. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

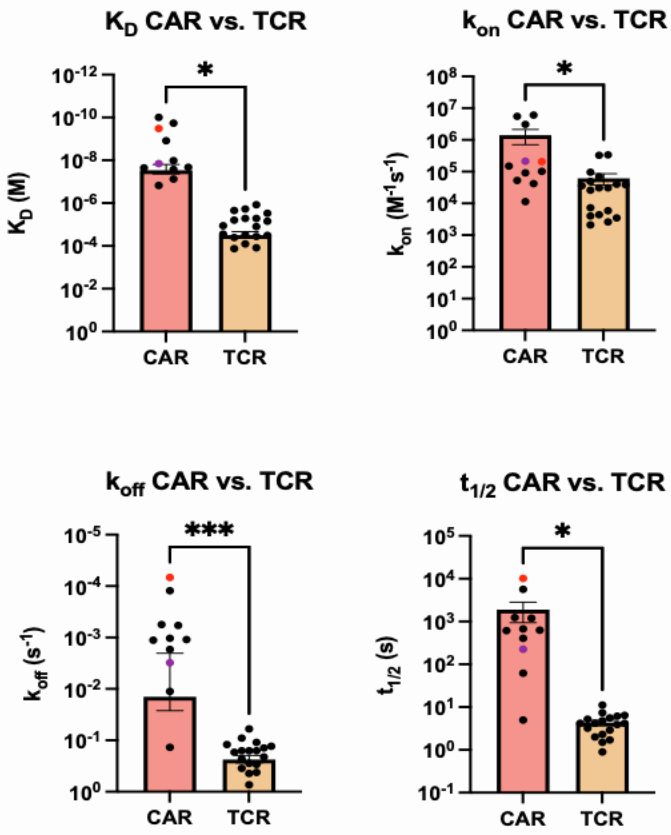

K_D CAR vs. TCR
k_{on} CAR vs. TCR
k_{off} CAR vs. TCR
$t_{1/2}$ CAR vs. TCR
K_D (M)
k_{on} ($M^{-1}s^{-1}$)
k_{off} (s^{-1})
$t_{1/2}$ (s)
CAR
TCR
*
***

Figure S8. Chimeric antigen receptors have a higher affinity to their cognate antigens than T cell receptors. Dissociation constant (K_d), association rate constant (k_{on}), dissociation rate constant (k_{off}), and residence time ($t_{1/2}$) values for commonly used chimeric antigen receptors (CARs) to their respective targets, and well-studied T cell receptors (TCRs) to their respective cognate peptide-MHC complexes from the literature. Red dots represent FMC63 CD19 CAR (CAR) and purple dots represent CAT-13.1E10 CD19 CAR (CAT). Bars represent mean ± standard error of the mean (SEM), with statistical significance assessed by unpaired Student's t test. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

SUPPLEMENTAL TABLES

Table S1. Differentially expressed genes in CAR Tregs compared with NoAct Tregs.

Table S2. Differentially expressed genes in TCR Tregs compared with NoAct Tregs.

Table S3. Differentially expressed genes in CAR Teff compared with NoAct Teff.

Table S4. Differentially expressed genes in TCR Teff compared with NoAct Teff.

Table S5. Differentially expressed genes in CAR Tregs compared with TCR Tregs.

Table S6. Genes upregulated in CAR Tregs, CAR Teff, and TCR Teff, but not in TCR Tregs.

Table S7. Genes upregulated only in TCR Tregs and not in CAR Tregs, CAR Teff or TCR Teff.

Table S8. Genes upregulated in PY3 Tregs and not in CAT Tregs.

Table S9. Flow cytometry antibodies and dyes used in this study.

Antigen	Clone	Fluorophore	Dilution	Vendor	Catalog #
CD4	SK3	FITC	1:100	BioLegend	980802
CD4	SK3	Pacific Blue	1:100	BioLegend	344619
CD4	SK3	Alexa Fluor 700	1:100	BioLegend	344621
CD4	SK3	PE-Cy7	1:100	BioLegend	344611
CD8	SK1	PE	1:100	BioLegend	344706
CD8	SK1	PerCP	1:100	BioLegend	344707
CD25	BC96	APC	1:100	BioLegend	302610
CD45RA	HI100	PE	1:100	BioLegend	304107
CD45RO	UCHL1	BV421	1:100	BioLegend	304223
CD71	CY1G4	PE	1:100	BioLegend	334105
CD80	2D10	APC	1:100	BioLegend	305220
CD83	HB15e	Brilliant Violet 421	1:100	BioLegend	305324
CD86	IT2.2	PE	1:100	BioLegend	305406
CD127	A019D5	PE	1:100	BioLegend	351304
CD127	A019D5	FITC	1:100	BioLegend	351311

CD154	24-31	APC	1:100	Biolegend	310809
CellTrace Violet	N/A	CellTrace Violet	1:1000	ThermoFisher	C34571
CellTrace Far Red	N/A	CellTrace Far Red	1:350	ThermoFisher	C34572
FOXP3	PCH101	eFluor 450	1:50	eBioscience	48-4776-42
FOXP3	PCH101	PE/Cy5.5	1:50	ThermoFisher	35-4776-42
Ghost	N/A	Red 780	1:500	Tonbo Biosciences	13-0865-T500
HELIOS	22F6	PE	1:50	BioLegend	137216
IFNG	4S.B3	BV510	1:50	Biolegend	502543
IL2	MQ1-17H12	Alexa Fluor 647	1:50	Biolegend	500315
Live-or-Dye	N/A	594/614	1:500	Biotium	32006
MYC-tag	9B11	Alexa Fluor 647	1:100	Cell Signaling Technologies	2233S
TIGIT	A15153G	Brilliant Violet 785	1:100	Biolegend	372735

Table S10. Primers used in this study.

Gene	Direction	Oligo sequence (5' to 3')
Human IFNG	FW	TCCCATGGGTTGTGTGTTTA
Human IFNG	REV	AAGCACCAGGCATGAAATCT
Human GZMB	FW	GGTGGCTTCCTGATACGAGACG
Human GZMB	REV	GGTCGGCTCCTGTTCTTTGAT
Human CD40LG	FW	GCGGCACATGTCATAAGTGAGG
Human CD40LG	REV	GTCCTTGTCTTTTAACGGTCAGC
Human TIGIT	FW	TGGTGGTCATCTGCACAGCAGT
Human TIGIT	REV	TTTCTCCTGAGGTCACCTTCCAC
Human TBX21	FW	ATTGCCGTGACTGCCTACCAGA
Human TBX21	REV	GGAATTGACAGTTGGGTCCAGG
Human GATA3	FW	ACCACAACCACACTCTGGAGGA
Human GATA3	REV	TCGGTTTCTGGTCTGGATGCCT
Human RORC	FW	GAGGAAGTGACTGGCTACCAGA
Human RORC	REV	GCACAATCTGGTCATTCTGGCAG
Human STAT1	FW	GGCAAAGAGTGATCAGAAACAA
Human STAT1	REV	GTTCAGTGACATTCAGCAACTC
Human RPL13a	FW	CATAGGAAGCTGGGAGCAAG
Human RPL13a	REV	GCCCTCCAATCAGTCTTCTG

Table S11. Sequences of constructs used in this study.