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Supplementary Materials for

Expression of Foxp3 by T follicular helper cells in end-stage germinal centers

Johanne T. Jacobsen†*, Wei Hu†, Tiago B. R. Castro, Sigrid Solem, Alice Galante, Zeran Lin, Samuel J. Allon, Luka Mesin, Angelina M. Bilate, Ariën Schiepers, Alex K. Shalek, Alexander Y. Rudensky, Gabriel D. Victora*

†These authors contributed equally to this work.
*Corresponding author. Email: jjacobsen@rockefeller.edu (J.T.J.); victora@rockefeller.edu (G.D.V.)

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MDAR Reproducibility Checklist (.pdf)
Movies S1 and S2 (.mp4)
Data S1 and S2 (.xlsx)

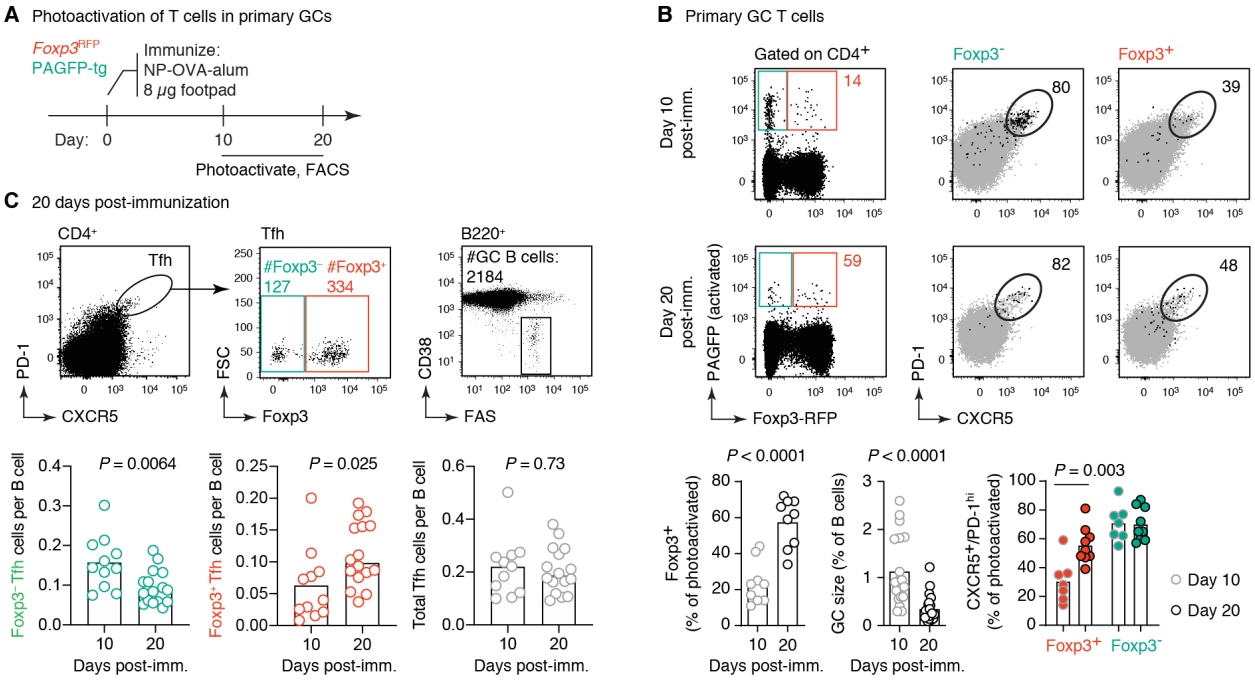

Fig. S1. Photoactivation of T cells from single GCs in a primary response model. (A) Experimental setup: *Foxp3*^{RFP} × PAGFP-tg mice were immunized with NP-OVA in alum adjuvant in the hind footpad, and single GCs were photoactivated for analysis by flow cytometry on either day 10 or day 20 post-immunization. GCs were identified by labeling follicular dendritic cells (FDCs) with anti-phycoerythrin (PE)/PE immune complexes prior to imaging. **(B)** Frequency and phenotype of photoactivated RFP$^+$ and RFP$^-$ CD4$^+$ T cells in single GCs. Each symbol represents one GC. Data are pooled from at least three independent experiments. Data for GC size are from 4 independent non-photoactivation flow cytometry experiments. Each symbol represents one mouse. **(C)** Ratios of Foxp3-RFP$^+$ or Foxp3-RFP$^-$ Tfh-phenotype (CXCR5$^+$PD-1^{hi}) T cells to GC B cells at early and late time points after primary immunization as in (A), but without photoactivation. Bar represents the mean. *P*-values are for Student's *t* test.

