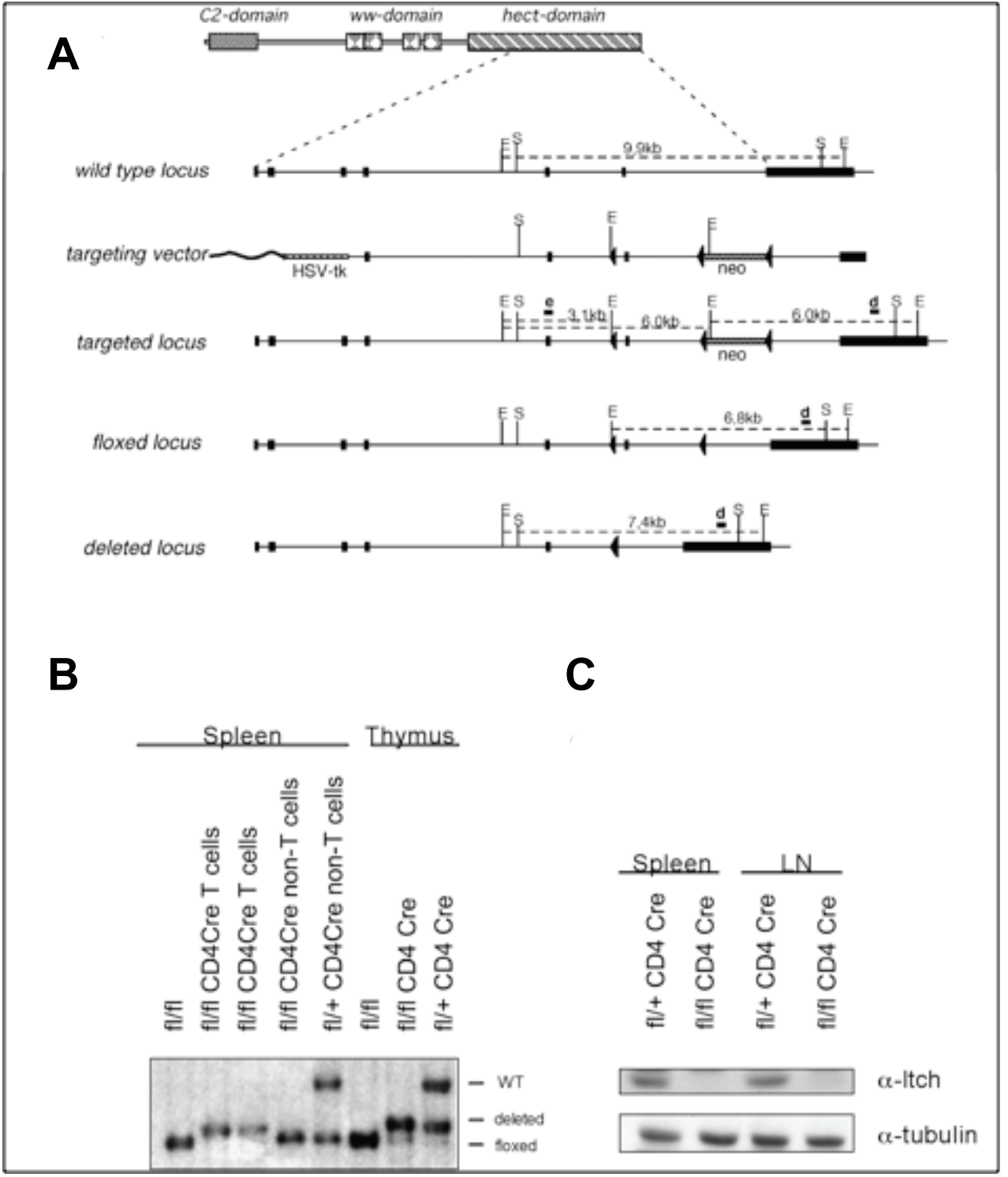
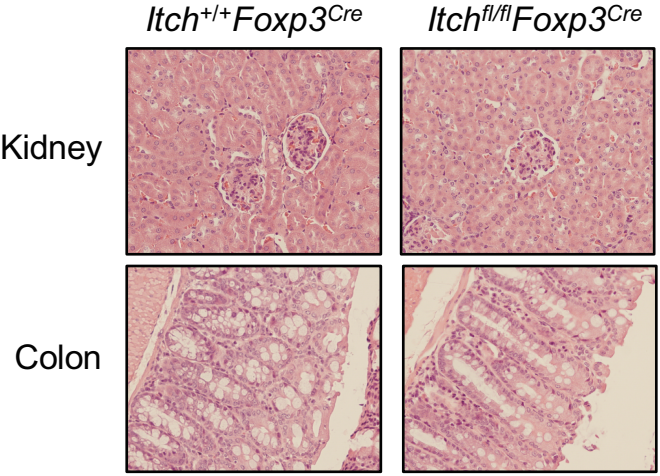
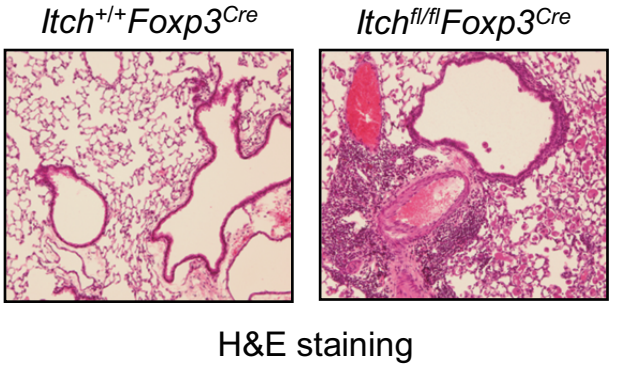


Supplemental Fig 1.



