PNAS

Supporting Information for

TGF-β broadly modifies rather than specifically suppresses reactivated memory CD8 T cells in a dose-dependent manner.

Alexis Taber, Andrew Konecny, Shannon Oda, James Scott-Browne, and Martin Prlic

Corresponding author: Martin Prlic
Email: mprlic@fredhutch.org

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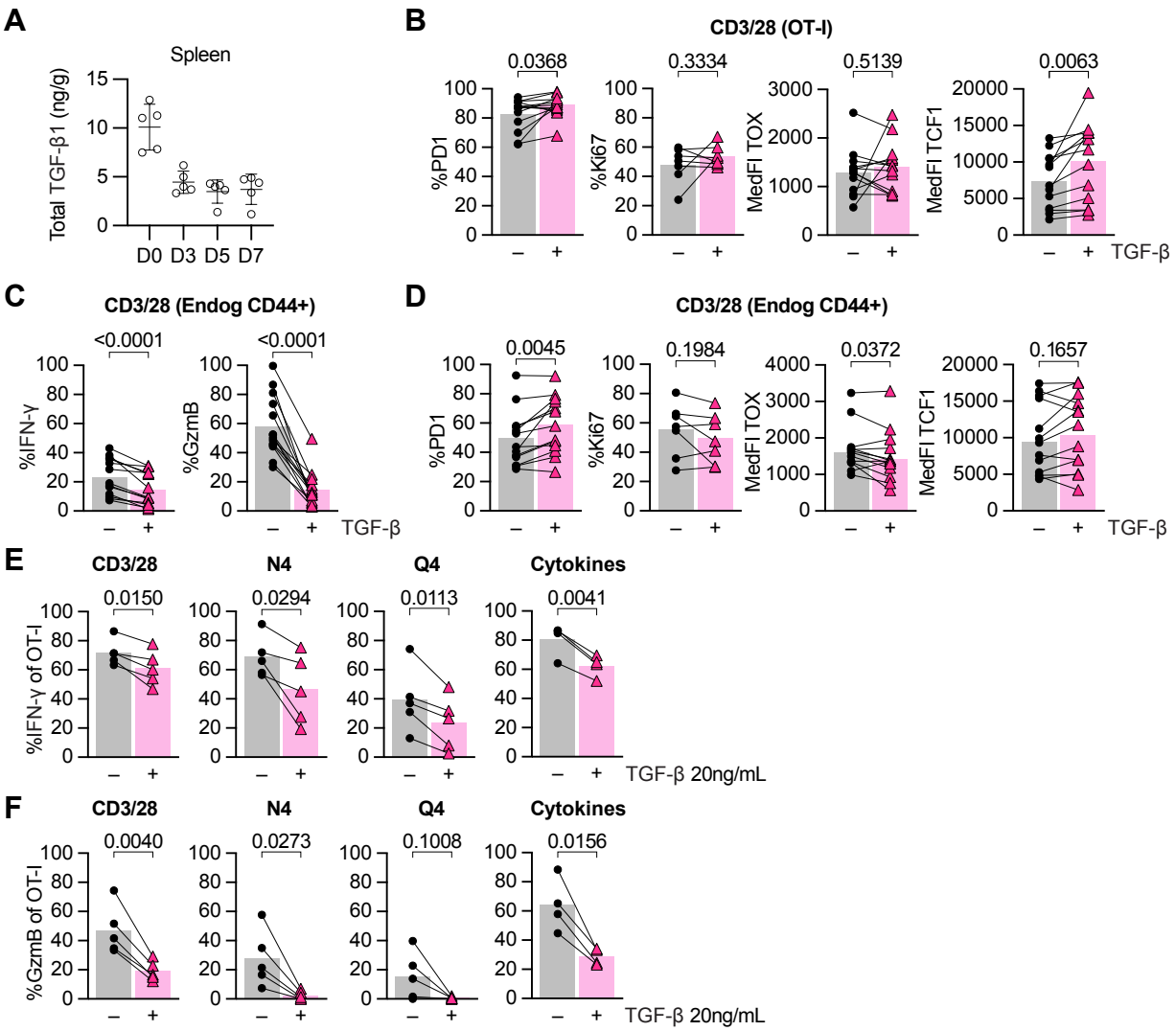

Figure S1: TGF-β increases PD-1 but not other markers of activation on memory CD8+ T cells

See Figure 1A for experimental setup. Enriched T cells were stimulated for 24 hours with CD3/28 in the presence or absence of TGF-β at 100ng/mL. **(A)** Total levels of extracellular TGF-β1 measured by ELISA from spleen of C67BL/6J mice at indicated infection timepoints (n = 5 animals). Infection was 4 × 10^3 CFU LM-OVA on Day 0. **(B)** Frequencies of PD1 and Ki67 and median fluorescence intensity (MedFI) of Tox and TCF1 in OT-1 T_{mem}. **(C)** Frequencies of IFN-γ, GzmB, **(D)** PD1, Ki67, and MedFI of Tox and TCF1 in endogenous CD44+ T cells. In **(B - D)** n = 13 animals for all markers except Ki67 n = 7. **(E)** Frequencies of IFN-γ and **(F)** GzmB of OT-I T_{mem} at 24h across indicated stimulation conditions with or without TGF-β at 20ng/mL. In **(E and F)** CD3/28, N4, and Q4 n = 5 animals, and Cyt n = 4. Statistical significances were calculated using paired t tests. Data shown are from 4 to 11 independent experiments.

