**Supporting Information**

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**SI Materials and Methods**


Dead cell discrimination was based on positivity for IR Live/Dead dye (Invitrogen).

Antibodies used for microscopy are shown in Table S1.

Flow Cytometry and Antibodies. Cells were stained at 4 °C for 30 min (1 h at 37 °C for CXCR5, biotin or CCR7). Intraacellular staining was carried out using an eBioscience Foxp3 staining kit according to the manufacturer’s instructions. Antibody clones are given in *SI Materials and Methods*. Tfh/Tfr cells used for neuropilin-1 staining were identified by PD-1 and BCL6 rather than by CXCR5-biotin and PD-1. IL-21 and BLIMP-1 expression was determined by reporter mice. Samples were collected on an LSR Fortessa (BD Biosciences), and compensation and data analysis were carried out using FlowJo vX.0.7 (TreeStar). For t-distributed stochastic neighbor embedding analysis, precompensated and gated data were exported with FlowJo and analyzed by ACCENSE (16).

Cell Sorting. CD4 T cells were enriched by CD4+ selection using a CD4+ selection kit (mouse) or CD4+ selection kit (human) and MACS manual cell separator columns according to the manufacturer’s instructions (Miltenyi). Cells were then stained and sorted on a BD FACSARia-SORP system (Becton Dickinson) (Fig. S5B).

GSEA. GSEA was performed with software from the Broad Institute (51). Fragments per kilobase million (FPKM) data from CD25+ Tfr and Tfr cells were used for enrichment of genes from our Tfh/eTreg DE gene set or from several published gene sets (22) [GEO accession no. GSE24574 (35), GEO accession no. GSE143500 (54)].

Vaccinations, Antibody Treatments, and Adoptive Transfer. Mice were vaccinated i.p. or s.c. with 100 μg of NP-Ova (Biosearch) in PBS mixed 1:1 for 30 min at room temperature with 100 μL of Imject alum (Pierce) in a total volume of 200 μL on day 0 of each experiment. For IL-2 blocking experiments, mice were treated i.p. at day −1 with 1 mg of anti–IL-2 (clone S4B6; BioXcell) or isotype control (rat IgG; Sigma) before vaccination at day 0.

For IL-2 complex experiments, recombinant murine IL-2 (eBioscience) was mixed with anti–IL-2 (Ultra-LEAF JES-1A12; Biorlegen) for 30 min at room temperature. Then, 1 μg per 5 μg or 2 μg per 10 μg (IL-2/anti–IL-2), 5 μg isotype control, or Ultra-LEAF Rat IgG2a (Biorlegen) was injected i.p. into mice at days −1, 1, 2, 3, 4, and 5, and mice were vaccinated on day 0.

For adoptive transfer, 1 × 10^6 CD62L+CD25+ nTconv cells from CD45.1 BALB/c mice and 1 × 10^6 CD62L+CD25–Foxp3+ nTreg cells from BALB/c eFox (Foxp3-Gfp) mice were transferred i.v. into athymic BALB/c nude mice. One day later, mice were vaccinated and draining LNs (dLNs), spleens, and Peyer’s patches were collected at day 14.

Immunohistochemistry. Immunohistochemical analysis was performed as described (52). Briefly, dLNs and spleens were removed and fixed with 4% parafomaldehyde overnight at 4 °C. Samples were then equilibrated in sucrose, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek), snap-frozen by hexane/ dry ice, and kept at −80 °C. Sections were cut with a cryostat (Leica) and mounted onto slides (Matsunami MAS-GP). After removing the Tissue-Tek O.C.T. Compound in PBS, sections were blocked for 30 min and washed with PBS, and antibodies were applied. Primary and secondary antibodies are listed in *SI Materials and Methods*. The sections were coverslipped with mounting medium and observed with a fluorescence microscope (Zeiss).