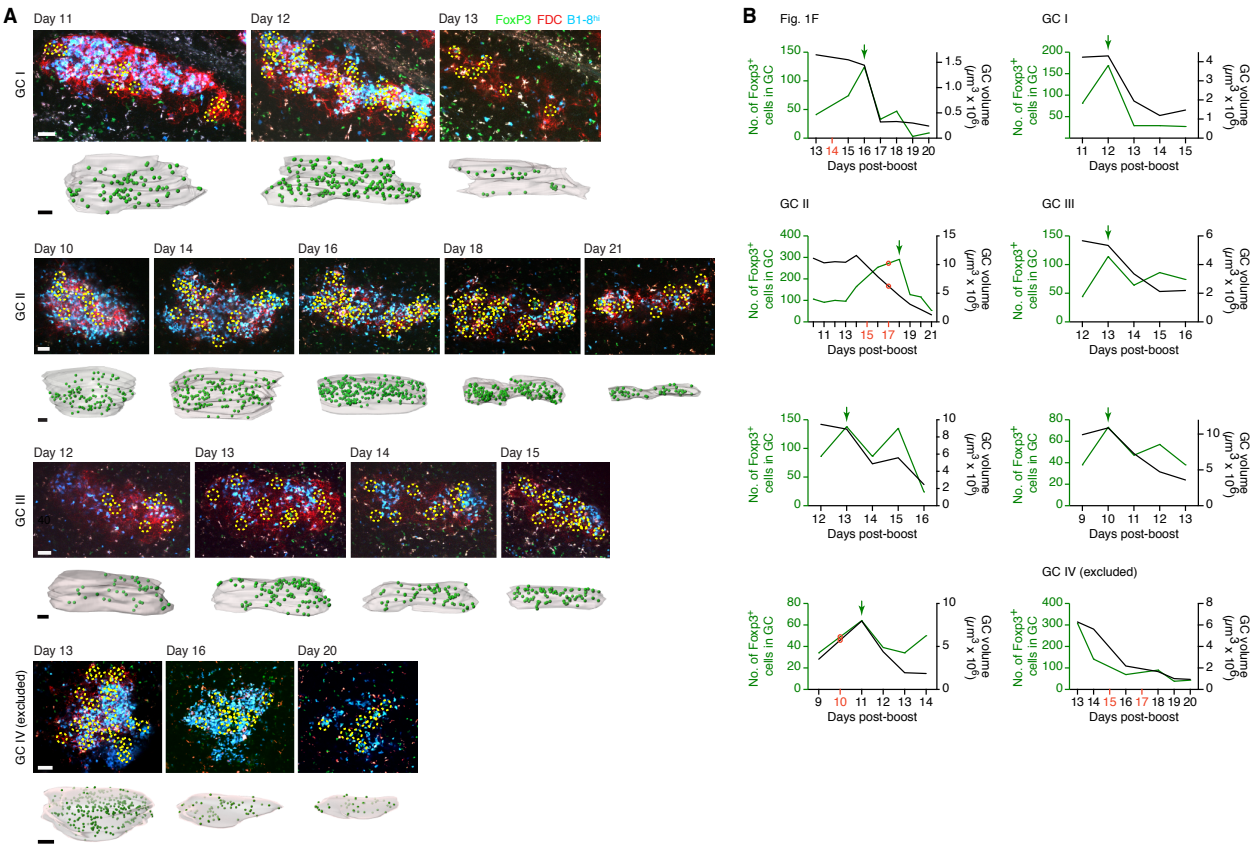

Fig. S2. Longitudinal imaging of GC contraction using iLN windows. (A) Additional examples of longitudinal GC imaging as described in Fig. 1F. Images are collapsed 10 μm, 3-slice *z*-stacks, renderings are entire GCs. Foxp3$^+$ cells are indicated by yellow circles (images) or green spheres (renderings). Scale bars: 30μm. **(B)** Quantification of data as in (A) for eight GCs. For completeness, quantification of the GC presented in Fig. 1F is reproduced from Fig. 1G. Timepoints not imaged are indicated by red ticks on the *x*-axis. When necessary for analysis, values for these timepoints were imputed as the mean of the two adjacent points (red circles). The peak number of Foxp3$^+$ cells is indicated by a green arrow. One GC (IV) in which the peak of Foxp3 cells could not be defined was excluded from the pooled analysis.