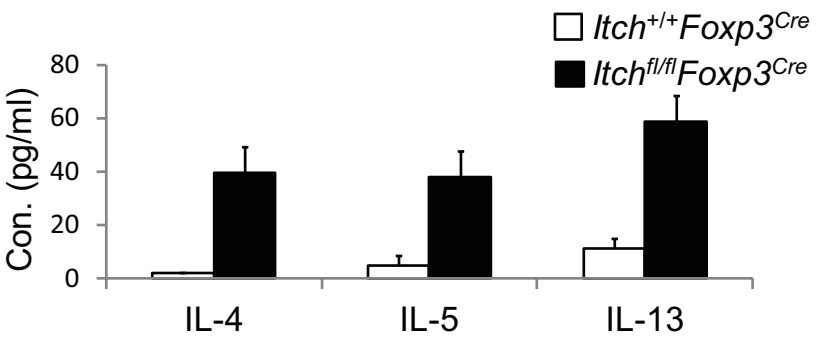
Supplemental Fig 2.

A



H&E staining

B



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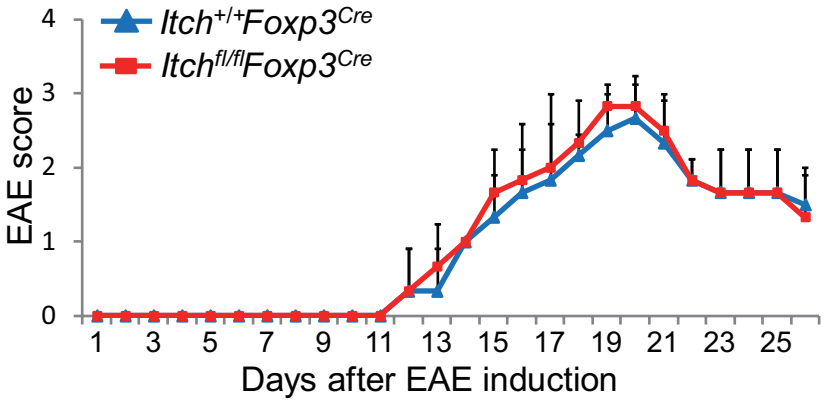

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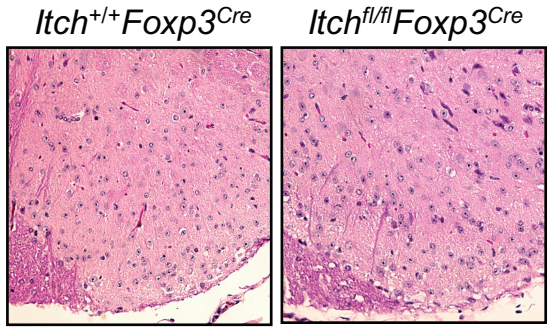

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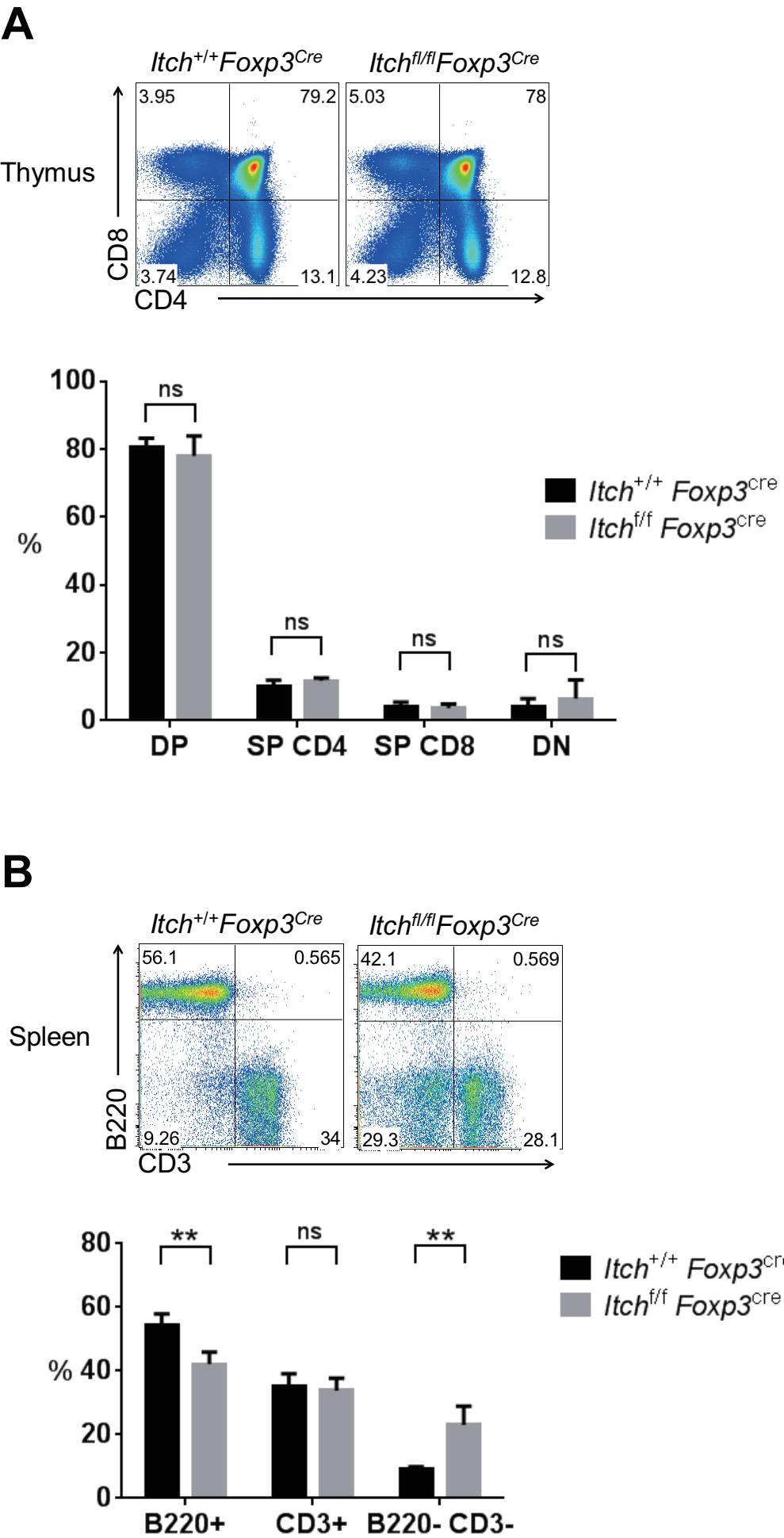

