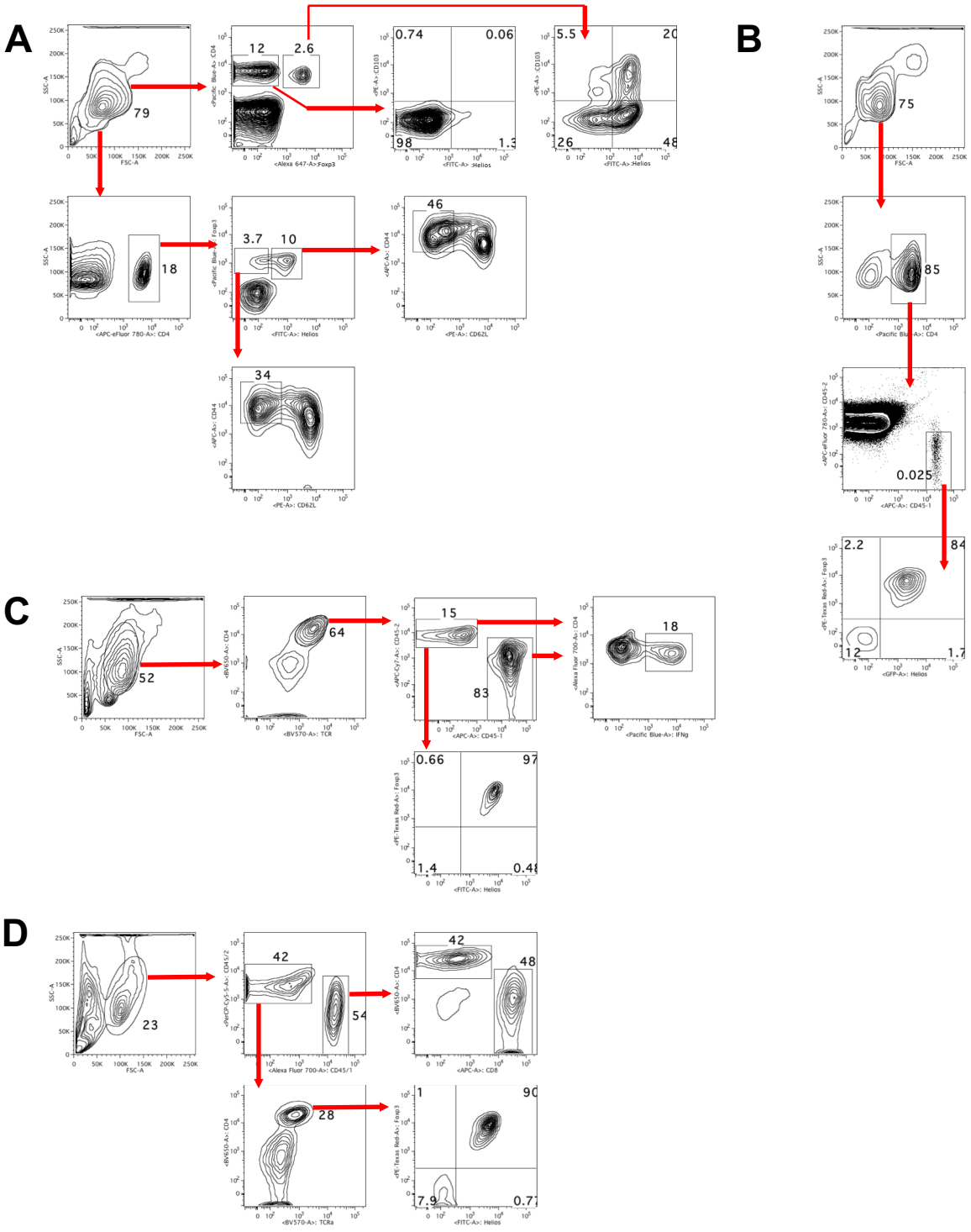

Fig. S1. Gating strategy for flow cytometry experiments. (A) Gating strategy for Fig. 1A, (quadrant gates for PE proteins-far right-were set on CD4+Foxp3- cells-2nd from right) Fig. 1B, Fig. 2A, Fig. 2B and Fig. 6D. **(B)** Gating strategy for Fig. 3. **(C)** Gating strategy for Fig. 4. **(D)** Gating strategy for Fig. 5.