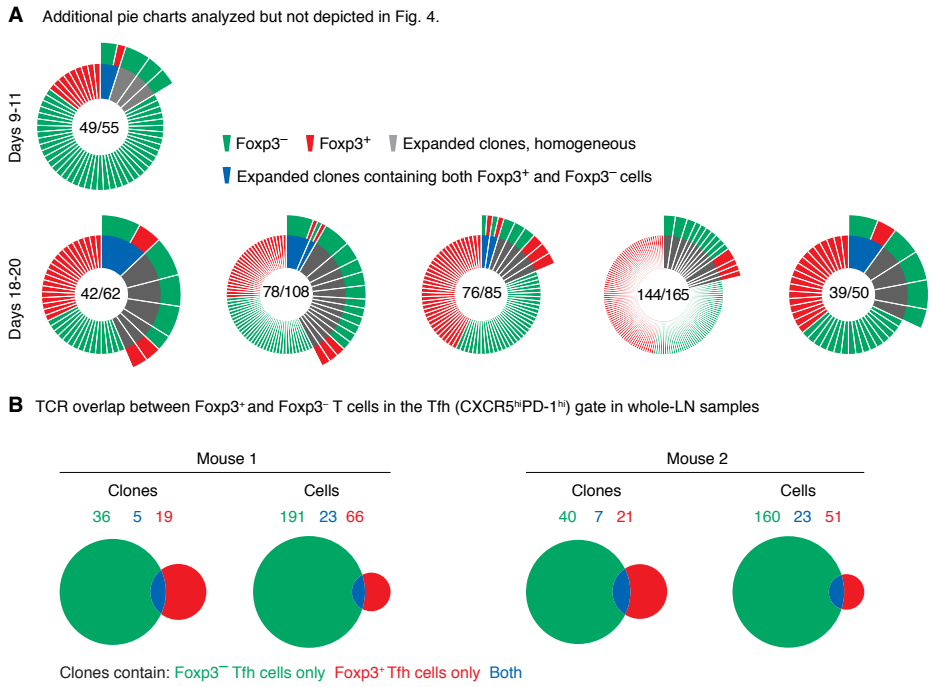

Fig. S3. TCR overlap between Foxp3$^+$ and Foxp3$^-$ GC T cells. (A) Additional pie charts showing TCR sharing between Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells sorted from the same photoactivated GC. Details as in Figure 4B. **(B)** TCR sharing between Foxp3$^+$ and Foxp3$^-$ Tfh-phenotype cells from the same LN. Foxp3^{RFP} mice were immunized with NP-OVA in alum. At 20 days post-immunization, and single RFP$^+$ or RFP$^-$ Tfh-phenotype cells (TCRβ$^+$B220$^-$ CD4$^+$CXCR5$^+$PD-1^{hi}) were sorted into 96-well plates for TCRβ sequencing. Venn diagrams show TCR overlap between RFP$^+$ or RFP$^-$ clones (*left*) or cells (*right*) for two mice from one experiment. Singletons (TCR sequences found only once) were excluded from the analysis. Clones containing cells in both RFP$^+$ or RFP$^-$ compartments are shown in blue.

