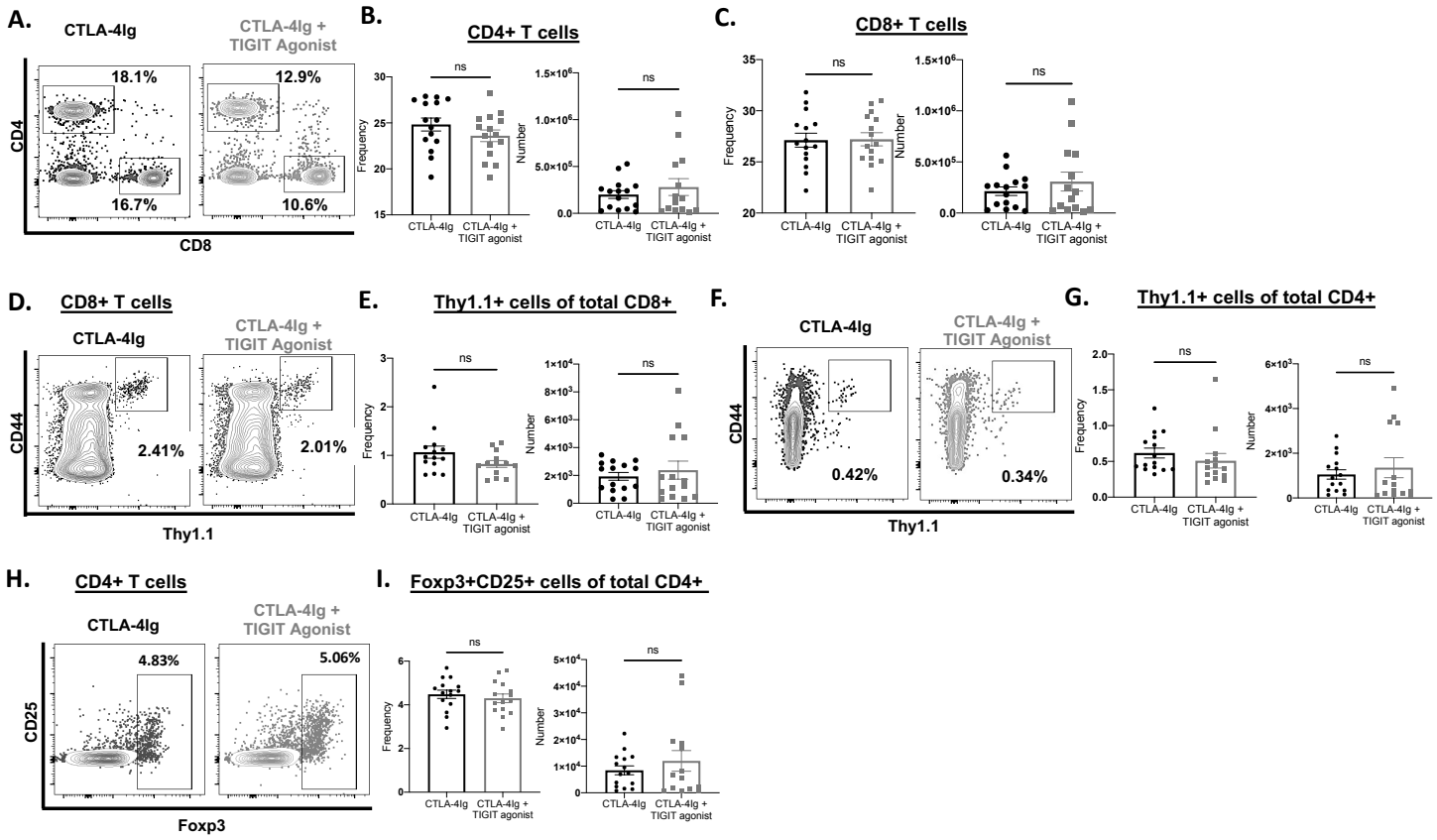
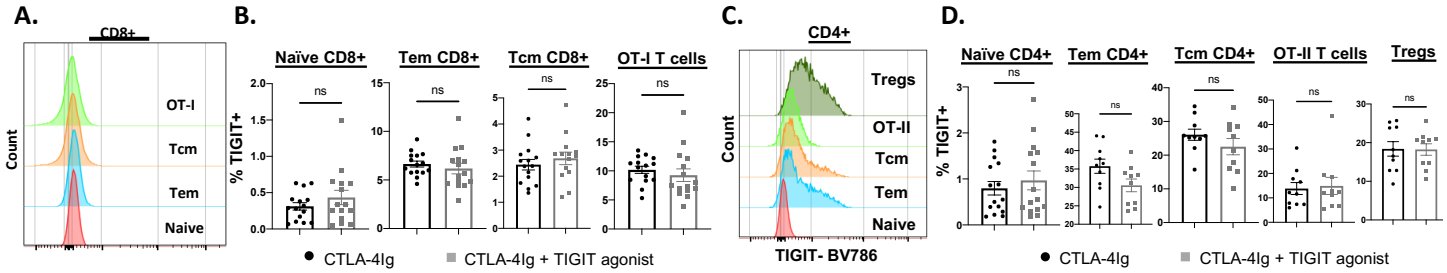
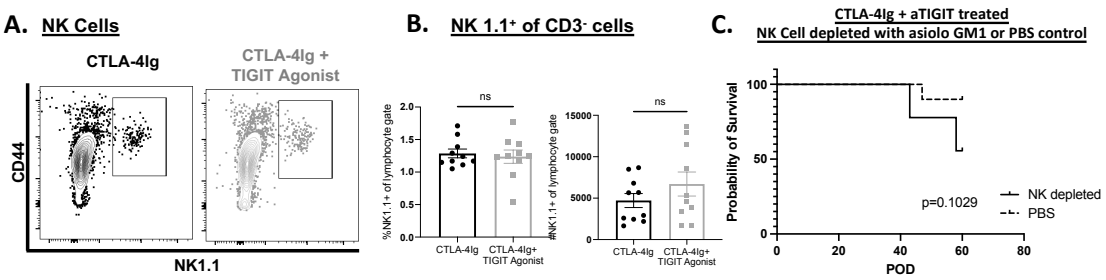
Supplemental Information



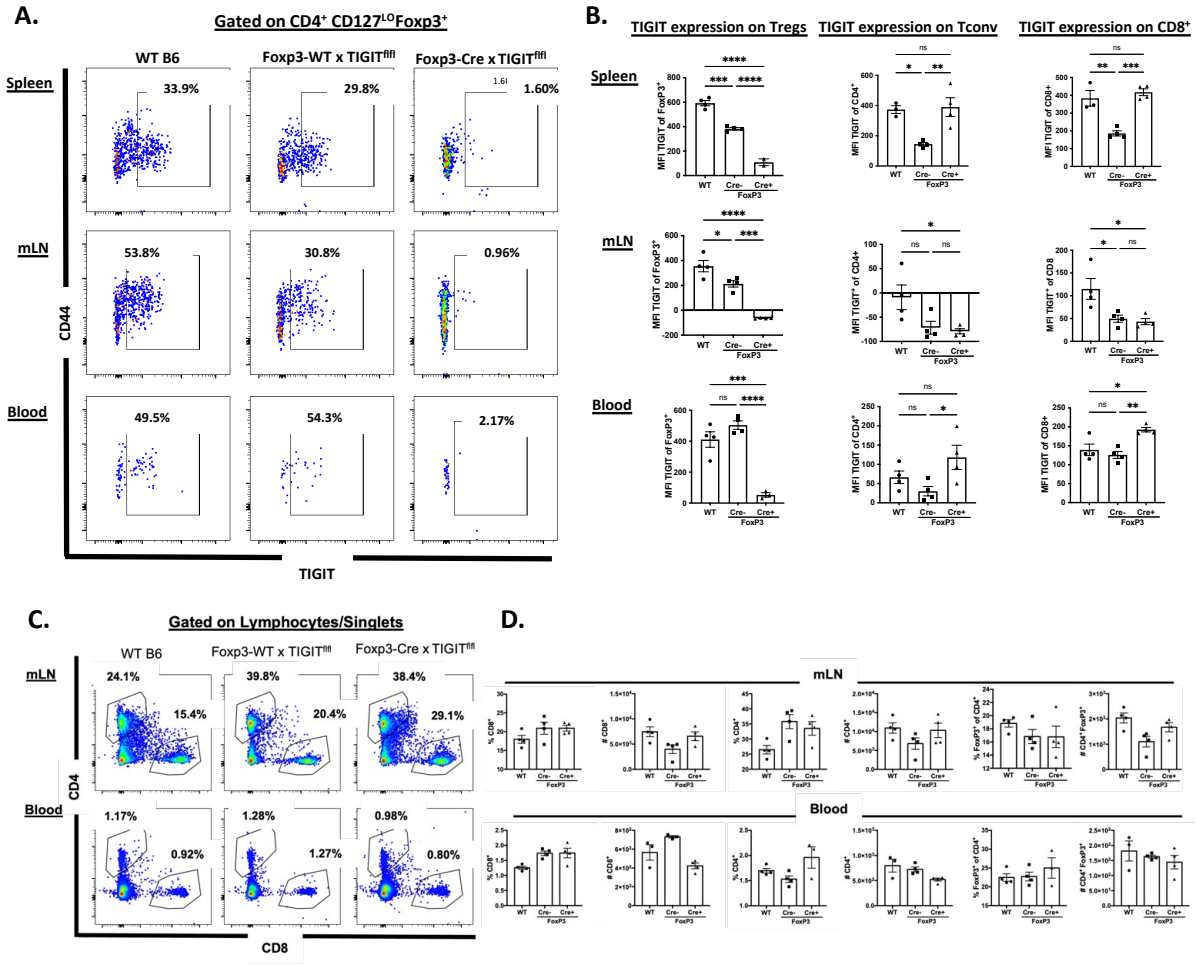
S1: Flow cytometric analysis of cells from draining lymph nodes 10 days after OVA graft challenge and treatment with CTLA-4Ig alone or CTLA-4Ig + TIGIT agonist. (A) Example flow and (B, C) summary data of the frequency and number of $CD4^+$ and $CD8^+$ T cells within the graft draining lymph nodes. Within the $CD8^+$T cell population graft-specific $Thy1.1^+$ T cells were measured (example flow D, summarized E) as well as the $CD4^+$ graft-specific $Thy1.1^+$ from the $CD4^+$ compartment (example flow F, summarized G). $Foxp3^+$ regulatory T cells were quantified within the $CD4^+$ T cell population (example