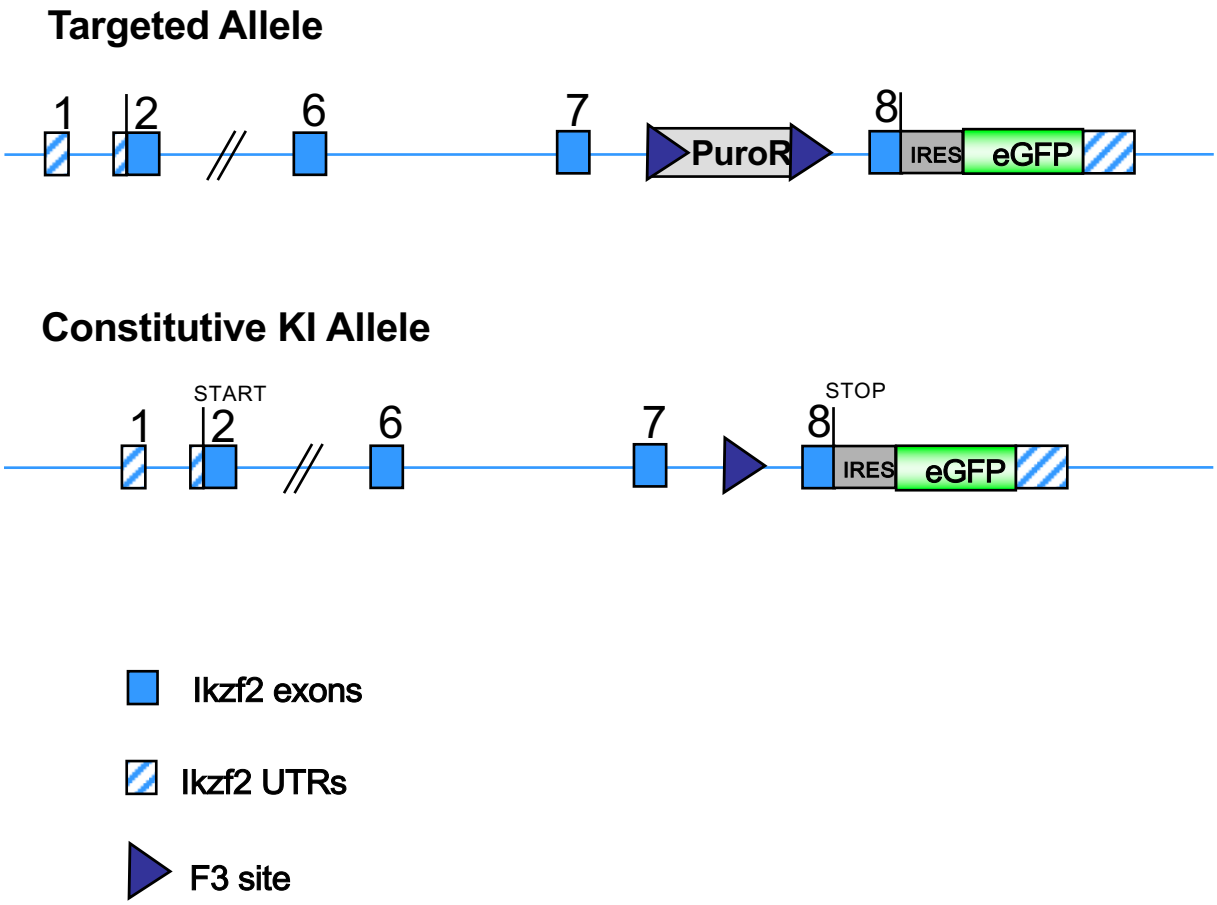

Fig. S2. Generation of the Helios-GFP reporter allele. The targeted allele after homologous recombination (top) and after Flp recombination (bottom).