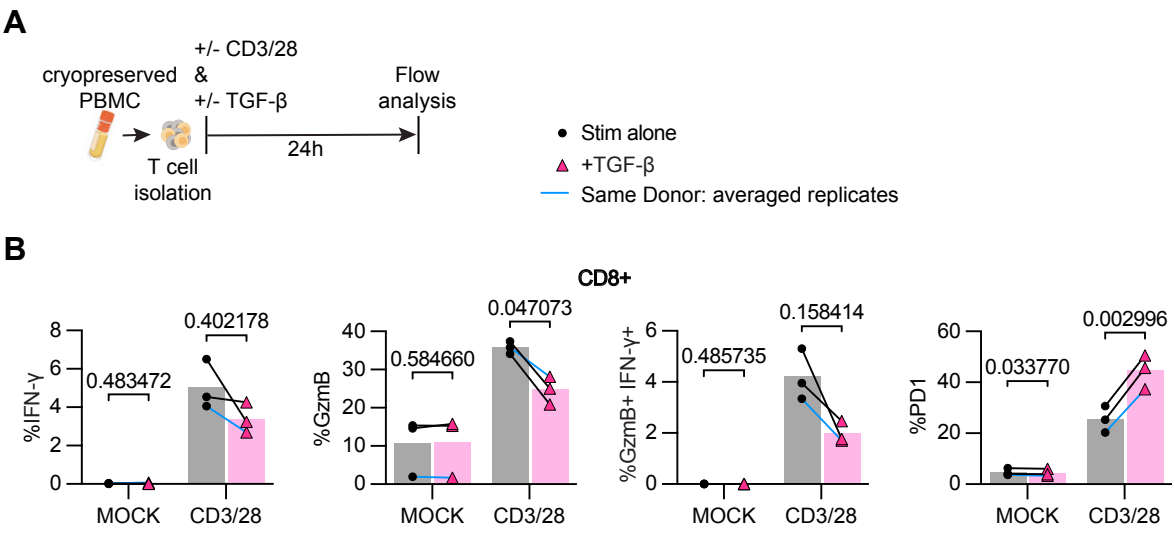

Figure S2: TGF-β inhibits cytotoxicity of CD8+ T cells from human PBMC
(A) Schematic of T cell isolation with magnet-activated cell sorting (MACS) from de-identified human PBMC and subsequent ex vivo stimulation and analysis. Human T cells were cultured in human RP10 media (RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, and 100 U/mL penicillin-streptomycin) with recombinant human TGF-β1 (PeproTech Cat #100-21), Dynabeads human T-Activator (Thermo Fisher) anti-CD3/CD28 beads (at a 1:1 bead/cell ratio), or with media alone. Stimulation was 24 hours with anti-CD3/CD28 microbeads in the presence or absence of TGF-β at 100ng/mL. **(B)** Frequencies of indicated markers in all CD8+ T cells (n = 3 donors). Experimental repeats belonging to the same donor are averaged and indicated by the blue line (4 replicates). Statistical significances were calculated using multiple paired t tests. Data shown are from 4 independent experiments.

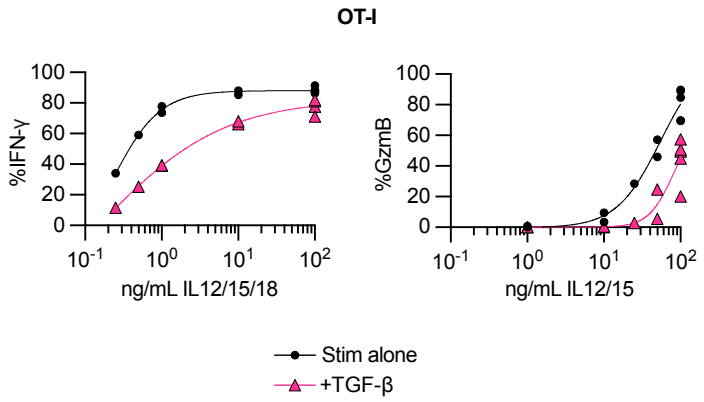

Figure S3: Pro-inflammatory cytokine abundance determines the susceptibility of OT-I T_{mem} to inhibition by TGF-β

See figure 1A for experimental setup. Stimulation was 24h with IL-12, IL-15, and IL-18 or IL-12 and IL-15 at the indicated (titrated) concentrations with or without TGF-β at 100ng/mL. Frequencies of IFN-γ and GzmB by OT-I T_{mem} (n = 3 animals). Data shown are from 3 independent experiments.