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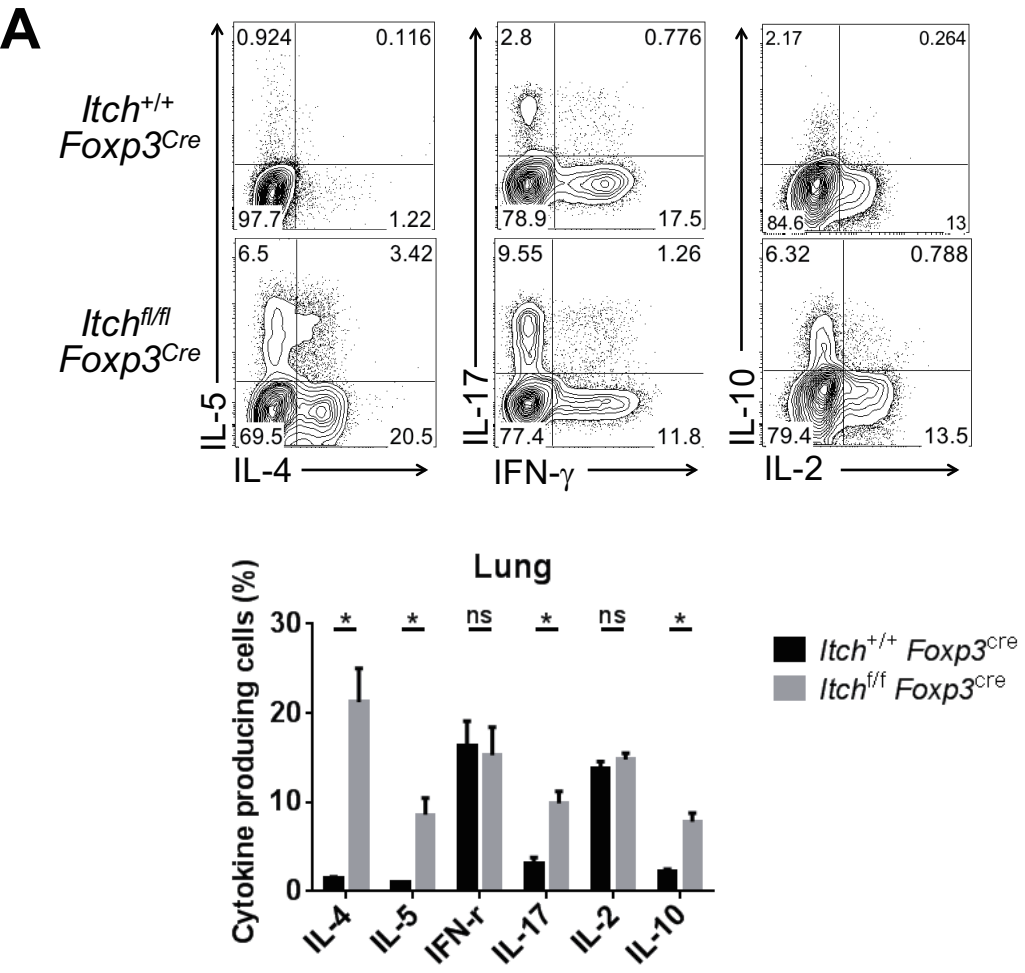