flow H, summarized in I). Experiments are representative of at least 2 independent experiments, mean ± SEM is shown. Non-parametric Mann-Whitney T tests were performed between groups for B and D, one way ANOVA were performed in F, G, I (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).



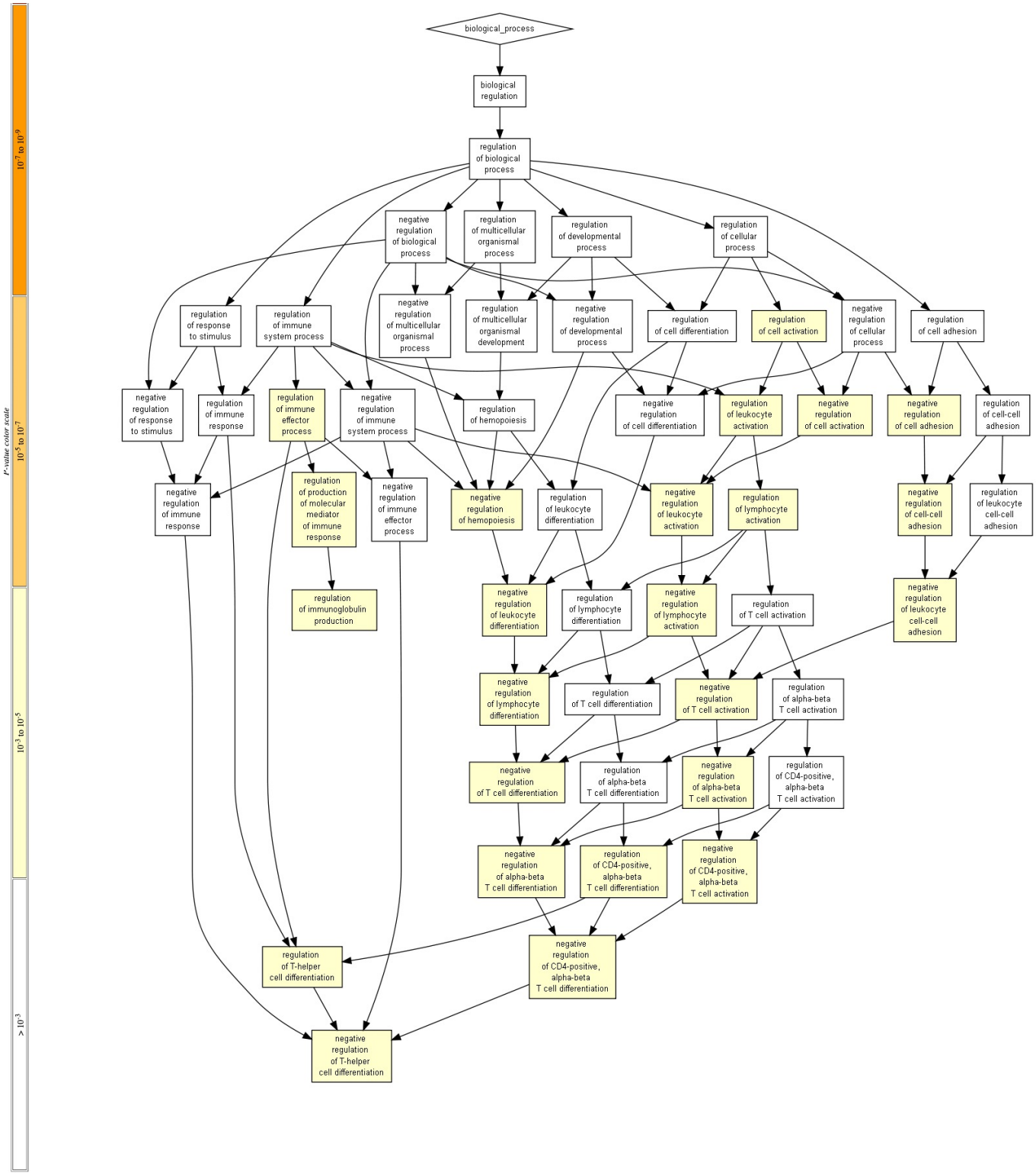
S2: (A-D) TIGIT expression on wild type CD8⁺ and CD4⁺ T cell subsets. CD8⁺ T cells from spleens 10 days after allograft challenge were gated on CD44 and CD62L to determine central memory, effector memory, and naïve cell subsets in addition to Thy1.1⁺ graft-specific cells and TIGIT expression on each subset was assessed (representative histograms A, summarized in B). CD4⁺ T cell subsets were also assessed (representative histograms C, summarized D). Experiments are