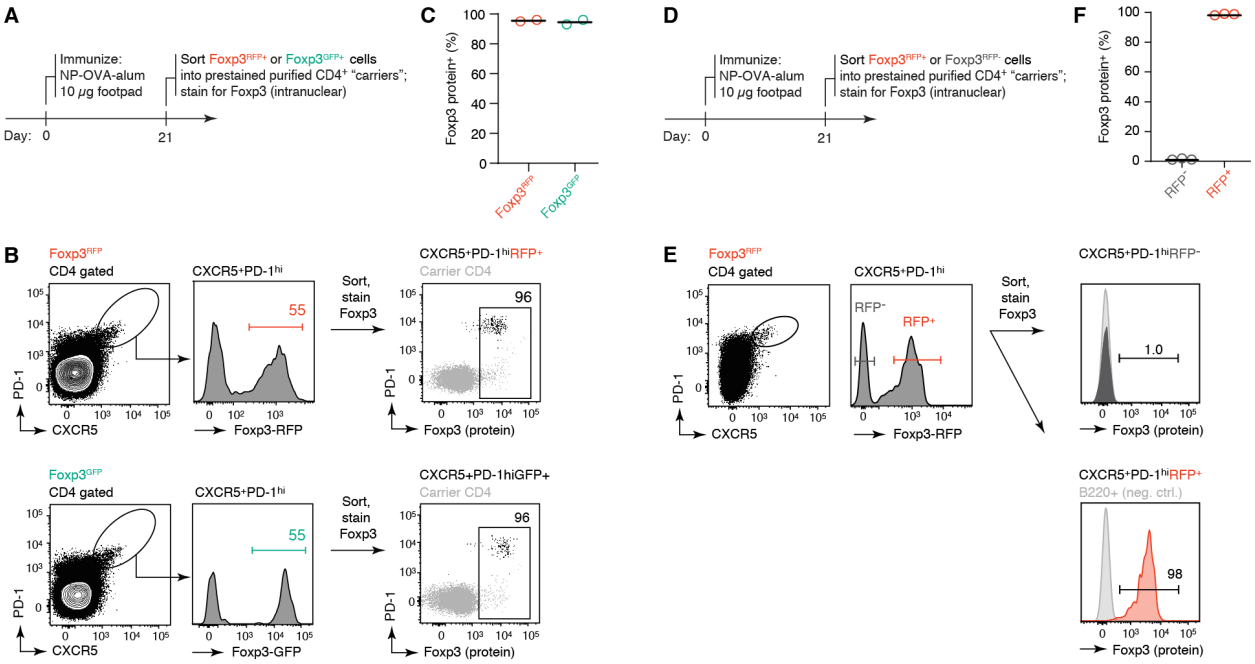

Fig. S4. Accuracy of reporting by Foxp3 alleles. (A) Experimental setup for panels (B,C). This protocol is aimed to circumvent the incompatibility of intranuclear staining for Foxp3 protein with fluorescent protein reporters. GCs were induced in the pLN of *Foxp3*^{GFP} or *Foxp3*^{RFP} reporter mice by footpad immunization with NP-OVA in alum. On day 21 post-immunization, pLN cells were stained for extracellular markers and reporter^{+} CXCR5^{+}PD-1^{hi} T cells were sorted into tubes containing "carrier" naïve CD4^{+} T cells pre-stained with CD4-PE and CD45.1 BV 421. This mix was assayed for Foxp3 protein expression by intranuclear staining. **(B)** Sort gates (left, center) and intranuclear Foxp3 staining for both reporter strains. **(C)** Quantification of percent positivity for Foxp3 protein for both strains as in (B). **(D-F)** As in (A-C) but showing absence of Foxp3 protein in reporter-positive cells. For (C), two independent experiments with one mouse each for *Foxp3*^{RFP} and *Foxp3*^{GFP} were performed. For (F), one experiment was performed using three *Foxp3*^{RFP} mice.

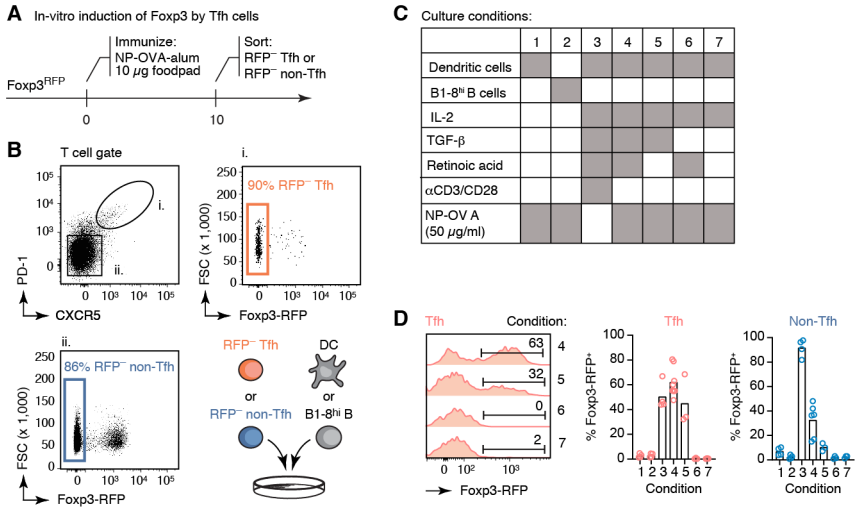

Fig. S5. Acquisition of Foxp3 by Tfh cells in vitro. (A-B) Experimental setup. RFP⁻ Tfh and non-Tfh cells were sorted from *Foxp3*^{RFP} mice 10 days after footpad immunization with NP-OVA in alum and incubated in vitro with the stimuli shown in **(C)** (gray boxes indicate stimuli added to the cultures). Expression of *Foxp3* was then assayed by RFP fluorescence on flow cytometry. **(D)** *Left*, representative flow plots showing RFP expression in selected culture conditions as in (C). *Center and right*, acquisition of RFP by T cells at day three of culture. Each symbol represents one replicate, bar indicates the mean. Data is from three independent experiments.