RNA-Seq and Analysis. For RNA-Seq, 1 × 10^6 cells were sorted using a BD FACSARia (Becton Dickinson). RNA was extracted using RLT buffer (Qiagen), subjected to library preparation using the Quartz-Seq protocol (53), and sequenced by Ion Proton (Life Technologies). After removing primers of Quartz-Seq using Cutadapt (version 1.7.1), sequences were mapped to mm9 using two-step mapping of TopHat2 (version 2.1.0) and bowtie2 (version 2.2.6). Normalized FPKM was generated with Cuffnorm (version 2.2.1). The FKPM list was used to define an expression cutoff value of 1.25 (log, FPKM); genes not expressed at this level by at least one pair of replicates were excluded from later analysis. Raw tag counts were obtained using HT-Seq (version 0.6.1). These counts were normalized by regularized log transformation and used for heat maps, principal component analysis, and Euclidean distance analysis in R software (version 3.3). Differential gene expression (based on raw tag counts) was performed in R using the TCC package (54). Normalization was by DEGES-modified DEseq2, followed by DE analysis by DESeq2. Genes with a false discovery rate of <0.01 and a fold change of ≥2 were considered DE.

CpG Methylation Analysis by Bisulfide Sequencing. Bisulfide sequencing was performed as previously described (30).

Retroviral Transfections. Retroviral transfections were performed as previously described (30). Briefly, 1 × 10^5 Treg cells were cultured in the presence of anti–CD3 anti–CD28 Dynabeads and 100 U/mL IL-2 for 24 h, transfected with retrovirus containing EV or ASC2 by spinfection with viral supernatants in the presence of 5 μg/mL Polybrene and IL-2 (100 U/mL) on consecutive days, and then cultured in the presence of anti–CD3 anti–CD28 Dynabeads and 100 U/mL IL-2 for 72 h.

In Vitro Suppression and Foxp3 Stability Assays. Murine T-cell suppression. A total of 1 × 10^7 B cells were cultured in the presence of 0.5 μg/mL anti–CD3, 10 μg/mL anti-IgM, and 5 × 10^3 Tfh cells with/without 5 × 10^3 of the indicated Treg population
for 6 d. Supernatant IgG1 concentrations were determined by ELISA.

**Foxp3 stability.** A total of $2.5 \times 10^3$ purified cells were stained with CellTrace Violet (CTV) and incubated with anti-CD3 anti-CD28 Dynabeads (Gibco) alone or with Dynabeads with 100 units of IL-2 or 20 ng/mL IL-4 for 3 d.

**Murine T-cell suppression.** A total of $5 \times 10^3$ nTconv cells were stained with CTV and incubated for 3 d with $5 \times 10^3$ B cells; 0.5 μg/mL anti-CD3; and $5 \times 10^3$, $2.5 \times 10^3$, or $1.25 \times 10^3$ of the indicated Treg population for 3 d.

**Human B-cell suppression.** A total of $5 \times 10^4$ B cells were cultured in the presence of anti-CD3 and anti-CD28 Dynabeads (Thermo Fisher Scientific) at a Dynabead/T-cell ratio of 1:32 with/without $1 \times 10^4$ Tfh cells and/or $1 \times 10^4$ eTreg or Tfr cells for 6 d. Supernatant IgG concentrations were determined by ELISA.

**Human T-cell suppression.** A total of $1 \times 10^4$ nTconv cells from PBMCs were stained with CTV cultured for 6 d with irradiated CD4$^+$ PBMCs with 1 μg of anti-CD3 with/without $5 \times 10^3$ of the indicated suppressor cells.
Identification of CD25^-Tfr cells. Mice were vaccinated s.c. and i.p. with 100 μg of NP-Ova in alum, and dLN, spleens (Spl), or Peyer’s patches (PP) were taken at day 7 or the indicated time. (A) Percentage of CD25^- within total Tfr cells from indicated organs. Data are pooled from 10 mice, representative of three separate experiments. (B) ACCENSE analysis of PP CD4^+ T cells. PD1, BCL6, CXCR5, Foxp3, and CD25 were used for mapping. CD44 and CD62L clustered on the basis of other parameters. Data are pooled from three mice concatenated into a single flow cytometry standard file, representative of two separate experiments. Scales are Z-scores (±SD from mean). (C) Time course of GC, Tfh, CD25^+ Tfr, and CD25^- Tfr cell numbers per 1 x 10^5 lymphocytes in dLN following vaccination at day 0.

Fig. S1.
Fig. S2. Phenotyping of CD25− Tfr cells. Mice were vaccinated s.c. with 100 μg of NP-Ova in alum, and dLNs were taken at day 7 or day 14. Expression of indicated markers by geometric mean fluorescence intensity (gMFI) or percent positive as assessed by flow cytometry. Mean ± SEM. Data are pooled from three mice, representative of two to four separate experiments (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). ns, not significant.

