Supporting Online Material for

**Stability of the Regulatory T cell Lineage in Vivo**

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Materials and Methods

Mice
B6.SJL-Ptprca Pep3b/BoyJ (Ly5.1+ B6) mice were purchased from the Jackson Laboratories. Foxp3<sup>GFP</sup> and TCRβδ-deficient mice were bred in UW and MSKCC. R26Y mice were kindly provided by Dr. Frank Costantini. RAG2p<sup>GFP</sup> spleens and thymi were kindly provided by Dr. Pamela Fink. Thy1.1 and BDC2.5 mice were obtained from the Modified Mouse Core of the JDRF Center to Immune Tolerance in Diabetes; Foxp3<sup>YFP</sup> (IRES-GFP knockin into the Foxp3 locus) on the NOD and B6 backgrounds were kindly provided by Dr. Vijay Kuchroo. All mice were maintained and handled at the University of Washington, MSKCC, Joslin Diabetes Center and Harvard Medical School SPF mouse facilities according to institutional guidelines.

Generation of Foxp3<sup>eGFP-Cre-ERT2</sup> mice
The Foxp3<sup>eGFP-Cre-ERT2</sup> knock-in allele was generated by replacement of the YFP-Cre coding sequence in the previously described targeting construct used to generate Foxp3<sup>YFP-Cre</sup> allele with a fragment encoding the eGFP-Cre-ERT2 fusion protein (24). Cre-ERT2 cDNA (plasmid kindly provided by Dr. Pierre Chambon) was cloned in frame with eGFP coding DNA. Gene targeting and generation of knock-in mice were carried out as previously described (24).

Tamoxifen treatment, IL-2 neutralization and CD40 cross-linking
Tamoxifen (Sigma) was dissolved in olive oil (Fluka) to a final concentration of 40mg/ml. Mice received three doses of tamoxifen (8 mg each) at days 0, 1, and 3 by oral gavage. Mice were analyzed at day 14 or later after the initiation of treatment. For some experiments mice were thymectomized two weeks prior to tamoxifen treatment. To induce IL-2 blockade tamoxifen-treated mice were injected i.p. with 1mg of rat anti–mouse IL-2 rat mAb (clone S4B6) (BioXCell) (15) or rat IgG (Caltag) (control) and analyzed 9 days later. Th1 responses in tamoxifen treated mice were induced by i.p. injection of 25ug of agonistic rat anti mouse CD40 mAb (clone FGK45) at days 0, 2 and 4 (BioXCell) or control rat IgG (Caltag) as described (23). Mice were analyzed 14 days and 5 months after the first injection.

Cell purification and flow cytometry
Cells were stained with the following directly conjugated antibodies: anti-CD4 (RM4-5), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (2E7), anti-CD25 (PC61.5), anti-CD8 (53-6.7), anti-CTLA-4 (UC10-4B9), anti-GITR (YGL-386), anti-ICOS (7E.17G9), anti-CXCR3 (CXCR3-173), anti-Foxp3 (FJK-16s), anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-5H4), anti-TNFα (MP6-XT22), anti-IL-17 (eBio17B7 or TC11-18H10.1 from Biolegend), anti-T-bet (4B10) (all from eBioscience)
and Ki-67 (B56) (BD Pharmingen). Flow cytometric analysis was performed using an LSRII flow cytometer (BD Bioscience) and FlowJo software (Tree Star). For cell isolation, CD4+ T cells were purified from total splenic or lymph node cell suspensions using magnetic beads and further sorted using an Aria II cell sorter (BD Bioscience) or MoFlo sorter (Dako). For intracellular cytokine staining lymph nodes and spleens were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) in the presence of Golgi Block (BD Pharmingen) for 5 hours at 37°C, 5% CO2. Cells were stained with antibodies for intracellular markers or cytokines according to manufacturer’s protocol.

**Listeria monocytogenes infection**
Mice were infected i.v. with *Listeria monocytogenes* (LM) (3000-5000 CFU per mouse) and analyzed 9 days later. For LM specific re-stimulation splenocytes from infected mice were incubated in the presence of 10^{-6}M LL0190-201 peptide (NEKYAQAYPNVS) and Golgi Block (BD Bioscience) for five hours at 37°C in CO2 incubator and IFN-γ and IL-17 production was assessed using flow cytometry.

**In vitro mRNA decay**
For assessment of mRNA decay rate, FACS sorted T cells were incubated in flat bottom 96 well plates for 0, 1, and 2 hours at 37°C, 5% CO2 in the presence of 5ug/ml of actinomycin D (Sigma). RNA was isolated using TRIzol® (Invitrogen) according to the manufacturer’s protocol and cDNA was synthesized using total RNA as a template with random hexamer primers and SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Relative abundance of Foxp3 cDNA was measured by qPCR. Real-time PCR primer sequences complementary to exon 11 of the Foxp3 gene and HPRT: hprt 5’-agctactgtgaatgatcagtcaacg and 5’-agaggtccttttcaccagca; Foxp3 Exon 11  5’-acatgaagagcctgccttggtaca and 5’-tggttccagatgttgtgggtgagt.