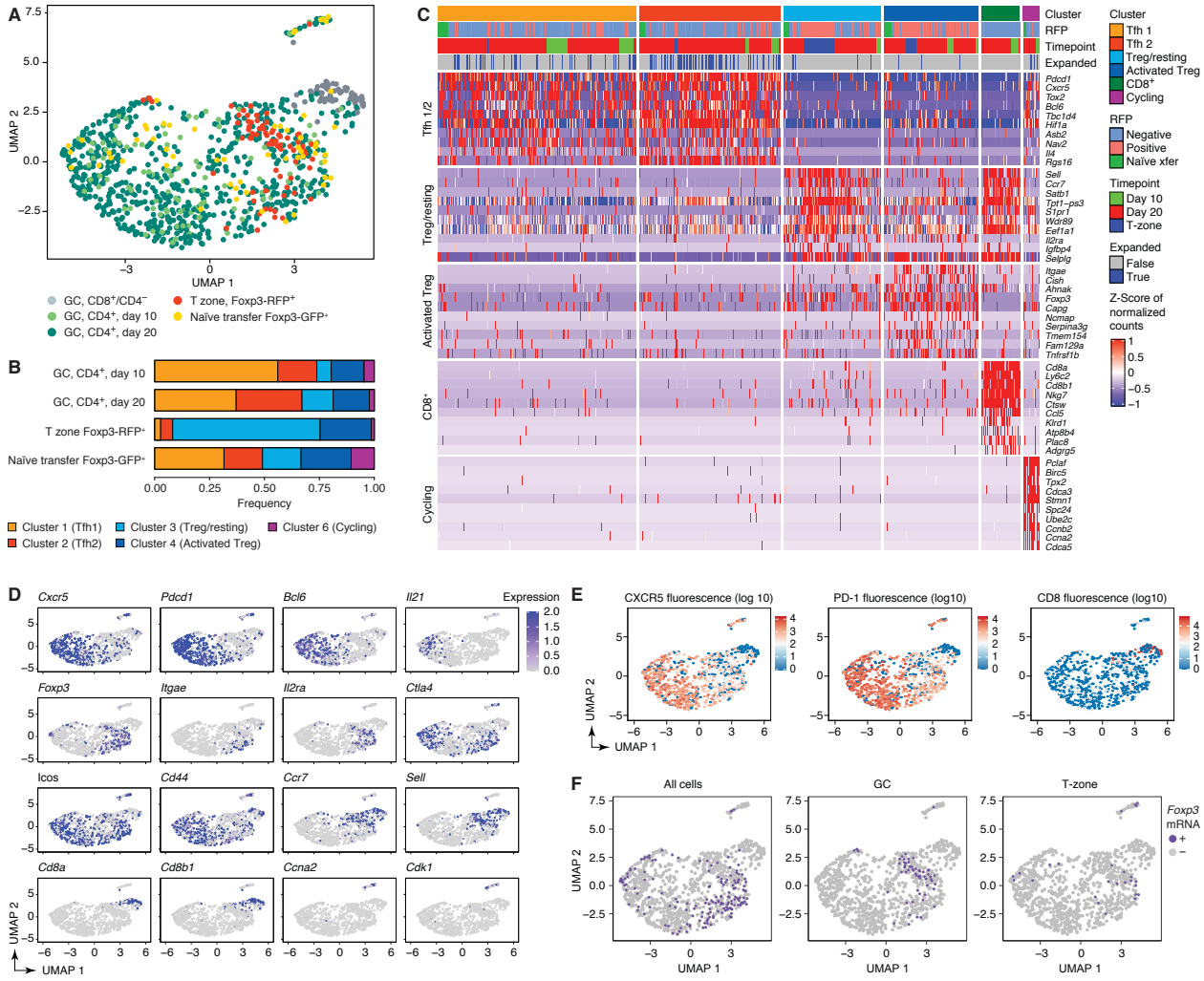

Fig. S6. Clustering of different populations of T cells by scRNA-seq. (A) tSNE distribution of sequenced T cells based on origin, showing total T cells from GCs photoactivated at 10 or 20 days post-immunization as well as RFP$^+$ T cells photoactivated within the T cell zone of a pLN from a naïve mouse. **(B)** Frequency of cells of each of the six clusters depending on the sorted population. **(C)** Heatmap showing expression of the top 10 (log fold-change) positive markers for each of the six clusters. Clusters 1 and 2 are treated as a single group in the heatmap. **(D)** Expression of selected mRNAs characteristic of each cluster. Clusters 1 and 2 contained T cells with a signature of Tfh cells including expression of the hallmark Tfh transcripts *Cxcr5*, *Pdcd1* (encoding PD-1), *Bcl6*. Major differences between *Tfh* Clusters 1 and 2 include higher expression of *Ctla4* and *Il21* in Cluster 1. Cluster 4 ("activated Treg") was enriched for *Foxp3* and other genes associated with Treg function, including *Itgae*, *Il2ra* (CD25), and *Ctla4*, but also expressed activation markers such as *Icos* and *Cd44*. Cluster 3 ("Treg/resting") was also enriched for Treg-associated genes such as *Foxp3 and Il2ra* (CD25), but co-expressed genes associated with naïve or resting status, including *Sell* (CD62L) and *Ccr7*. Cluster 5 expressed genes associated with the CD8$^+$ lineage, including *Cd8a* and *Cd8b*, and was enriched in cells with high expression of CD8 by flow cytometry. Cells in this cluster also expressed high levels of naïve-associated genes *Sell* and *Ccr7,* but also showed expression of the activation marker *Cd44*. This was the only cluster completely lacking clonal expansions. Cluster 6 ("cycling") included a small number of cells with strong expression of proliferation-associated genes such as *Ccna2* (cyclin A2) and *Cdk1* (Fig. S4B-D). **(E)** Expression of CXCR5, PD-1, RFP, and CD8 by flow cytometry, obtained from index-sorting files. **(F)** Cluster distribution of cells expressing *Foxp3* mRNA, as a complement to RFP expression shown in Fig. 4B. Cells in blue are those that are *Foxp3*$^+$ in the sample indicated in the graph title. All other analyzed cells are shown in gray. Number of GCs included and number of independent experiments are indicated in table S1.