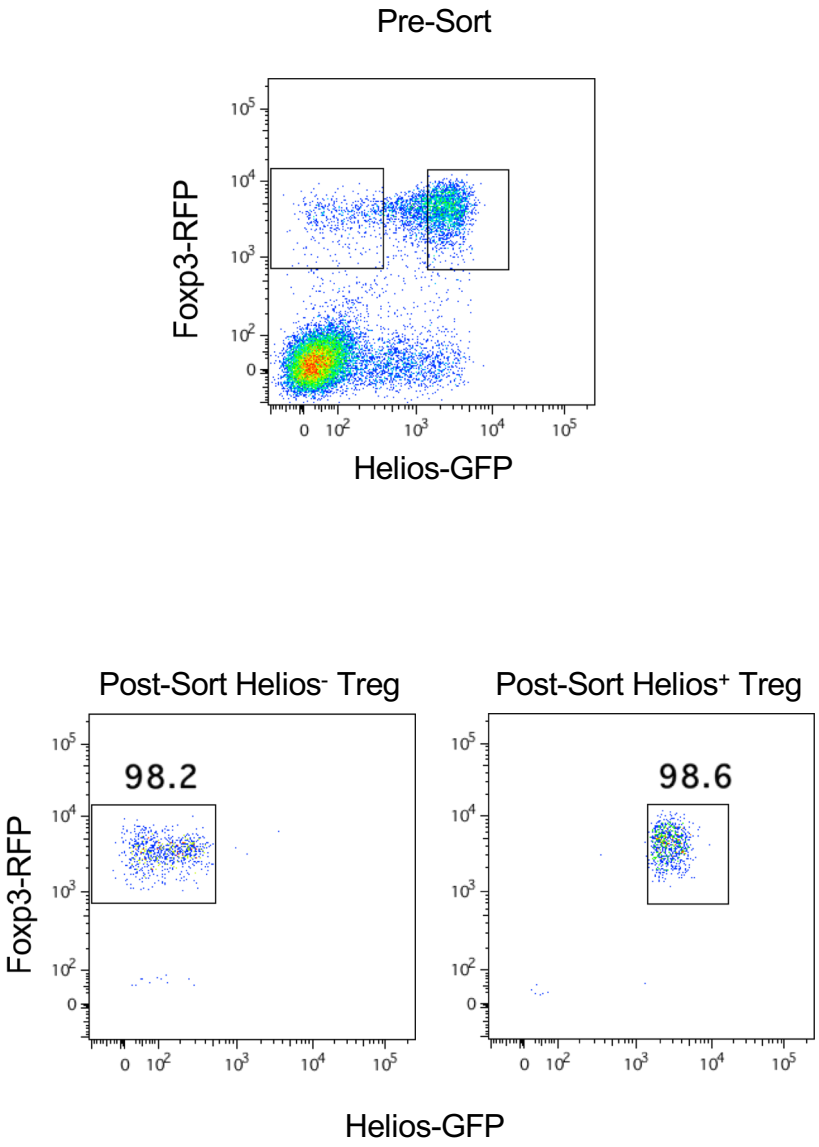

Fig. S3. Sorting strategy for CD4$^+$Foxp3$^+$Helios$^-$ and CD4$^+$Foxp3$^+$Helios$^+$ cells. Lymphocytes were stained with Pacific Blue anti-CD4 and cell sorted by gating on CD4$^+$ cells. The typical gates for CD4$^+$Foxp3$^+$Helios$^-$ are indicated on the left and CD4$^+$Foxp3$^+$Helios$^+$ cells on the right. Post sort analysis is shown below.