representative of at least 2 independent experiments, mean ± SEM is shown. Non-parametric Mann-Whitney T tests were performed between groups for B and D, one way ANOVA were performed in F, G, I (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



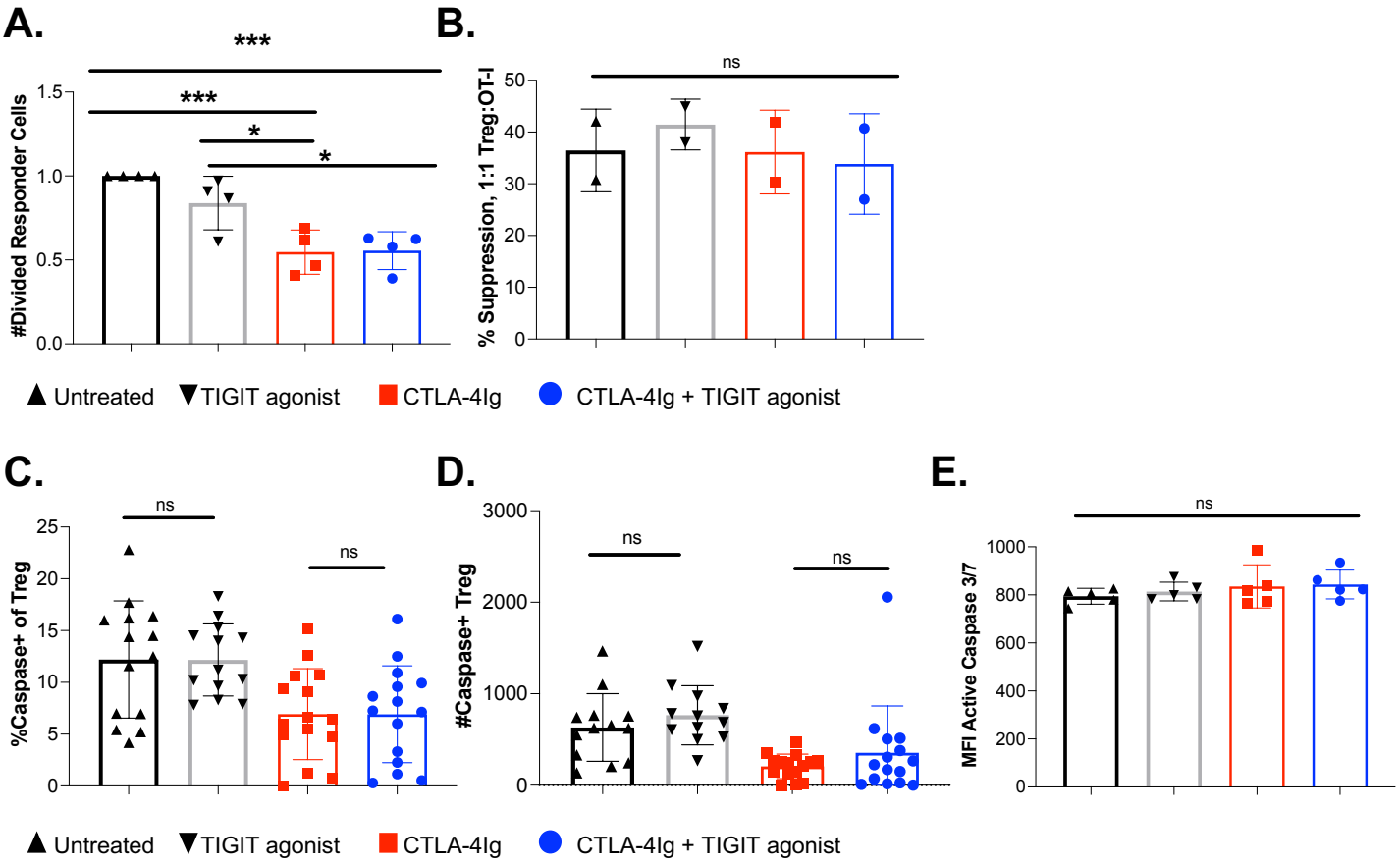
S3: NK cells were quantified in the draining lymph nodes (represented in A, summarized B). NK cells were depleted prior to OVA allograft challenge and treatment with CTLA-4Ig+ TIGIT agonist. Graft survival was assessed (*p=0.1029,* C). Experiments are representative of at least 2 independent experiments, mean ± SEM is shown. Non-parametric Mann-Whitney T tests were performed between groups for B and D, one way ANOVA were performed in F, G, I (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