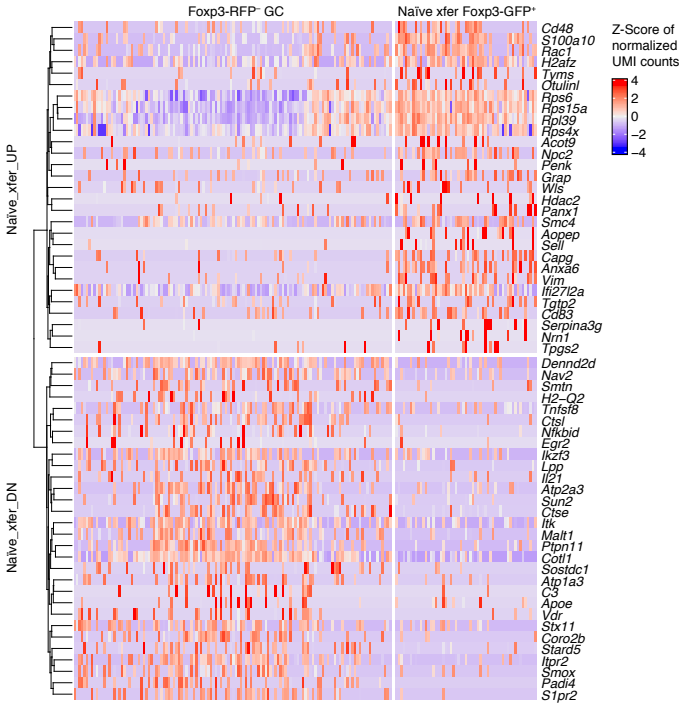

Fig. S7 Naïve transfer signatures. Heat map showing genes included in the "Naïve_xfer_UP" and "Naïve_xfer_DN" signatures. The top 30 genes up or downregulated in the comparison of Foxp3^{+} Tfh cells derived from transferred naïve precursors compared to photoactivated Foxp3^{-} Tfh cells are included in the signature. *Foxp3* itself was excluded from the list of positive markers, leaving only 29 genes in the "UP" signature.