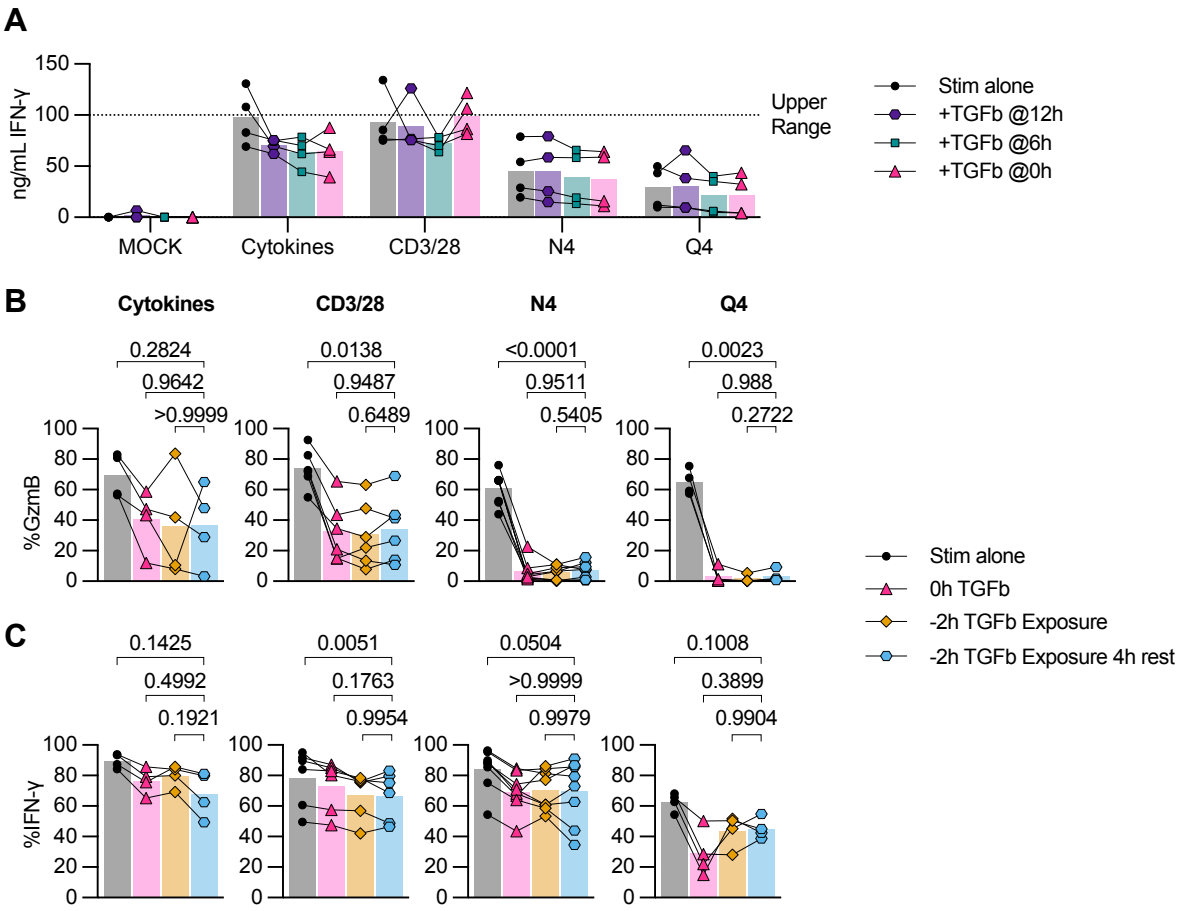

Figure S4: TGF-β exposed OT-I T_{mem} do not regain full cytotoxic function after short rest period

(A) IFN-γ levels measured by ELISA from supernatant of ex vivo experiments described in Figure 3A (n = 4 animals). **(B and C)** Stimulated OT-I T_{mem} from experiments outlined in Figure 3D including an additional condition of a 4-hour rest in fresh media between TGF-β exposure and activating stimulation. **(B)** Frequencies of GzmB and **(C)** IFN-γ in OT-I T_{mem} compared across stimulation conditions (CD3/28 n = 6 animals, N4 n = 8, Q4 and Cyt n = 4). All indicated statistical significances were calculated using one-way ANOVA. Data shown are from 3 to 7 independent experiments.