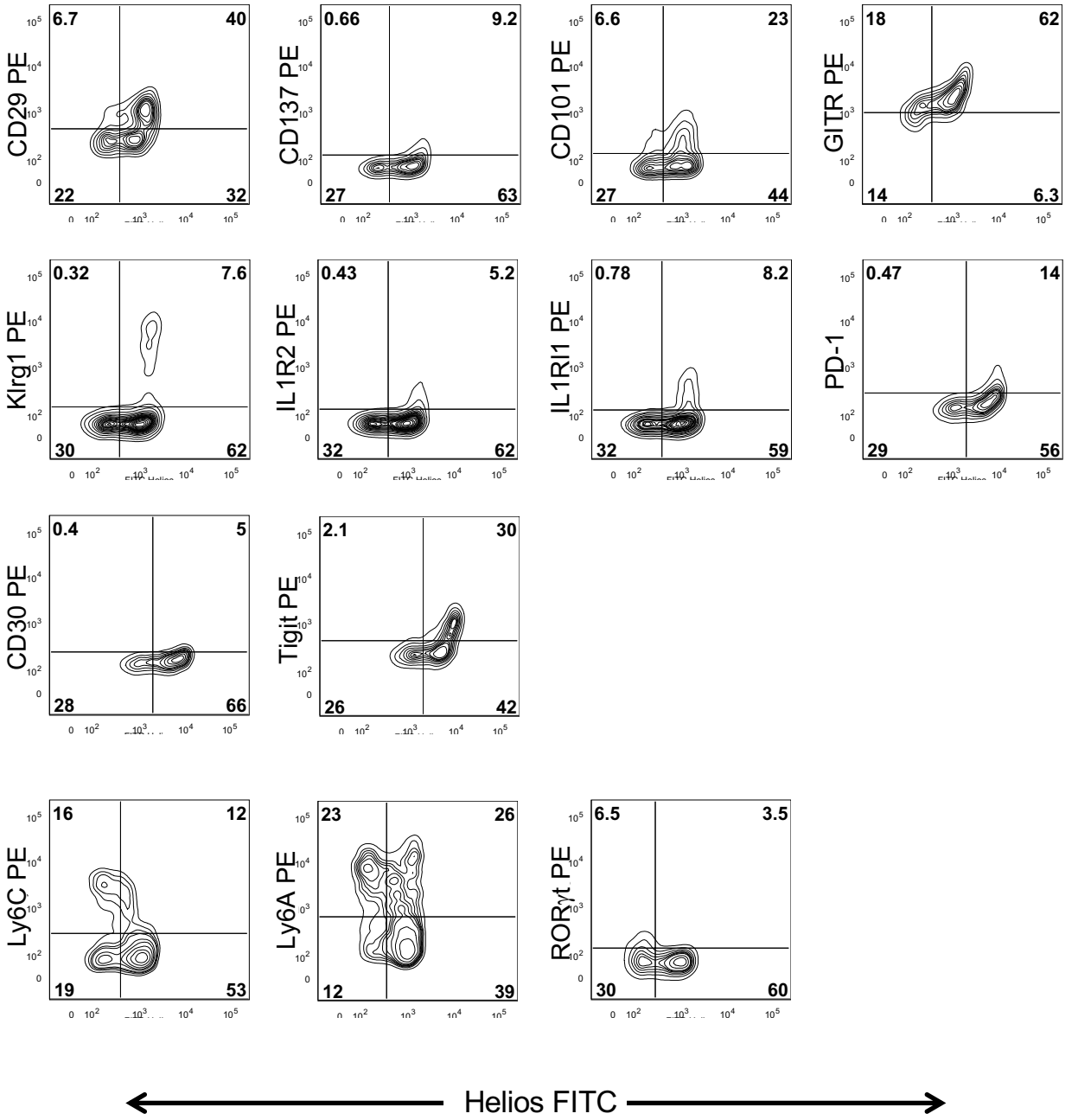

Fig. S4.Validation of gene expression profiles by flow cytometry. Splenic $CD4^{+}Foxp3^{+}$ Treg from C57BL/6 mice were analyzed by flow cytometry for the indicated markers and intracellular expression of Helios. Data is representative of at least three independent for each marker with n=1 or 2 mice per experiment.