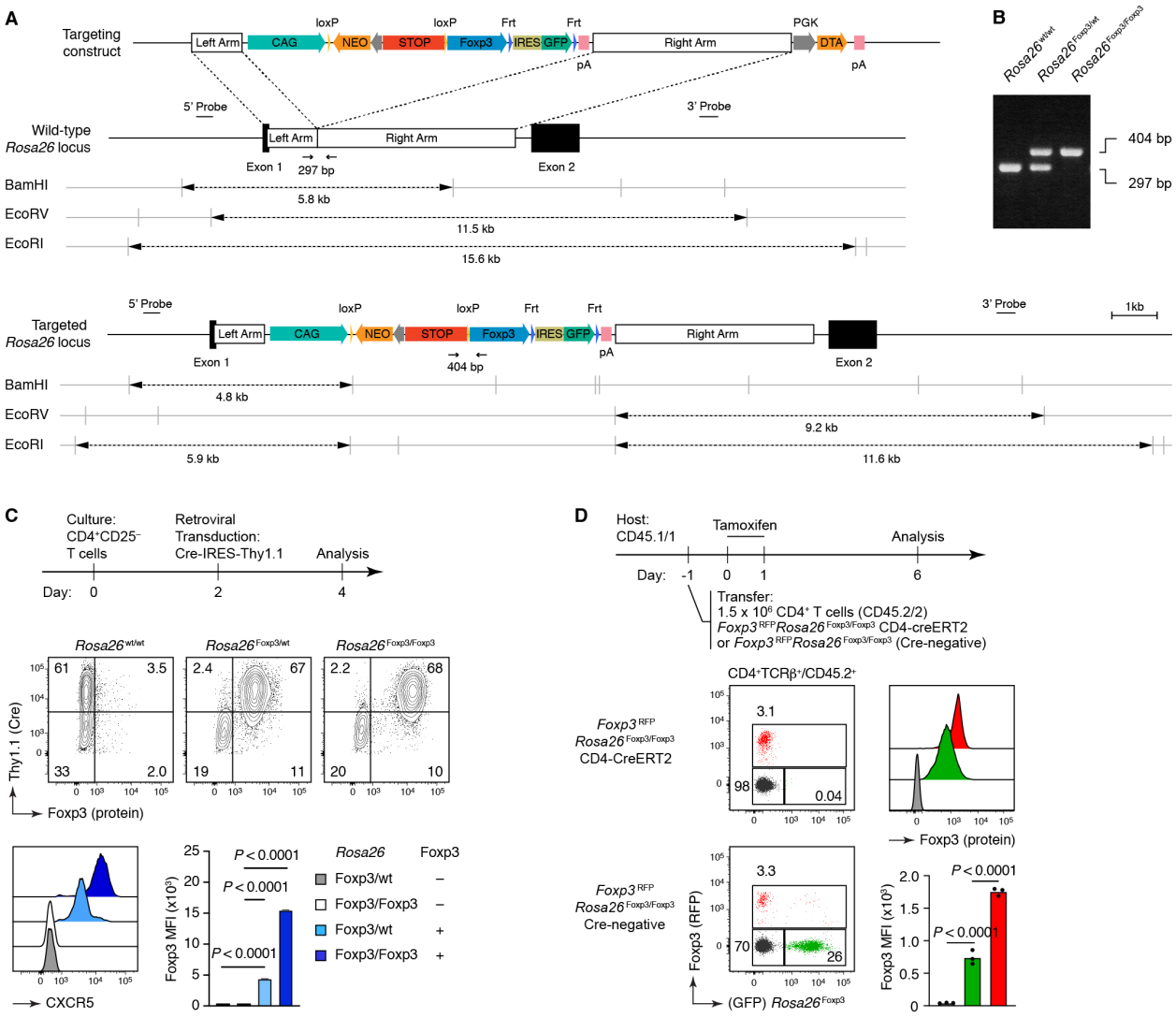

Fig. S8. Design and characterization of the *Rosa26*^{Foxp3} allele. (A) Detailed design of the targeting vector, Southern blotting, and PCR genotyping strategy. All elements are drawn to scale. **(B)** PCR genotyping of *Rosa26* ^{Foxp3} and WT mice using the primers indicated in (A). **(C)** Expression of Foxp3 protein in vitro by CD4^{+}CD25^{-} T cells from the indicated strains transduced with a Cre-expressing retrovirus that co-expresses a Thy1.1 reporter. Data are representative of two independent experiments with two mice per group. Bar graph shows mean±SEM, *p*-values are for one-way ANOVA. **(D)** Expression of the GFP reporter and of Foxp3 in CD4^{+} T cells from *Foxp3*^{RFP}*Rosa26* ^{Foxp3} positive or negative for the CD4-CreERT2 transgene transferred into allelically marked recipients. Mice were treated with tamoxifen on for two consecutive days after transfer and analyzed six days after the first dose. Data are representative of two independent experiments with three mice per group. Bar graph shows mean±SEM, *P*-values are for one-way ANOVA.

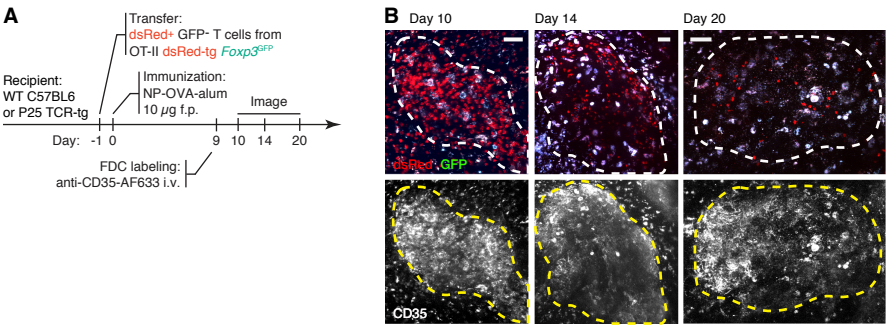

Fig. S9. OT-II Tfh cells fail to upregulate Foxp3 in late GCs. (A) Experimental setup: 5 x 10^5 dsRed$^+$ GFP$^-$ T cells from *Foxp3*^{GFP} dsRed-tg OT-II donors were adoptively transferred into either WT C57BL6 or P25 TCR-tg recipients. Mice were immunized with NP-OVA in alum and injected with CD35-Alexa Fluor 633 to label the FDC network one day prior to analysis. **(B)** Representative images of single GCs at days 10, 14 and 20 post-immunization are shown. In total, two and three C57BL6 and two and three P25 TCR-tg mice were analyzed at days 10 and 20 respectively in two independent experiments, and two C57BL6 mice were analyzed at day 14 in one experiment. No GFP-positive cells were detected in any of the time points. Images are a 12-µm *z*-stack for day 10 and 17-µm *z*-stacks for days 14 and 20. Scale bars: 30 µm.