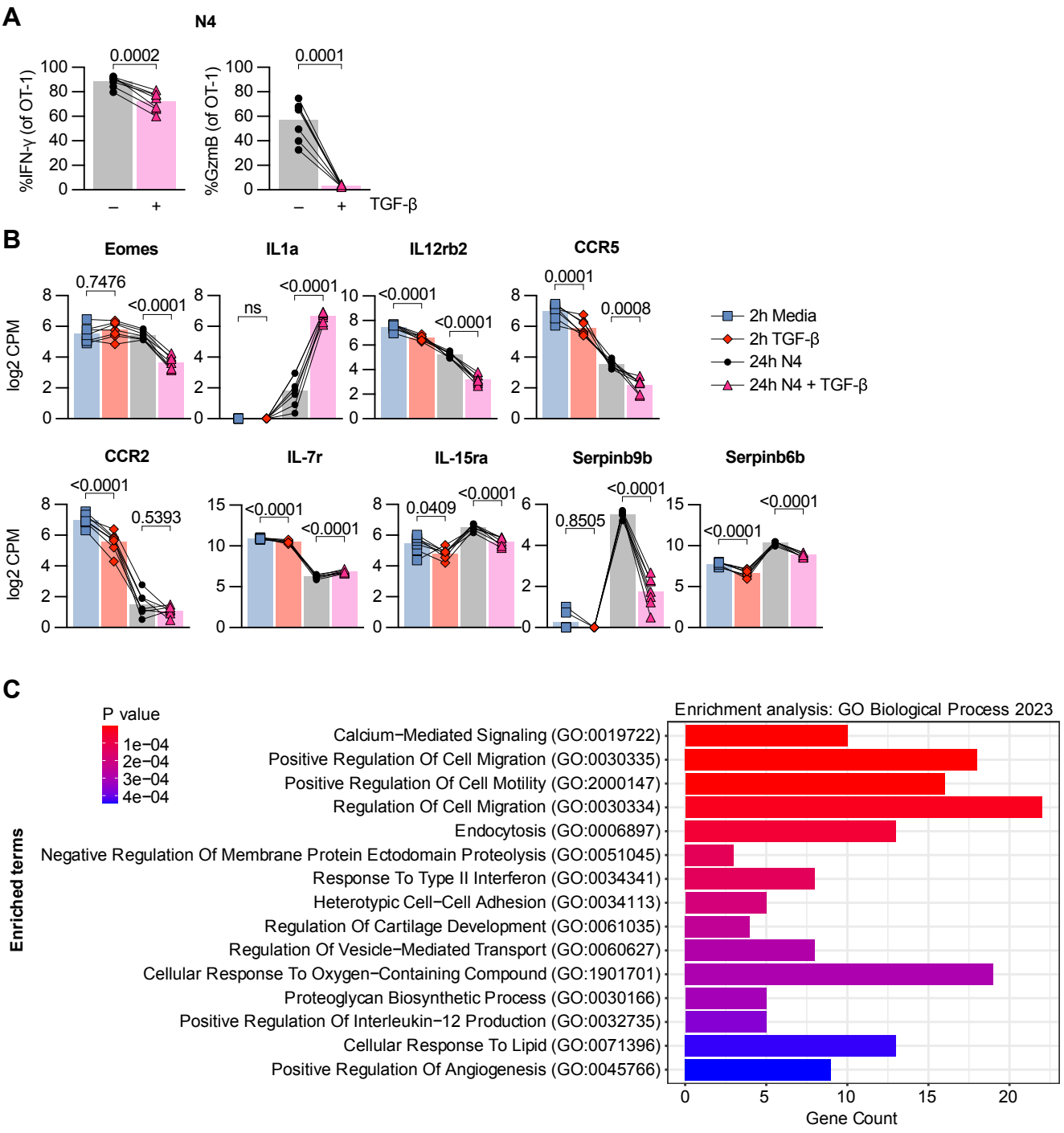

Figure S5: TGF-β induces transcriptional changes associated with cell migration in OT-I T_{mem}
(A) Frequencies of IFN-γ and GzmB in OT-I T_{mem} from mice used for ATAC- and RNA-seq after 24h N4 stimulation with or without TGF-β at 100ng/mL (n = 7 animals). Statistical significances were calculated using paired t tests. **(B)** Selected DE genes from RNA-seq and calculated adj. P values. **(C)** Enrichment analysis of RNAseq data from GO Biological Process 2023 database. Data shown are from 2 independent experiments.