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Fig. S3. RNA-Seq of CD25− Tfh cells. Mice were vaccinated with NP-Ova in alum, and dLNs were taken at day 7. A total of 1 × 10⁴ cells were sorted by FACS before RNA-Seq. RNA was extracted using RLT buffer, and then subjected to library preparation using the Quartz-Seq protocol and sequenced by Ion Proton. Heat maps, hierarchical clustering, and Euclidean distance analysis were produced using R software. Differential gene expression analysis was performed in R by TCC/DEseq2. Genes with a false discovery rate of <0.01 and a fold change of ≥2 were considered DE. (A) Z-scored heat map of a full list of Tfh vs. eTreg DE genes, with columns and rows hierarchically clustered. (B) Z-scored heat map of selected Tfh-related genes. (C) GSEA of CD25− Tfr vs. CD25+ Tfr cells with the BCL6hi Tfh vs. BCL6lo Tfh gene list from ref. 22 (GEO accession no. GSE24574). Positive enrichment shows enrichment in CD25− Tfr cells, and negative enrichment shows enrichment in Tfr cells. (D) Euclidean distance analysis of gene expression.
**Fig. S4.** Suppressive function and stability of CD25− Tfr cells. Mice were vaccinated with NP-Ova in alum, and dLNs were taken at day 7. Cells were sorted by BD FACSAria-SORP (Becton Dickinson) after negative selection of CD4 by magnetic beads. (A) Total of 1 × 10^6 cells were cultured in the presence of 0.5 μg/mL anti-CD3, 10 μg/mL anti-IgM, and 5 × 10^5 Tfh cells with/without 5 × 10^3 of the indicated Treg population for 6 d. Intracellular IgG1 expression is shown. Data are representative of two separate experiments. (B) Total of 5 × 10^6 nTconv cells were stained with CTV and incubated for 3 d with 5 × 10^5 B cells; 0.5 μg/mL anti-CD3; and 5 × 10^3, 2.5 × 10^3, or 1.25 × 10^3 suppressor cells. Data are representative of two separate experiments. (C) Total of 2.5 × 10^6 purified cells were stained with CTV and incubated with Dynabeads with/without the indicated cytokines (100 units of IL-2 + 20 ng of IL-6 or 20 ng of IL-4 + 20 ng of IL-6) for 3 d. Foxp3 and CD25 expression is shown. Data are representative of two separate experiments. (D) CD4-enriched T cells from dLNs before and after sorting by BD FACSAria-SORP. (E and F) Total of 1 × 10^5 CD45.1 nTconv cells and 1 × 10^5 CD45.2 eFox CD25− Tfr cells were transferred i.v. into nude mice. One day later, mice were vaccinated, and dLNs were collected at day 14. (E) CD25 and CXCR5 expression by Foxp3− CD45.1 cells (Right) and Foxp3−GFPCD45.2 cells (Left). (F) Percentage of CD45.2 Foxp3+Foxp3−GFP− and CD45.2 Foxp3−GFP+ cells in eTreg, Tfr, and CD25− Tfr populations. Data are representative of two separate experiments.
Effect of IL-2 KO and antibody blockade on CD25− Tfr cells. (A–D) Four-week-old IL-2 KO homozygous (−/−), heterozygous (−/+), or wild-type (+/+), or wild-type (+/+). Littermates were killed. (A) Foxp3 and CD25 expression by CD4+ cells in LNs. (B) Summary data of GC and Tfh cells from LNs of indicated mice. (C) Summary data of Treg, Tfr, and CD25− Tfr cells within Tfr cells from LNs of indicated mice. (D) Summary data of Treg, Tfr, and CD25− Tfr cells within Tfr cells from Peyer’s patches of indicated mice. Mean ± SEM. Data are pooled from three mice, representative of two separate experiments. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001). ns, not significant.