S4: (A-B) Assessment of TIGIT expression on cells in WT versus Treg-TIGIT conditional knockout animals. Representative flow cytometry plots of TIGIT expression on Treg in WT B6 mice, Foxp3-WT xTIGIT$^{fl/fl}$ mice, and Foxp3-Cre x TIGIT$^{fl/fl}$ mice in mesenteric lymph nodes and peripheral blood (A). Comparison summary data of TIGIT expression (MFI) between WT B6 mice, Foxp3-WT x TIGIT$^{fl/fl}$ mice, and Foxp3-Cre x TIGIT$^{fl/fl}$ mice on Treg, Tconv, or CD8^{+} T cells from mesenteric lymph nodes and peripheral blood (B). (C-D) Quantification of lymphocyte populations in WT B6 mice, Foxp3-WT x TIGIT$^{fl/fl}$ mice, and Foxp3-Cre x TIGIT$^{fl/fl}$ mice in mesenteric lymph nodes and peripheral blood. Representative flow cytometry plot of CD4 and CD8 expression on lymphocytes from the spleen, mesenteric lymph nodes and peripheral blood of WT B6, Foxp3-WT x TIGIT$^{fl/fl}$ mice, and Foxp3-Cre x TIGIT$^{fl/fl}$ mice (C). The frequency and number of CD8^{+}, CD4^{+}, and Foxp3^{+} Treg for WT B6, Foxp3-WT x TIGIT$^{fl/fl}$ mice, and Foxp3-Cre x TIGIT$^{fl/fl}$ mice are summarized for mesenteric lymph nodes and blood (D). Experiments are representative of at least 2 independent experiments, mean ± SEM is shown. Non-parametric Mann-Whitney T tests were performed between groups for B and D, one way ANOVA were performed in F, G, I (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).



S5: Visualization of GO biological processes enriched in WT Treg compared to TIGIT cKO Treg. GO terms, enrichment, p values, and network visualization were generated in GORILLA. Included all terms with a p value $<10^{-4}$.