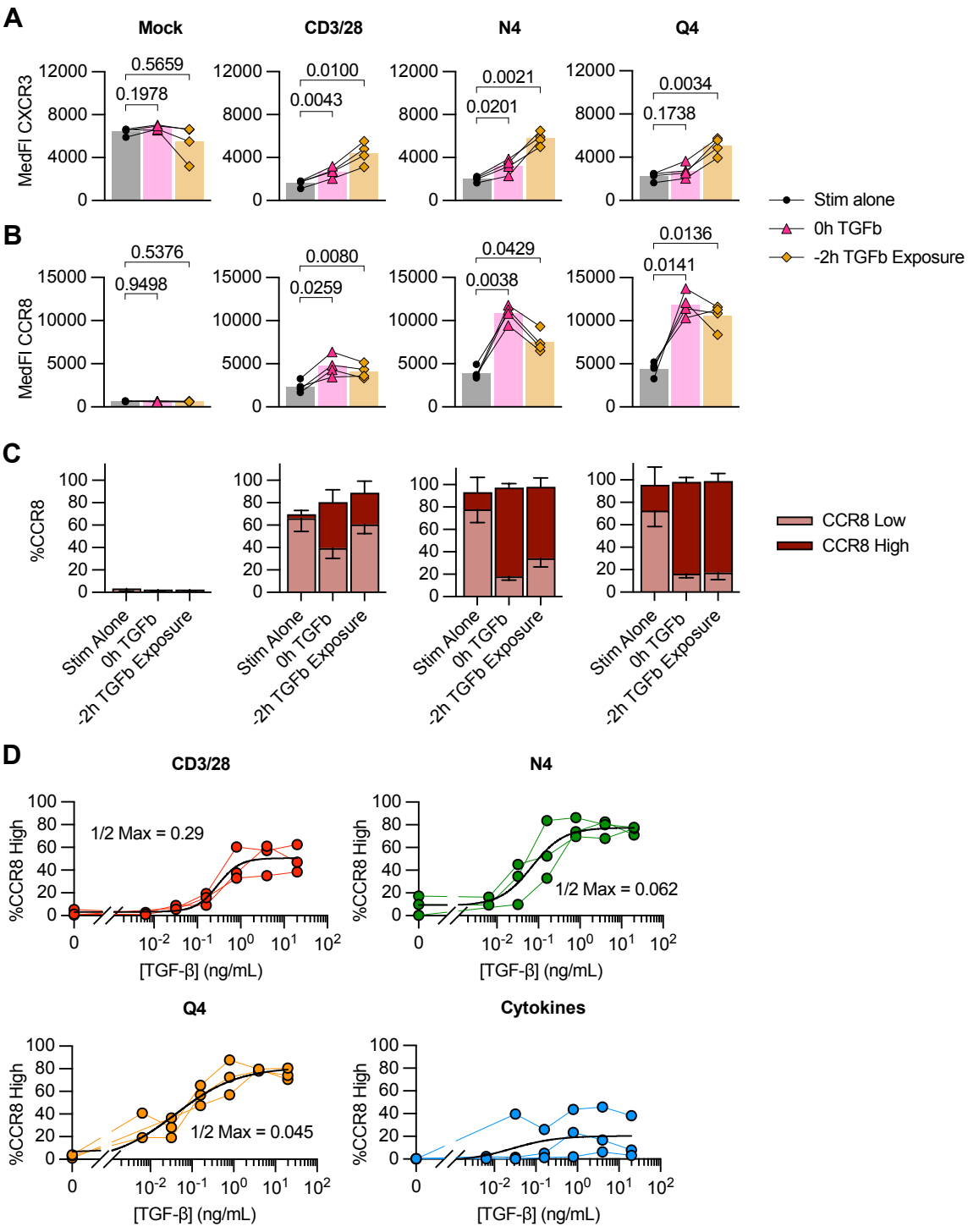

Figure S6: TGF-β induces chemokine receptor expression in OT-I T_{mem} in a dose-dependent manner

(A - C) See Figure 3D for experimental setup. **(A)** MedFI CXCR3 and **(B)** MedFI CCR8 in OT-1 T_{mem}. **(C)** Frequency of low and high CCR8 expression by flow cytometry in OT-I T_{mem} (n = 4 animals). **(D)** Frequency of CCR8-high expression by flow cytometry across stimulation conditions in the presence of titrated TGF-β (n = 3). TGF-β was titrated in five-fold dilutions starting with 20ng/mL and ending at 0.032ng/mL. Data shown are from 2 to 3 independent experiments.