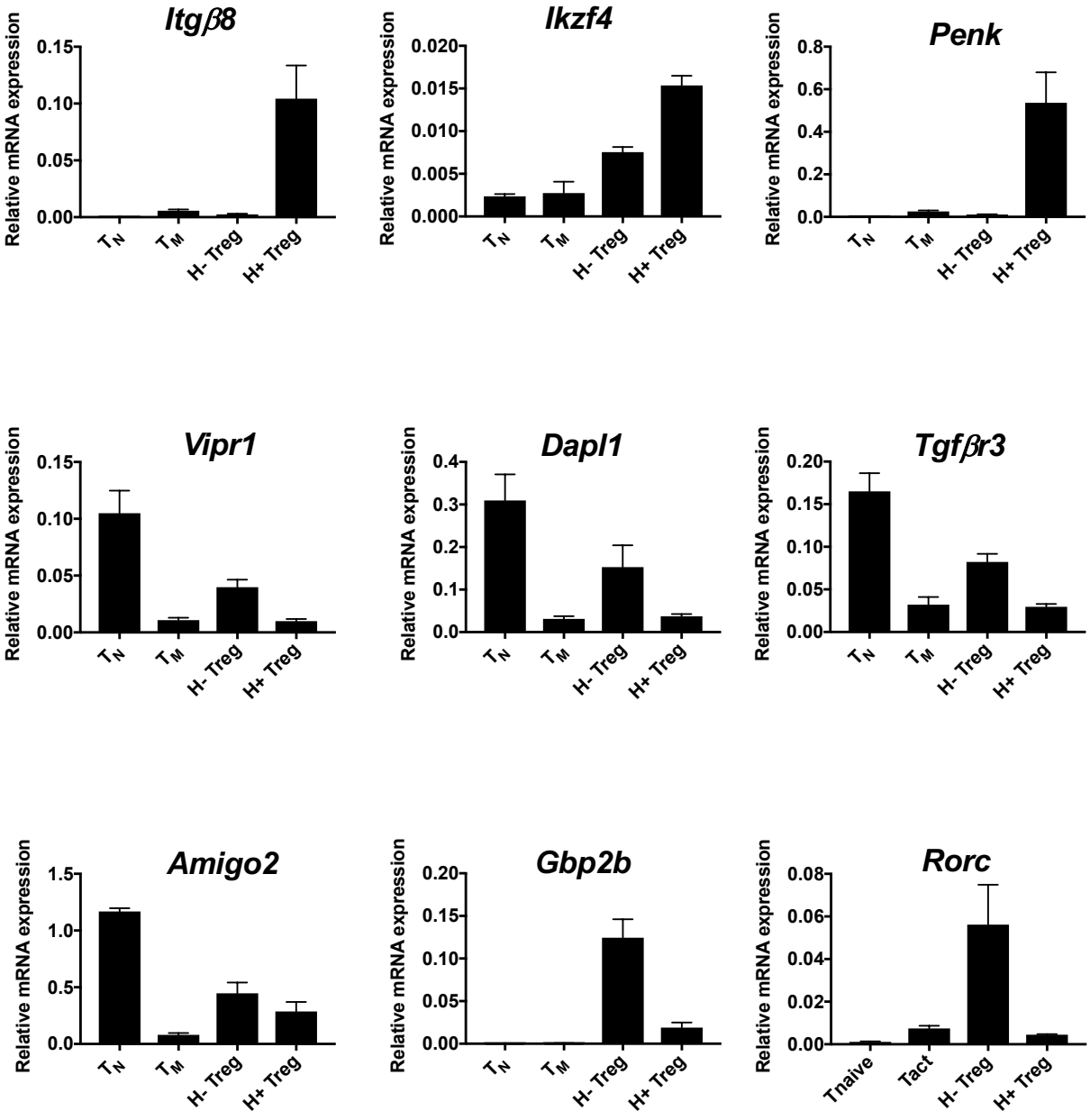

Fig. S5. Validation of gene expression profiles by qPCR. T_N cells ($CD4^+RFP^-CD44^-$), T_M cells ($CD4^+RFP^-CD44^+$), $Helios^-$ Treg ($CD4^+RFP^+GFP^-$) or $Helios^+$ Treg ($CD4^+RFP^+GFP^+$) were sorted from double reporter mice and analyzed by qPCR in triplicate for the indicated genes. Data is representative of at least three independent experiments for each gene.