S6: CTV labeled OT-I T cells were stimulated with 10nM of SIINFEKL peptide and treated with CTLA-4Ig, TIGIT agonist, or CTLA-4Ig+TIGIT agonist and cultured for 3 days. The number of divided cells were quantified for each treatment condition and compared to untreated controls (A). Stimulated OT-I T cells incubated with the 4 treatments were co-cultured at a 1:1 ratio with Treg and the percent suppression was calculated for untreated, CTLA-4Ig, TIGIT agonist, and CTLA-4Ig+ TIGIT agonist treated cells (B). In the draining lymph nodes of WT C57/BL6 mice the frequency (C), number (D), and MFI (E) of caspase 3/7 activity in Treg was measured. One-way ANOVA analysis with multiple comparisons was performed (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Supplemental Table 1: Antibodies used for flow cytometry

Antibody	Clone	Fluorophore	Dilution	Purchased From
CD4	GK1.5	BUV395 APC	1:200	BioLegend
CD8	53-6.7	BUV737 BV786	1:200	BioLegend
CD45		BV421 Alexa700	1:200	BioLegend
GITR	DTA-1	BV605 BV711	1:100	BD
TIGIT	1G9	BV711 BV786	1:100	BioLegend
CD25	3C7	PE FITC	1:100	Biolegend
Foxp3	FJK-16s	APC PE	1:100	Thermo Fisher
Thy1.1	OX-7	PercP	1:200	BioLegend
CD44	IM7	BV605	1:300	BioLegend
NK1.1	PK136	APC-Cy7	1:200	BioLegend
CXCR3	CXCR3-173	BV711 FITC	1;200	Biolegend
DNAM	TX42.1	BV650	1:200	BioLegend
CD38	90	BV421	1:100	Biolegend

CCR4	2G12	PE	1:100	BioLegend
CCR5	J418F1	PE-Cy7	1:100	BioLegend
CCR7	4B12	PE-Dazzle	1:100	Biolegend
VLA4	9C10 (MFR4.B	BV650	1:100	BD
LFA1	M17/4	BV786 PE	1:100	Biolegend
CD69	H1.2F3	APC-Cy7	1:200	BioLegend
CD62L	MEL-14	BV510	1:200	BioLegend
CellTrace Violet	C34557		2.5uM	Thermo Fisher
Live/Dead Fixable Aqua	L34957	BV510	1:1000	Thermo Fisher
Cell Event Caspase 3/7 Green Flow Kit	C10427		1:1000	Thermo Fisher

Supplemental Table 2: GO terms for biological processes identified using PANTHER and GORILLA

GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)	Genes
GO:0045581	negative regulation of T cell differentiation	1.17E-05	1.57E-01	27.86 (10566,37,41,4)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5 Prdx2 - peroxiredoxin 2
GO:0050868	negative regulation of T cell activation	1.89E-05	1.26E-01	14.98 (10566,86,41,5)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5 Tigit - t cell immunoreceptor with ig and itim domains Prdx2 - peroxiredoxin 2
GO:0045620	negative regulation of lymphocyte differentiation	2.16E-05	9.63E-02	23.97 (10566,43,41,4)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5 Prdx2 - peroxiredoxin 2
GO:1903038	negative regulation of leukocyte cell-cell adhesion	2.62E-05	8.78E-02	14.01 (10566,92,41,5)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5 Tigit - t cell immunoreceptor with ig and itim domains Prdx2 - peroxiredoxin 2
GO:0045623	negative regulation of T-helper cell differentiation	3.55E-05	9.50E-02	45.48 (10566,17,41,3)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5
GO:0043371	negative regulation of CD4-positive, alpha-beta T cell differentiation	5.91E-05	1.32E-01	38.66 (10566,20,41,3)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5
GO:0002637	regulation of immunoglobulin production	6.19E-05	1.18E-01	18.41 (10566,56,41,4)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Ighm - immunoglobulin heavy constant mu Tnfrsf4 - tumor necrosis factor receptor superfamily, member 4
GO:0051250	negative regulation of lymphocyte activation	6.21E-05	1.04E-01	11.71 (10566,110,41,5)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5 Tigit - t cell immunoreceptor with ig and itim domains Prdx2 - peroxiredoxin 2
GO:0046639	negative regulation of alpha-beta T cell differentiation	6.87E-05	1.02E-01	36.82 (10566,21,41,3)	Foxp3 - forkhead box p3 Il2 - interleukin 2 Socs5 - suppressor of cytokine signaling 5