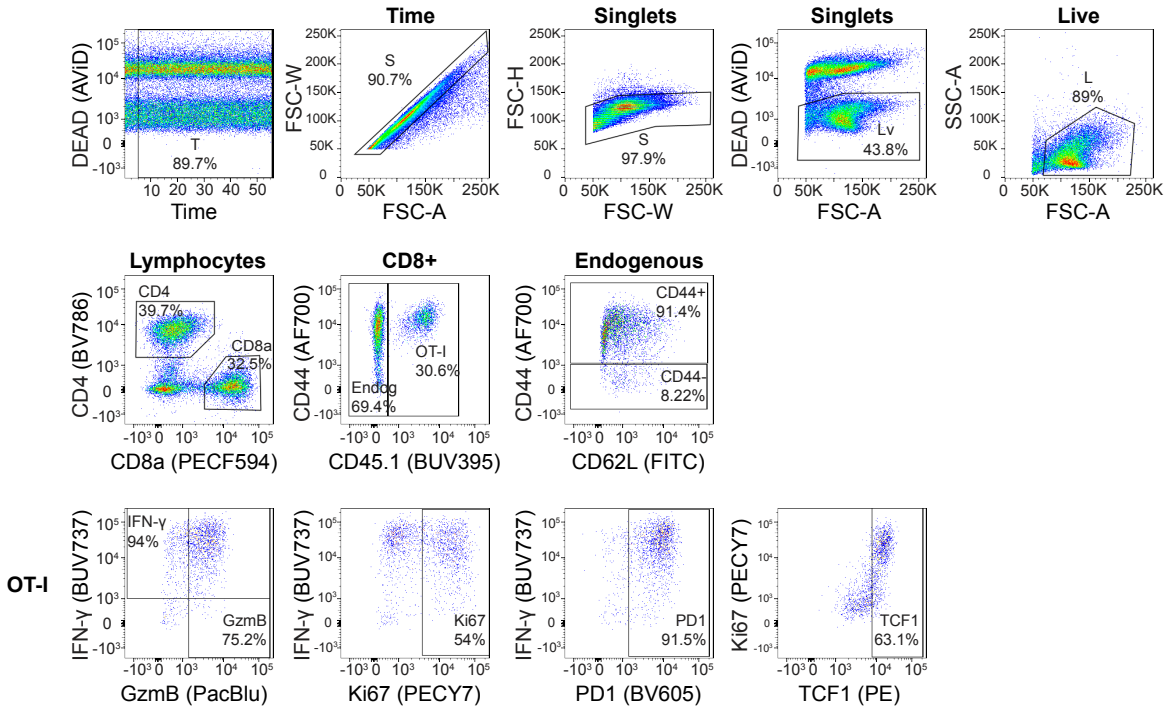

Figure S7: Representative flow gating for T cells isolated from OT-I memory mice
Related to Fig.1, 2, 3, Supplemental Fig. 1, 3, 4. Stimulation was 24h with plate-bound CD3/28.

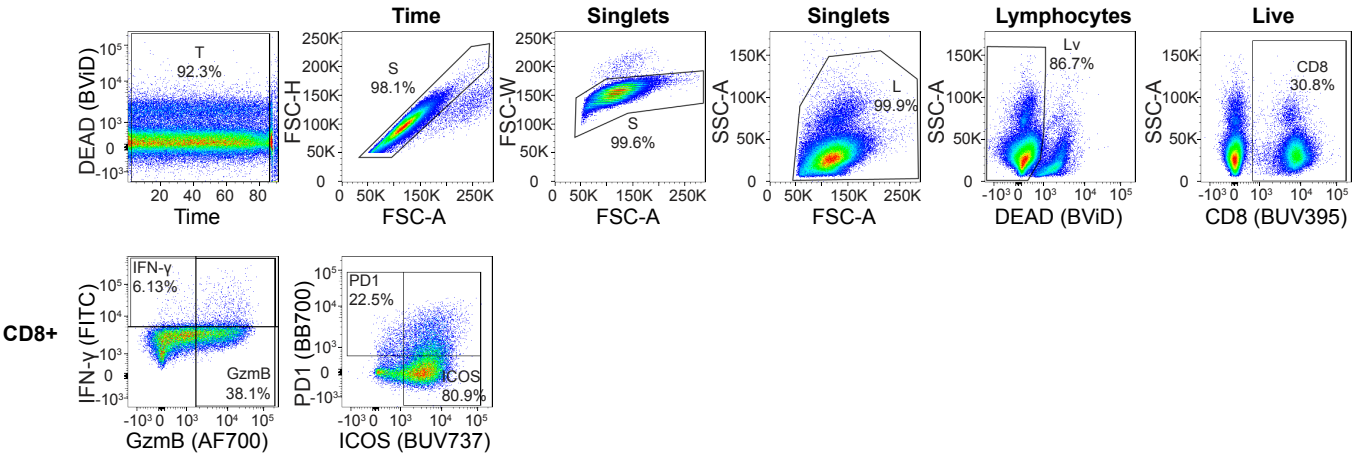

Figure S8: Representative flow gating for T cells isolated from human PBMC
Related to Supplemental Fig. 2. Stimulation was 24 hours with anti-CD3/CD28 microbeads.