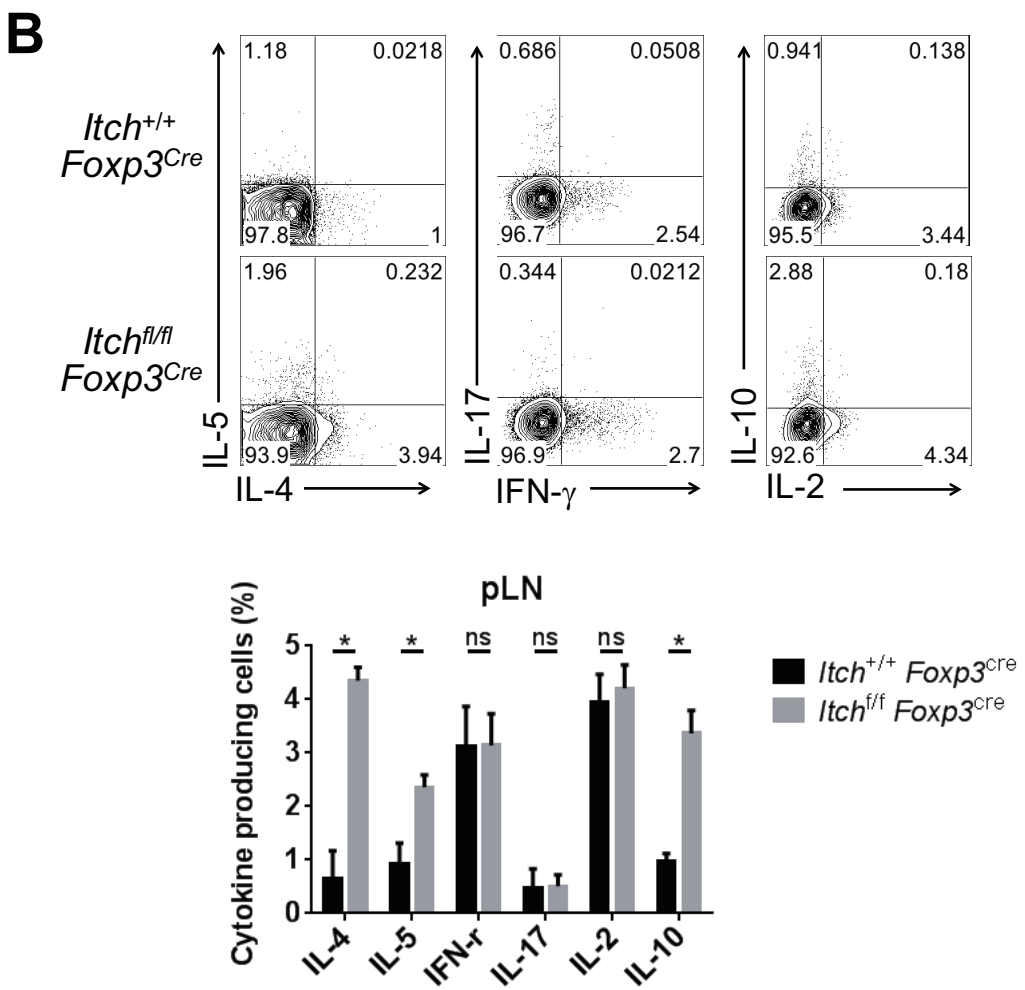

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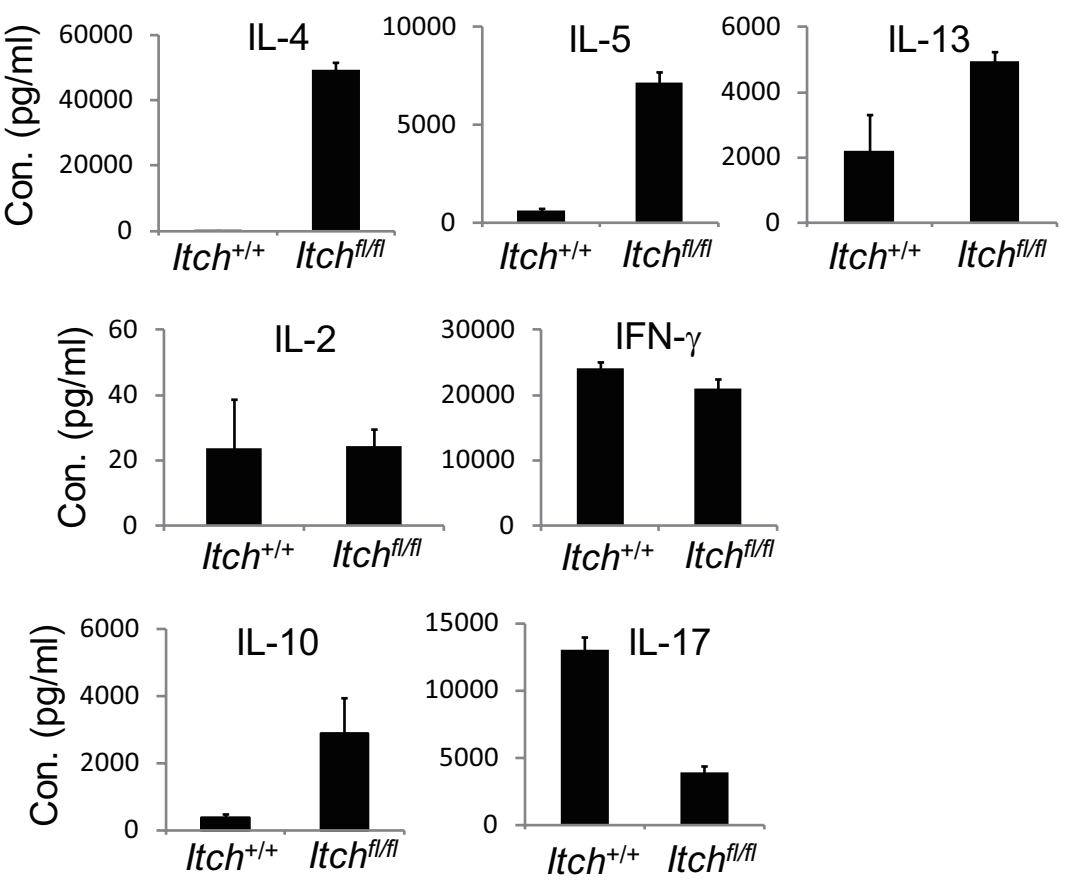