**Adoptive T cell transfer**
For adoptive transfers, GFP+CD4+ Treg cells were sorted twice by FACS from Ly5.2+ Foxp3^{GFP} mice or Foxp3^{eGFP-Cre-ERT2} x R26Y mice (purity > 98%). Total Ly5.1+ CD4+ T cells were isolated from C57/B6 mice using magnetic beads (Invitrogen). 5x10^{5} Ly5.2+GFP+ Treg cells and 1.5x10^{6} Ly5.1+CD4+ T cells were injected i.v. into TCRβδ-deficient recipient mice and Ly5.2+ T cells were analyzed for Foxp3 expression three weeks after transfer. For in vivo Treg cell induction assays, GFP-CD4+CD62Llow cells and GFP+CD4+ were sorted by FACS from Ly5.2+ Foxp3^{eGFP-Cre-ERT2} x R26Y mice and co-transferred with GFP+CD4+ or GFP-CD4- cells, respectively, that were isolated by FACS from Ly5.1+ Foxp3^{GFP} mice (purity > 98%). 5x10^{5} GFP’ Treg cells and 1.5x10^{6} GFP+CD4+ T cells were injected i.v. into TCRβδ-deficient recipient mice, which were treated with tamoxifen two weeks after transfer. Ly5.2+ T cells were analyzed for YFP and Foxp3 expression six weeks after transfer. For adoptive transfers into the diabetes model, 1-2x10^{5} CD4+GFP+ cells were double sorted from NOD.Foxp3^{GFP}.BDC2.5 mice (purity >99%) and transferred i.v. into congenic 12-week-old NOD.Thy1.1 recipient mice. Analysis of the pancreas, spleen, and pancreatic lymph node was performed 4 weeks post-transfer. Thy1.2 donor cells were analyzed by flow cytometry for GFP.
expression, and after intracellular staining for IFN-γ, IL-17A or FoxP3 expression. For the arthritis model, 1-2x10^5 CD4^+GFP^+ cells were double sorted from K/BxN.Foxp3^{300} mice, and transferred i.p. into 20 day old K/BxN.CD45.2 mice. Tissues were collected from 35-day-old recipient K/BxN mice, when clear signs of clinical arthritis were exhibited, evidenced by thickening of ankles and front paws. CD45.1^+/CD45.2^- donor cells were analyzed for GFP or intracellular IL-17A expression.
Supplemental Figure 1. *Foxp3<sup>GFP-Cre-ERT2</sup> x R26Y* mice allow for inducible labeling and tracing of Foxp3 expressing cells *in vivo.*

Schematic of knock-in and breeding strategy used to generate *Foxp3<sup>GFP-Cre-ERT2</sup> x R26Y* mice. Administration of tamoxifen to *Foxp3<sup>GFP-Cre-ERT2</sup> x R26Y* mice results in three populations of CD4<sup>+</sup> cells defined by expression of YFP and GFP.
Supplemental Figure 2. Normal thymic and peripheral T cell subsets in Foxp3<sup>GFP-Cre-ERT2</sup> x R26Y mice

Flow cytometric analysis of thymocytes, splenocytes, and lymph node cells in Foxp3<sup>GFP-Cre-ERT2</sup> x R26Y mice demonstrates normal frequencies of CD4, CD8, and Foxp3 expressing cells.
Supplemental Figure 3. YFP and GFP expression in Foxp3<sup>Cre-ERT2</sup> x R26Y mice
(A) GFP expression correlates with expression of Foxp3 in Foxp3<sup>Cre-ERT2</sup> x R26Y mice. GFP<sup>-</sup> cells were sorted by FACS to ≥99% purity and stained for intracellular Foxp3. (B) Flow cytometric analysis of YFP and GFP expression by splenocytes from untreated Foxp3<sup>Cre-ERT2</sup> x R26Y mice demonstrates that YFP is not expressed prior to tamoxifen administration. (C) Tamoxifen induced expression of YFP is restricted to Foxp3<sup>+</sup> cells. Labeling was performed as in (A) and YFP<sup>+</sup> cells were sorted by FACS to ≥99% purity and stained for intracellular Foxp3.
Supplemental Figure 4. Inducible labeling of Foxp3+ cells in Foxp3GFP-Cre-ERT2 x R26Y mice.
Administration of tamoxifen results in three populations of CD4+ cells defined by expression of YFP and GFP. Flow cytometric analysis of CD25 and Foxp3 expression in YFP+, YFP-GFP+, and YFP-GFP- cells is shown. Data are representative of three independent experiments with ≥3 mice per group in each experiment. All mice were 6-8 weeks of age at the time of tamoxifen administration.
Supplemental Figure 5. YFP-tagged Treg cells are phenotypically similar to untagged Foxp3-expressing Treg cells

Expression of GITR, ICOS, CD103, CD62L and CD44 in YFP+, YFP-GFP+ and YFP-GFP- cells (red, blue, and black lines, respectively).
Supplemental Figure 6. Maintained expression of Foxp3 and Treg cell phenotype in YFP+ cells.
(A) YFP+ cells from purified by FACS from spleens and lymph nodes of Foxp3<sup>CreERT2</sup> x R26Y mice and stained for intracellular Foxp3 5 months post tamoxifen labeling. (B) Expression of ICOS and GITR in YFP+GFP- and YFP-GFP+ Treg cells. Data are representative of three independent experiments (n≥3 per group). Mice were 6-8 weeks of age at the time of labeling.
Supplemental Figure 7. Proliferation profiles of YFP labeled and unlabeled GFP+ cells in Foxp3GFP-CreERT2 x R26Y mice.