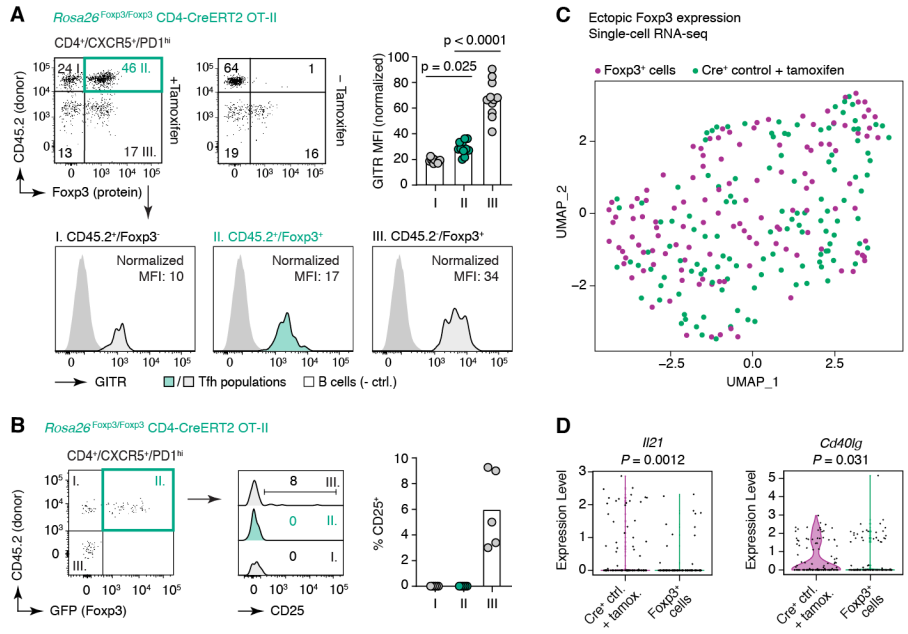

Fig. S10. Forced expression of Foxp3 in Tfh. Experimental layout as in Fig. 4K. **(A)** Expression of GITR in OT-II cells induced to express Foxp3. Dot plots are reproduced from Fig. 4L. Expression of GITR, normalized to the levels found on B220$^+$ B cells in the same sample, is quantified across multiple mice in the top-right panel. Each symbol represents one mouse from three independent experiments. **(B)** Expression of CD25 in OT-II cells induced to express Foxp3. Dot plots show gating strategy, based on expression of GFP, the surrogate reporter for Foxp3 expression in the *Rosa26*^{Foxp3} strain. Percentage of CD25$^+$ cells in each gate is quantified across multiple mice in the rightmost panel. Each symbol represents one mouse from two independent experiments. *P*-values are for Student's *t* test. **(C)** UMAP plot showing distribution of Foxp3$^+$ OT-II Tfh cells (GFP$^+$CXCR5$^+$PD-1^{hi}) from *Rosa26*^{Foxp3} mice and Foxp3$^-$ OT-II Tfh cells from Cre$^+$ control mice. **(D)** Expression by scRNA-seq of selected genes by the same cell populations represented in (C). Each symbol represents one cell, pooled from four experimental and four control mice sorted from a single experiment. *P*-values are for Wilcoxon signed-rank test.

Table S1. Characteristics of cells used in the scRNA-seq experiment.

Plate	Experiment	Source	$CD8^+$ T cells	Cells passing threshold
1	1	T-zone	Excluded	77
2	1	Day 20	Included	77
3	1	Day 20	Included	86
4	1	Day 20	Included	82
5	2	Day 20	Excluded	38
6	2	Day 20	Included	67
7	2	Day 10	Included	68
8	2	Day 20	Excluded	53
9	3	Day 20	Excluded	66
10	3	Day 20	Excluded	74
11	3	Day 10	Excluded	58
12	4	Naïve transfer	Excluded	40
13	4	Naïve transfer	Excluded	41
14	4	Day 20	Excluded	70
15	4	Day 20	Excluded	71

Table S2. Reagents used for flow cytometry

Surface molecule	Fluorochrome	Clone	Manufacturer	Final conc. (µg/ml)
B220	BV785	RA3-6B2	Biolegend	0.5
B220	BV421	RA3-6B2	Biolegend	0.5
CD4	V500	RM4-5	BD Biosciences	0.5
CD4	BV785	RM4-5	Biolegend	0.5
CD16/32 (Fc-block)	---	2.4G2	Bio-X-Cell	1.0
CD38	PerCP-Cy 5.5	90/CD38	BD Biosciences	0.5
CD45.2	BV 605	104	Biolegend	0.5
CD80	PE	2D10.4	eBioscience	0.5
CD152 (CTLA-4)	BV 421	UC10-4B9	Biolegend	0.5
CD185 (CXCR5)	BV650	L138D7	Biolegend	1.0
CD279 (PD-1)	APC/Cy7	29F.1A12	Biolegend	0.5
CD357 (GITR)	biotin	DTA-1	Biolegend	0.5
FAS	Pe-Cy7	Jo2	BD Biosciences	0.25
Foxp3	APC	FJK-16s	eBioscience	1.0
Streptavidin	PE	---	Biolegend	0.25

Movie S1. Foxp3^{+} T cell surge precedes the onset of GC regression. Collapsed 4D dataset showing longitudinal iLN window imaging at days 13, 14, and 15 after subcutaneous boosting with NP-OVA (experimental setup as in Fig. 1A). Green, GFP signal from the *Foxp3*^{GFP} recipient; blue/cyan, CFP from adoptively-transferred B1-8^{hi} NP-specific B cells; red, anti-CD35-Alexa Fluor 594 (FDCs). Original images were acquired with the GC in different angular orientations. GCs were rotated post-acquisition so that orientation in the video remains fixed, causing areas outside the original acquisition to be visible at the later timepoints. Videos are displayed at 210× real time.

Movie S2. Long-lived interactions between Foxp3^{+} T cells and a CD11c^{+} TBMs in GCs at day 10 post-boosting. Collapsed 4D dataset of pLN intravital imaging showing endogenous Foxp3-GFP^{+} T cells (blue), CD11c-YFP^{+} myeloid cells, including DCs and TBMs (yellow-green), and FDCs stained with Alexa Fluor 594-conjugated antibody to CD35 (red), 10 days after footpad boosting with NP-OVA (experimental setup as in Fig. 1A). The first (overview) movie is shown twice, once as raw data and the second time with long-lived interactions between CD11c^{+} TBMs and Foxp3^{+} T cells indicated by tracks. This is followed by a magnified example a long-lived interaction between these two cell types. Videos displayed at 210× real time.

Data S1. Sequences of all T cell receptor genes analyzed in Figures 3 and 4.

Data S2. Primers used for T cell receptor sequencing.