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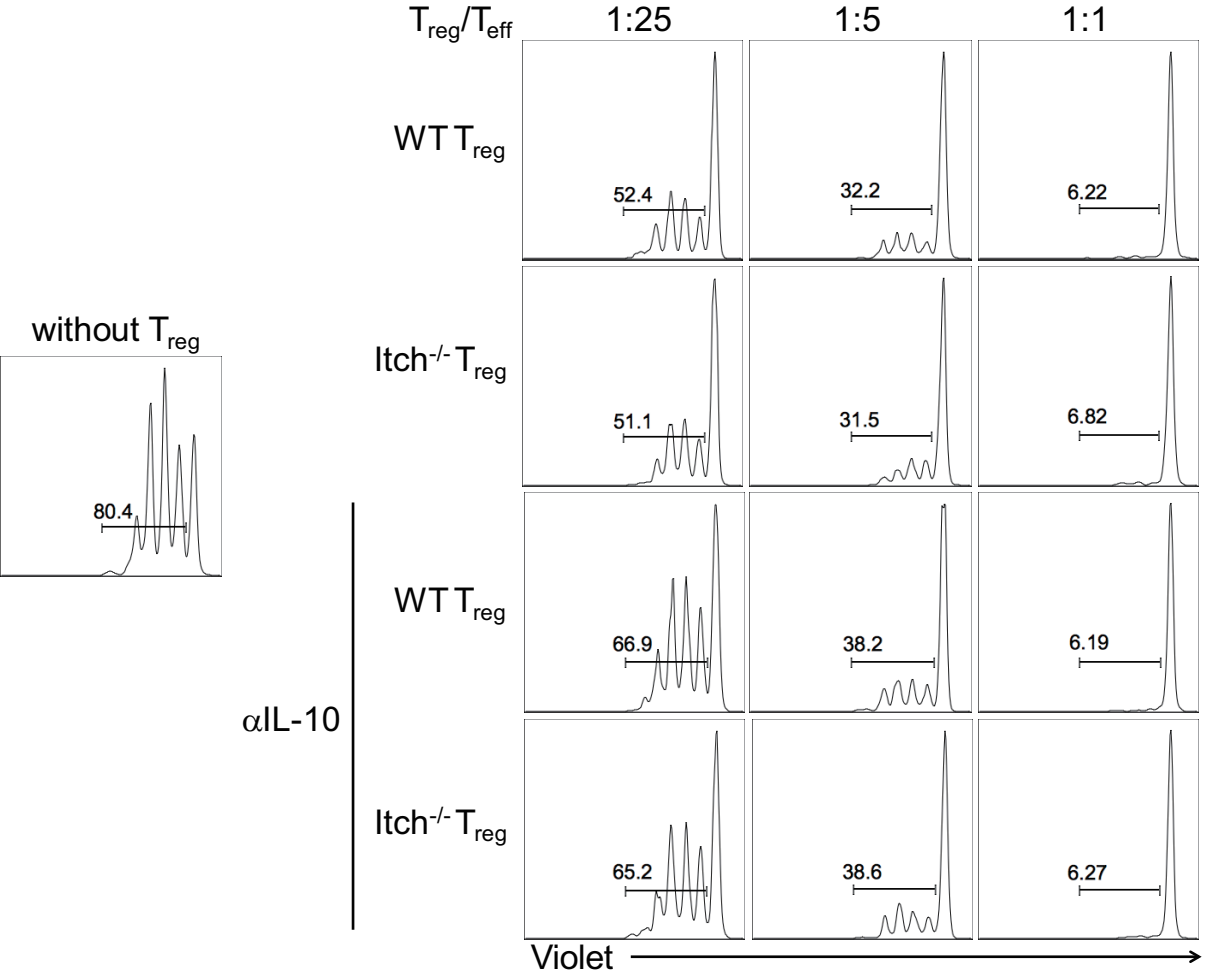

Supplemental Fig 8.

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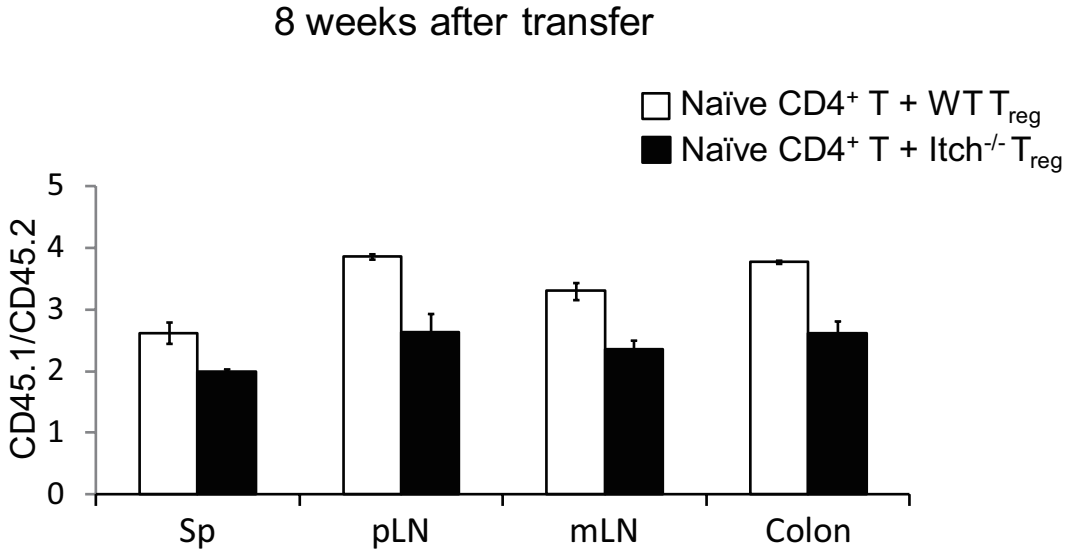