YFP+ and YFP-GFP+ Treg cells proliferate to a similar extent in the steady state as indicated by intracellular Ki67 staining of CD4+ cells isolated from spleens and lymph nodes of Foxp3GFP-CreERT2 x R26Y mice 5 months after tamoxifen administration.
Supplemental Figure 8. IL-2 deprivation results in a small, but detectable decrease in Foxp3 expression.

(A–C) Tamoxifen-treated Foxp3<sup>GFP-Cre</sup> x R26Y mice were injected with IL2 blocking antibody or control IgG and Foxp3 expression in FACS-sorted YFP<sup>+</sup> cells was assessed 9 days later. (A) Intracellular Foxp3 staining of YFP<sup>+</sup> cells from lymph nodes. Numbers represent percentages of cells in the indicated gate. (B) Percentages of YFP<sup>+</sup> cells expressing Foxp3. Each symbol represents an individual mouse. (C) Relative expression of Foxp3 in YFP<sup>+</sup> cells from lymph nodes of mice treated with IL2 blocking antibody. (D) Representative intracellular staining for IFNγ, IL-17, IL-2, and TNFα. Data are representative of three independent experiments (n≥2 per group). Mice were 6-8 weeks of age at the time of antibody administration.
Supplemental Figure 9. Stability of Foxp3 mRNA in *Foxp3*^GFP-Cre-ERT2^ x R26Y mice

Relative amounts of Foxp3 mRNA at 0 and 2 hours of culture in the presence of actinomycin D in YFP^+ and GFP^+ cells from sorted *Foxp3*^GFP-Cre-ERT2^ x R26Y and *Foxp3*^GFP^ mice, respectively. Data are representative of two independent experiments.
Supplemental Figure 10. Foxp3 expression is stably maintained following lymphopenic transfer. (A) Representative plots of Foxp3 expression and percentages of cells remaining Foxp3\(^+\) among transferred GFP\(^+\) cells from Foxp3\(^{3GFP}\) and Foxp3\(^{3GFP-Cre-ERT2}\) x R26Y mice. (B) Percentage of Foxp3\(^+\) cells among adoptively transferred CD45.2\(^+\) T cells. Error bars represent standard deviation of the mean. Data are representative of two independent experiments with \(\geq 3\) mice per group.
**Supplemental Figure 11.** Infection with *L. monocytogenes* leads to upregulation of T-bet in Foxp3+ and Foxp3- cells and antigen-specific IFNγ production by Foxp3- cells

(A) Representative flow-cytometric analysis of T-bet expression in uninfected and infected mice 9 days after infection with *L. monocytogenes.* (B) Production of IFNγ in cells from infected and uninfected mice in response to restimulation with LLO190-201 peptide *in vitro.*
Supplemental Figure 12. Foxp3 expression is stably maintained in Th1 inflammation.
Tamoxifen-treated Foxp3GFP-CreERT2 x R26Y mice were injected with agonistic CD40 antibody or control IgG and analyzed 14 days and 5 months post injection. (A) CD40 cross-linking induces expression of CXCR3 and Tbet in YFP+ CD4+ T cells in lymph nodes. Lymph nodes were stained for Foxp3 14 days after injection of antibody and analyzed for expression of CXCR3 and T-bet among YFP+ cells. (B) IL-2 and IFNγ production 14 days post treatment with CD40 antibody or control IgG. (C) Flow cytometric analysis of Foxp3 expression by splenic YFP+ cells 5 months after treatment with CD40 antibody (red) or control-IgG (grey) (3 mice per group). (D) Percentages of Foxp3 expressing cells among YFP+ cells 14 days and 5 months after treatment with CD40 antibody or rat-IgG. Each symbol represents an individual mouse. Data are representative of three independent experiments (n≥2 per group). Mice were 6-8 weeks of age at the time of infection.
Supplemental Figure 13. Peripheral Treg cell generation in a lymphopenic host is accompanied by some transient Foxp3 expression

Proportion of Foxp3 expressing cells among CD45.2+ YFP-labeled cells derived from YFP-GFP- or YFP-GFP+ cells following co-transfer of these cells with CD45.1+ GFP+ or GFP- cells, respectively, into lymphopenic animals. Recipients received tamoxifen 2 weeks after transfer and were analyzed 4 weeks later.