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Effect of IL-2 complex on CD25 Tfr cells. (A–D) Mice were treated at days −1, 1, 2, 3, 4, and 5 with IL-2 complex at a 1:5 or 2:10 ratio of 1 μg of murine IL-2 (55-μg of anti-IL-2 (4E6 1A12), or with 5 μg of isotype (iso) control alone. Mice were vaccinated with NP-Ova in alum at day 0, and spleens taken at day 7. (A) Zebra plots and histograms of Foxp3 and CD25 expression by CD4 cells (left), CXCR5 and PD1 expression by Foxp3 Treg cells (center), and CD25 expression by Tfr cells (right). (B) Summary data of Treg, Tfr, and CD25 Tfr cells. Data are pooled from seven mice, representative of two separate experiments. The line indicates mean. (C) Summary data of GC-B cells, Tfh cells, total number of CD25 Tfr cells, and gMFI of BCL6 expression by Tfr cells in spleens (n = 3–7 as indicated). (D) Summary data of Tfr, CD25 Tfr, and CD25 Tfr cells as a proportion of CD4 in Peyer's patches (one outlier removed from 2:10 group as outlined in Materials and Methods). (E) GC-B cells and Tfh cells in Peyer's patches (n = 7, line denotes mean). (F) Z-score heat map and clustering dendrogram of microarray gene expression by CD25 Tfr cells from naive mice or Treg cells from IL-2−/− mice with and without peritoneal injections of recombinant IL-2 every 8 h for the 24 h before euthanasia. Microarray data are from Fontenot et al. (12) (GEO accession no. GSE4179). (G) GSEA of CD25 Tfr vs. CD25 Tfr cells with the gene list from IL-2 KO Treg vs. wild-type Treg cells. Data are from Yu et al. (34), (GEO accession no. GSE14350). Positive enrichment shows enrichment in CD25 Tfr cells, and negative enrichment shows enrichment in CD25 Tfr cells (**P < 0.05, ***P < 0.01, ****P < 0.001, *****P < 0.0001); ns, not significant.
Fig. S7. Function and phenotype of Tfr cells in blood and tonsils. (A–D) PBMCs were purified from the blood of healthy donors. (A) Expression of HLA-DR and Ki-67 by nTreg (Left), cTfr (Center), and eTreg (Right) cells. Data are representative of three separate experiments. (B and C) Total of $5 \times 10^4$ B cells were cultured in the presence of anti-CD3 and anti-CD28 Dynabeads at a 1:32 Dynabead:T-cell ratio (Thermo Fisher Scientific) with/without $1 \times 10^4$ Tfh cells and/or $1 \times 10^4$ eTreg cells or indicated Tfr cells for 6 d. (B) CD20^lo^CD38^hi^ plasma cell formation. (C) Total number of CD20^lo^CD38^hi^ plasma cells. Mean ± SEM of duplicates. Data are representative of two separate experiments (*P ≤ 0.05; ns, not significant). (D) Total of $1 \times 10^4$ nTconv cells from PBMCs were stained with CTV cultured for 6 d with irradiated CD4^-^PBMCs with 1 μg of anti-CD3 with/without $5 \times 10^3$ of the indicated suppressor cells. Data are representative of two separate experiments. (E and F) Fresh human tonsils were obtained from the National Disease Resource Interchange. (E) BCL6 and CXCR5 expression by Foxp3^+^Helios^-^ cells. (F) ACCENSE analysis of gated CD45RA^-^Foxp3^+^ Helios^-^ Treg cells. Cells were mapped by CD25, BCL6, CXCR5, and PD1. K-means were used to identify separate regions (Upper Left). (G) Histograms of ACCENSE-identified populations. Population 2, CD25^-^ Tfr cells; population 8, Tfr cells; population 1, 3–7 was recombined by concatenation of flow cytometry standard files.
Table S1. Primary and secondary antibodies for immunohistochemistry

<table>
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<tr>
<th>Antibody</th>
<th>Primary</th>
<th>Secondary</th>
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<tbody>
<tr>
<td>Foxp3</td>
<td>Rat anti-mouse Foxp3 Biotin (clone FJK-16s; eBioscience)</td>
<td>Donkey anti-rat Alexa Fluor 488 or 594</td>
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<tr>
<td>CD25</td>
<td>Goat anti-mouse CD25 antibody (AF2438, Accession no. Q54412; R&amp;D Systems)</td>
<td>Donkey anti-goat Alexa Fluor 568</td>
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<tr>
<td>IgD</td>
<td>Rat anti-mouse IgD AF647 (clone 11-26c.2a; Biolegend)</td>
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<tr>
<td>GL7</td>
<td>Rat anti-human/mouse GL7, eFluor660 (clone GL-7; eBioscience)</td>
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Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)