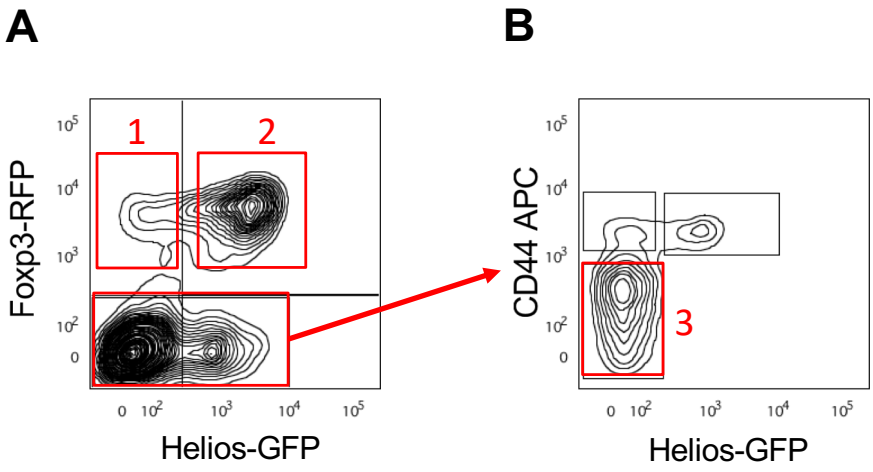

Fig. S6. Sorting strategy for TCR repertoire analysis. mLN from double reporter mice were stained for CD4 and CD44 and sorted for the following: **(A)** Population 1: Helios$^-$ Treg (CD4$^+$RFP$^+$GFP$^-$), Population 2: Helios$^+$ Treg (CD4$^+$RFP$^+$GFP$^+$), **(B)** Population 3: T_N cells (CD4$^+$RFP$^-$CD44$^-$).