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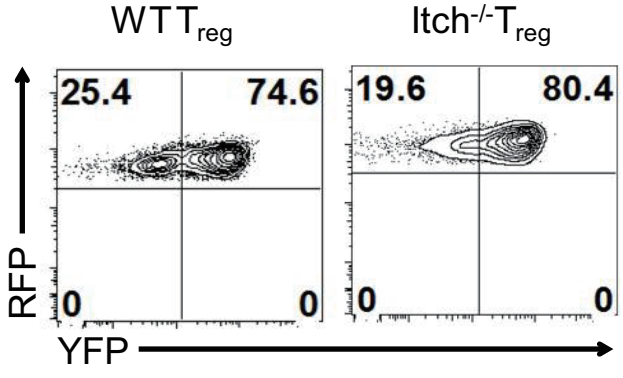

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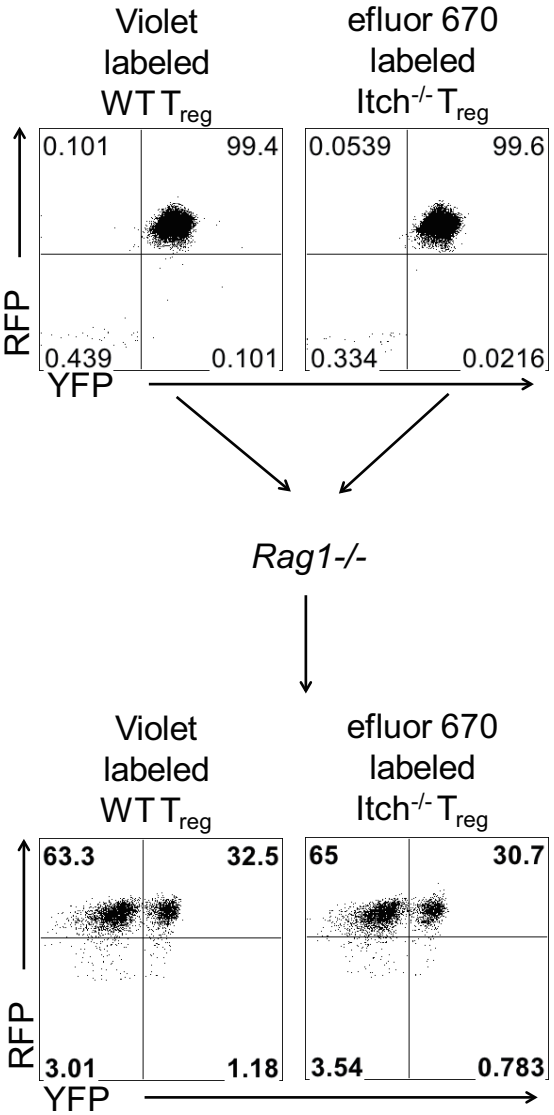

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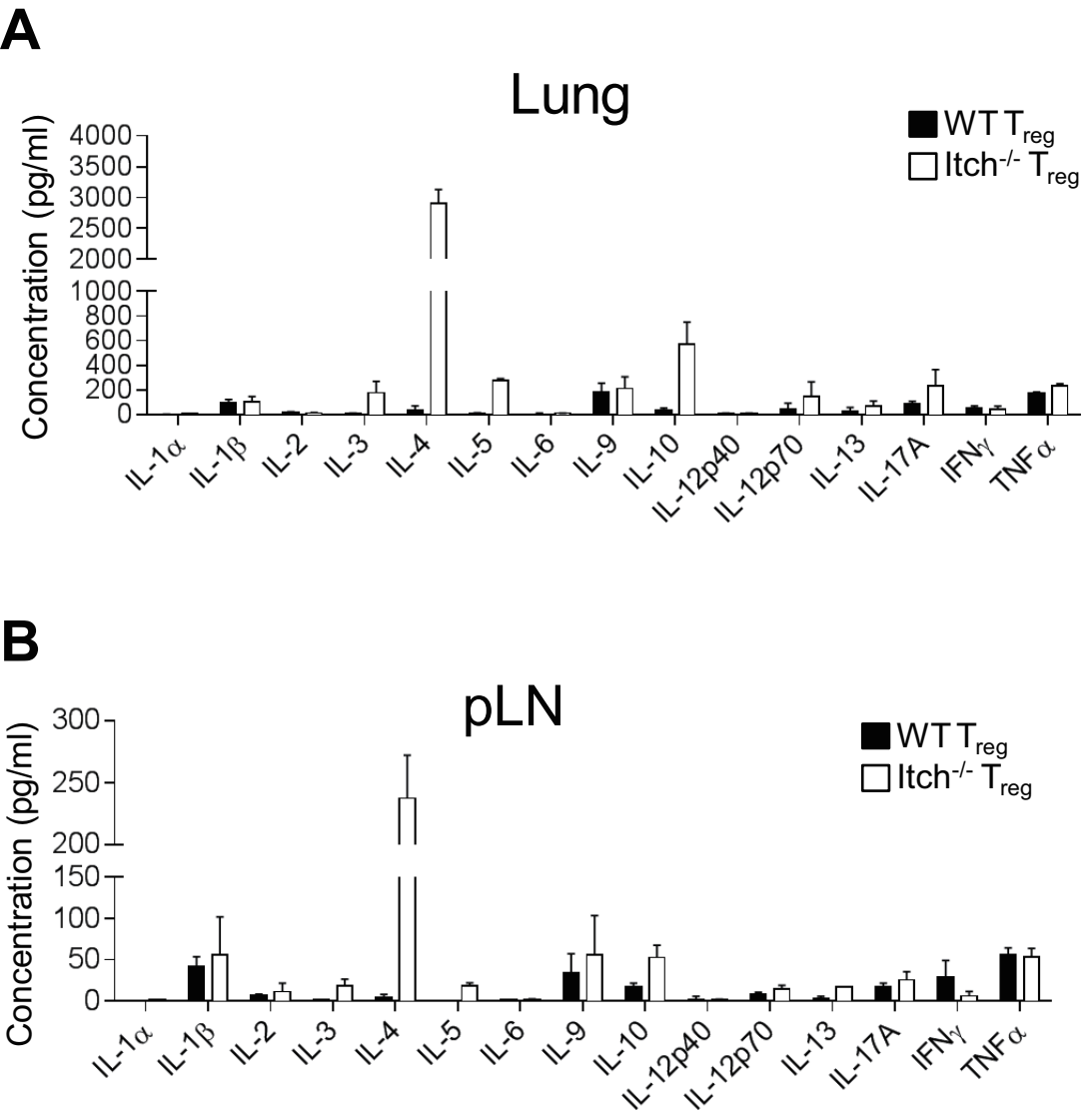