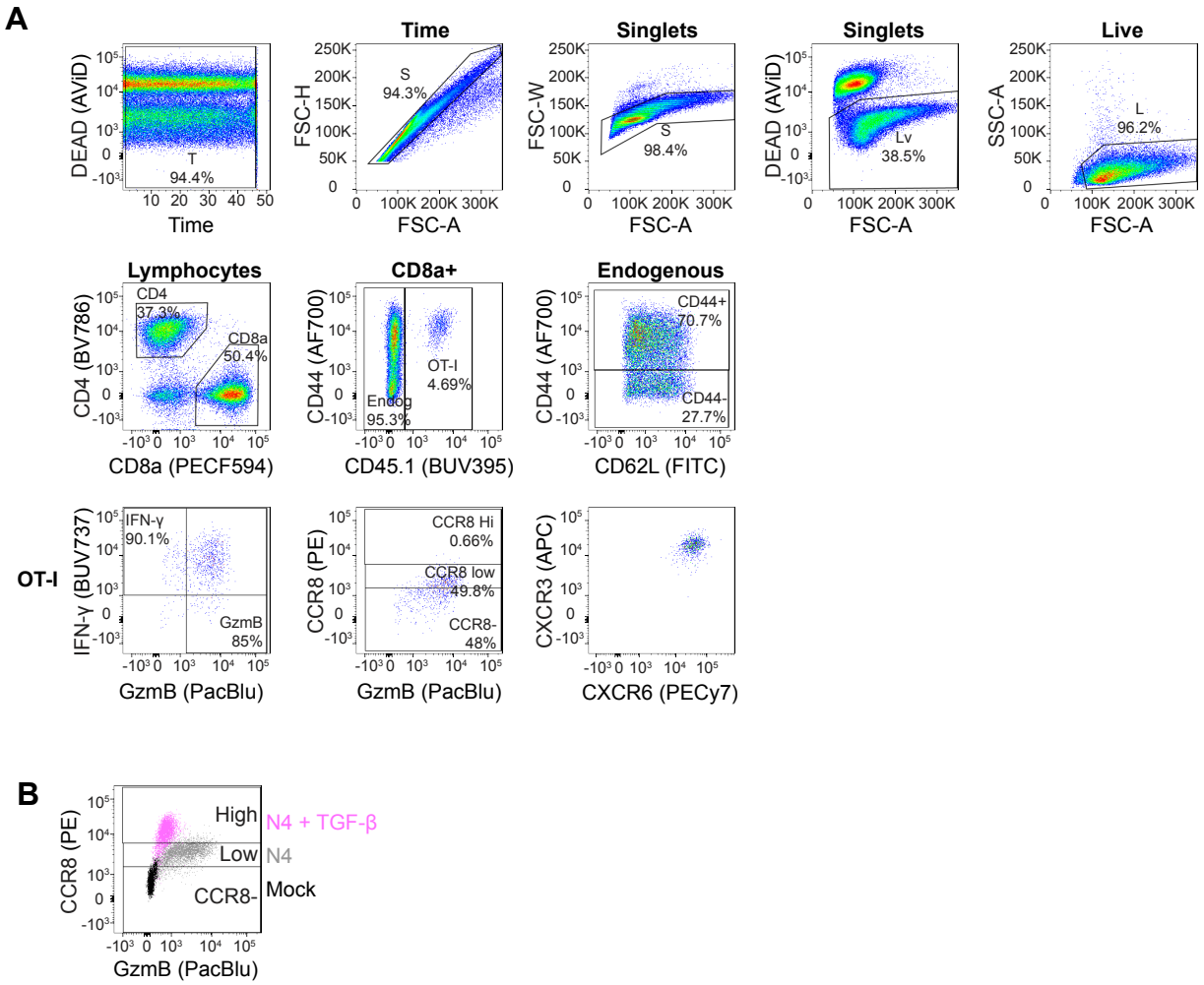

Figure S9: Representative flow gating for T cells isolated from OT-I memory mice
Related to Fig. 5, Supplemental Fig. 6. **(A)** Stimulation was 24h with plate-bound CD3/28. **(B)** Representative gating of CCR8 expression in OT-I T_{mem} across stimulation conditions: Media alone (Mock), N4, and N4 + TGF-β.

TGF-β Mouse Panel					
Reagent	Fluor	Dilution	Clone	Vendor	Cat.no
Viability stain (PBS diluent) 20 min, on ice					
LIVE/DEAD fixable aqua viability dye (AViD)	V510	1:250	NA	Thermo Fisher	Cat#L34966
Surface stain (FACS Wash diluent) 20 min, on ice					
FC block (CD16/32)	Purified	1:200	2.4G2	BD Biosciences	Cat# 553141
CD4	BV786	1:200	GK1.5	BD Biosciences	Cat#563331
CD8a	PECF594	1:300	53-6.7	BD Biosciences	Cat#562283
CD45.1	BUV395	1:200	A20	BD Biosciences	Cat#565212
CD44	AF700	1:200	IM7	Thermo FIsher	Cat#560567
PD-1	BV605	1:100	29F.1A12	Biolegend	Cat#135220
CD62L	FITC	1:200	MEL-14	eBioscience	Cat#11-0621-85
Fix (eBioscience FOXP3 fixation buffer) 20 min, on ice					
Intracellular stain (eBioscience FOXP3 perm buffer diluent) 30 min, on ice					
Ki-67	PECy7	1:800	SolA15	BioLegend	Cat#52426
TOX	APC	1:100	REA473	Miltenyi Biotec	Cat#130-118-335
IFN-γ	BUV737	1:200	XMG1.2	BD Biosciences	Cat#612769
GzmB	PacBlu	1:100	GB11	BioLegend	Cat#515408
TCF1/7	PE	1:40	C63D9	Cell Signaling	Cat#144565