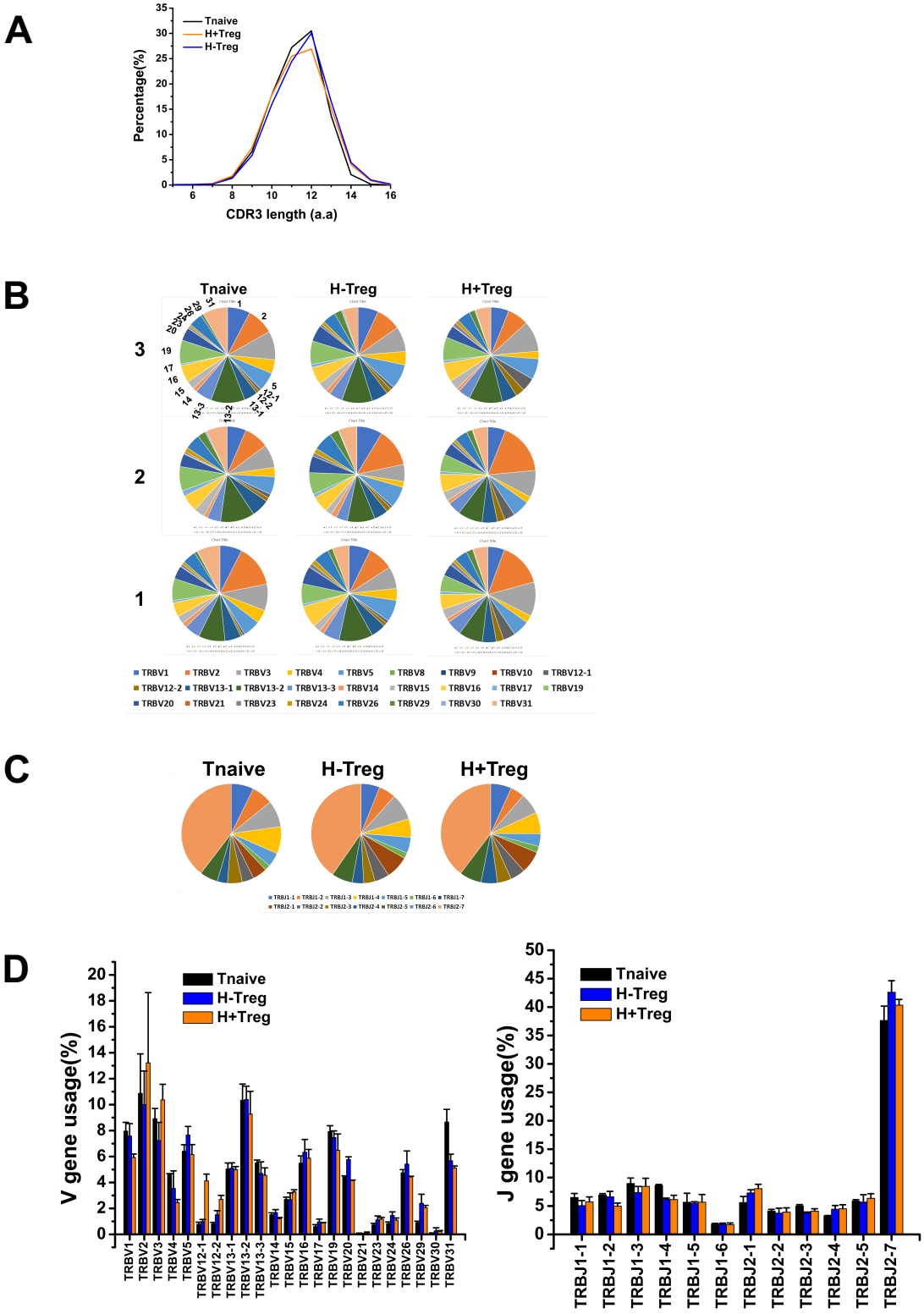

Fig. S7. TCR repertoire analysis. DNA was isolated from cells sorted as in. Fig. S5 and the *TCRB* CDR3 regions were amplified and sequenced by Adaptive Biotechnologies, Inc. ImmunoSeq software was used to analyze **(A)** the CDR3 length **(B)** V gene usage and **(C)** J gene usage from a representative mouse. **(D)** Summary of V and J gene usage from all three mice.

	# Cells	Sequencing Level	Total Templates	Total Productive Templates	Unique Rearrangements
Exp 1					
#1 Helios$^-$ Treg	136,000	Deep	39,289	26,128	18,422
#1 Helios$^+$ Treg A	520,000	Deep x 2	123,852	87,123	57,673
#1 Helios$^+$ Treg B			119,397	83,107	54,960
#2 Helios$^-$ Treg	170,000	Deep	70,914	48,597	32,350
#2 Helios$^+$ Treg A	640,000	Deep x 2	144,529	101,302	66,092
#2 Helios$^+$ Treg B			135,406	94,550	63,422
Exp 2					
#1 Tnaive	$3.7x10^6$	Max	2,284,171	1,668,176	1,141,034
#1 Helios$^-$ Treg	168,000	Deep	51,762	37,612	28,146
#1 Helios$^+$ Treg	866,000	Ultra	506,464	372,511	234,063
#2 Tnaive	$2.8x10^6$	Max	1,340,467	967,896	755,770
#2 Helios$^-$ Treg	133,000	Deep	51,457	37,465	28,201
#2 Helios$^+$ Treg	568,000	Ultra	332,569	243,214	152,522
#3 Tnaive	$2.8x10^6$	Max	1,183,699	857,077	674,670
#3 Helios$^-$ Treg	124,000	Deep	42,404	30,831	22,832
#3 Helios$^+$ Treg	640,000	Ultra	200,447	145,717	99,948

Table. S1. Cell populations used for TCR repertoire analysis. For Experiment #1, the indicated cell populations from the mLN of two individual mice were obtained by FACS sorting. The number of cells sorted from each population, the sequencing level, total templates, total productive templates and the number of unique rearrangements are indicated. For Experiment #2, the indicated cell populations from the mLN of three individual mice were obtained by FACS sorting.