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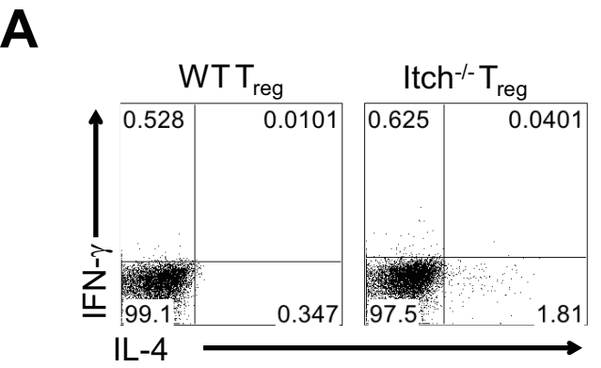

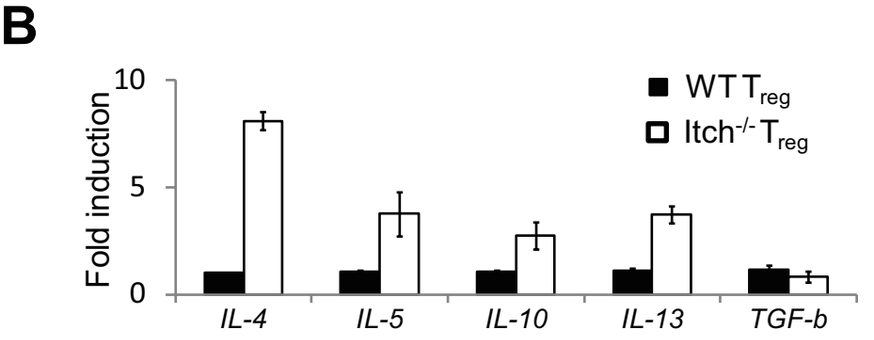

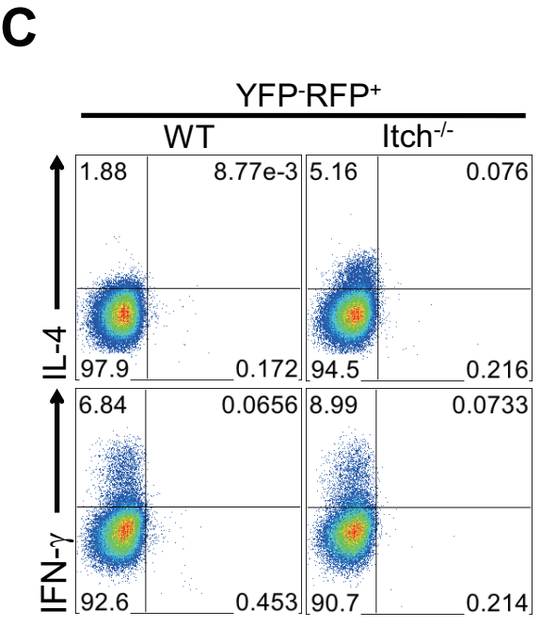

Supplemental Fig 12.

Supplemental Figure legends

Supplemental Figure 1. Generation of *Itch*$^{fl/fl}$ mice

(A) Strategy for *Itch* gene targeting. (B) Deletion efficiency in *Itch*$^{fl/fl}$*CD4*^{Cre} mice. Southern blot analysis of genomic DNA from splenocytes or thymocytes digested with EcoRV. (C) Western blot analysis of the wild-type Itch protein in CD4^{+} cells purified from the spleen and lymph nodes of *Itch*$^{fl/fl}$ *CD4*^{Cre} mice and littermate controls.

Supplemental Figure 2. Hematoxylin and eosin-stained tissue sections from *Itch*$^{+/+}$*Foxp3*^{Cre} and *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice

The tissue sections of kidney and colon of *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice did not exhibit noticeable lesions.