Table S1: TGF-β mouse panel
Related to Fig.1, 2, 3, Supplemental Fig. 1, 3, 4. Mouse flow cytometry panel for T cells isolated from OT-1 memory mice.

TGF-β Human Panel					
Reagent	Fluor	Dilution	Clone	Vendor	Cat.no
Viability stain (PBS diluent) 20 min, room temp					
Human TruStain FcX (Fc-Block)	NA	1:25	NA	Biolegend	Cat#422302
LIVE/DEAD fixable blue viability dye (BViD)	UV450	1:500	NA	Thermo Fisher	Cat#L34962
Surface stain (Brilliant Stain Buffer diluted 10x in FACS Wash) 20 min, room temp					
CD8	BUV395	1:80	RPA-T8	BD Biosciences	Cat#563795
CXCR6	BUV563	1:20	13B 1 E5	BD Biosciences	Cat#748450
CCR7	BUV661	1:80	2-L1-A	BD Biosciences	Cat#749824
ICOS	BUV737	1:20	DX29	BD Biosciences	Cat#749665
CD25	BV421	1:40	2A3	BD Biosciences	Cat#564033
CD28	BV480	1:40	CD28.2	BD Biosciences	Cat#566110
CD45RA	BV570	1:160	HI100	BioLegend	Cat#304132
CD39	BV605	1:40	A1	BioLegend	Cat#328236
CD69	BV650	1:20	FN50	BD Biosciences	Cat#310933
CD103	BV750	1:160	Ber-ACT8	BD Biosciences	Cat#747099
CCR5	BV785	1:20	3A9	BD Biosciences	Cat#565001
PD1	BB700	1:20	EH12.1	BD Biosciences	Cat#566460
IL-1R1	PE	1:20	polyclonal	R&D Systems	Cat#FAB269P
CXCR3	PE-CF594	1:20	1C6/CXCR3	BD Biosciences	Cat#562451
CD137	PE-Cy5	1:20	4B4-1	BD Biosciences	Cat#551137
IL18R1	PE-Cy7	1:40	H44	BioLegend	Cat#313812
Fix (eBioscience FOXP3 fixation buffer) 20 min, room temp					
Intracellular stain (eBioscience FOXP3 perm buffer diluent) 30 min, room temp					
IFN-γ	FITC	1:40	B27	BD Biosciences	Cat#554700
GzmB	AF700	1:80	GB11	BD Biosciences	Cat#560213
CCR8	BV711	1:20	433H	BD Biosciences	Cat#747575

Table S2: TGF-β human panel
Related to Supplemental Fig. 2. Human flow cytometry panel for T cells isolated from human PBMC.

Chemokine Receptor Panel: Mouse					
Reagent	Fluor	Dilution	Clone	Vendor	Cat.no
Viability stain (PBS diluent) 20 min, on ice					
LIVE/DEAD fixable aqua viability dye (AViD)	V510	1:250	NA	Thermo Fisher	Cat#L34966
Surface stain (FACS Wash diluent) 20 min, on ice					
FC block (CD16/32)	Purified	1:200	2.4G2	BD Biosciences	Cat#553141
CD4	BV786	1:200	GK1.5	BD Biosciences	Cat#563331
CD8a	PECF594	1:300	53-6.7	BD Biosciences	Cat#562283
CD45.1	BUV395	1:200	A20	BD Biosciences	Cat#565212
CD44	AF700	1:200	IM7	BD Biosciences	Cat#560567
CD62L	FITC	1:200	MEL-14	eBioscience	Cat#11-0621-85
CXCR3	APC	1:100	CXCR3-173	BD Biosciences	Cat#562266
CXCR6	PECy7	1:200	SA051D1	BioLegend	Cat#151118
Fix (eBioscience FOXP3 fixation buffer) 20 min, on ice					
Intracellular stain (eBioscience FOXP3 perm buffer diluent) 30 min, on ice					
CCR8	PE	1:200	SA214G2	Biolegend	Cat#150311
IFN-γ	BUV737	1:200	XMG1.2	BD Biosciences	Cat#612769
GzmB	PacBlu	1:100	GB11	Biolegend	Cat#515408

Table S3: Chemokine receptor mouse panel
Related to Fig. 5, Supplemental Fig. 6. Mouse flow cytometry panel for T cells isolated from OT-1 memory mice.