Supplemental Figure 3. Enhanced antigen-induced airway inflammation in mice harboring Itch-deficient T_{reg} cells

(A) Twenty-four hours after the last OVA challenge, lung tissue sections from *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice were stained with H&E to visualize the inflammatory infiltrates. (B) IL-4, IL-5 and IL-13 concentrations in BAL fluid were measured by ELISA 24 hr after the last OVA challenge. Error bars indicate the mean (± SD).

Supplemental Figure 4. Normal EAE development in *Itch*$^{+/+}$*Foxp3*^{Cre} and *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice

(A) Groups of *Itch*$^{+/+}$*Foxp3*^{Cre} and *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice were immunized s.c. with MOG35–55 in CFA. Pertussis toxin was given i.p. at the time of immunization and 48 h later. Mice were monitored daily

for 25 days for clinical signs of EAE and were scored using an arbitrary scale of 0–5 as described in Methods. (B) Histopathology of spinal cord tissue section on day 25. Sections were stained with H&E. All data are representative of two independent experiments with ≥ 2 mice per group.

Supplemental Figure 5. Impaired immune homeostasis in mice harboring Itch-deficient T_{reg} cells

(A) Thymocyte development in *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice. Total thymocytes from 8-week-old *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice and *Itch*$^{+/+}$*Foxp3*^{Cre} littermates were analyzed by flow cytometry for CD4 and CD8 expression. (B) Flow cytometry analysis of the percentage of B220^{+} and CD3^{+} T cells from the spleen of 8-week-old *Itch*$^{fl/fl}$*Foxp3*^{Cre} mice and *Itch*$^{+/+}$*Foxp3*^{Cre} littermates. All results are representative of at least three experiments. Error bars indicate the mean (± SD).

Supplemental Figure 6. Augmented T_H2 cytokine production in mice harboring Itch-deficient T_{reg} cells

Total cells were isolated from collagenase-digested lungs (A) or peripheral lymph nodes (B) of *Itch*$^{fl/fl}$ *Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice, and then stimulated with PMA and ionomycin in the presence of Golgi-stop for 4 hr. The frequency of the indicated cytokine-producing CD4^{+} effector T cells (gated on CD4^{+} cells) was determined by flow cytometry. A representative of two independent experiments is shown.

Supplemental Figure 7. Increased production of T_H2 cytokines by splenic T cells from *Itch*$^{fl/fl}$ *Foxp3*^{Cre} mice

Splenocytes from *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice were cultured in a 96-well plate (1x10^{6} cells/well), and stimulated with anti-CD3 antibody (1 μg/ml) for 4 days. Supernatants were collected and cytokine levels were measured by ELISA. Results were representative of two experiments (mean ± SD of triplicates done in one experiment).

Supplemental Figure 8. Normal in vitro suppressive activity of Itch-deficient T_{reg} cells

Naive $CD4^+CD25^-$ T cells ($1x10^5$) labeled with violet dye were cultured together in 96-well plates for 4 d with an increasing ratio of sorted T_{reg} cells in the presence of anti-CD3 (1 μg/ml) plus irradiated splenocytes ($5x10^4$). Cell proliferation was measured by violet dilution. Where indicated, anti-IL-10 (10 μg/ml; BD pharmingen) antibody was added. Results were representative of two experiments.

Supplemental Figure 9. Analysis of Itch-deficient T_{reg} cells.

(A) Abnormal expansion of Itch-deficient T_{reg} cells under lymphopenic conditions. *Rag1*$^{-/-}$ mice were given sorted $CD4^+YFP^+$ ($CD45.2^+$) T_{reg} cells from *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice, together with $CD4^+CD45RB^{hi}$ ($CD45.1^+$) naïve T cells. The ratios of $CD4^+CD45RB^{high}$ ($CD45.1^+$) to $CD4^+YFP^+$ ($CD45.2^+$) T_{reg} cells in the spleen (SP), peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN) and colonic lamina propria (colon) of the *Rag1*$^{-/-}$ recipient mice at 8 weeks after transfer. (B) T_{reg}-specific Itch deletion has no effect on Foxp3 stability in T_{reg} cells. YFP^+RFP^+ T_{reg} cells sorted from *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice were cultured for 5 days in 96 well plates coated anti-CD3/CD28 antibodies in the presence of IL-2. The percentage of ex-T_{reg} cells was determined by analyzing YFP and RFP expression using FACS. Representative plots from 3 experiments.

Supplemental Figure 10. T_{reg}-specific Itch deletion has no effect on Foxp3 stability in T_{reg} cells

Itch-sufficient $CD4^+YFP^+RFP^+$ T_{reg} cells labeled with violet dye and Itch-deficient $CD4^+YFP^+RFP^+$ T_{reg} cells labeled with eFluor670 dye were adoptively co-transferred into *Rag1*$^{-/-}$ mice. 4 weeks after the transfer, the percentage of ex-T_{reg} cells was determined by analyzing YFP and RFP expression using FACS.

Supplemental Figure 11. Cytokine profiles of Itch-deficient T_{reg} cells

YFP^+ T_{reg} cells were isolated by flow cytometry from lungs (A) or peripheral lymph nodes (B) of *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice. They were stimulated with anti-CD3/CD28 antibodies for 2 days. Supernatants were collected, and cytokine concentrations were measured by Bio-plex array.

Error bars indicate the mean (± SD).

Supplemental Figure 12. Increased T_H2 cytokine production in Itch-deficient T_{reg} cells

(A) FACS-sorted $CD4^+YFP^+$ T_{reg} cells from *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice were stimulated in vitro for 16 hr, and Golgi-stop was added during the last 4 hr of culture. IL-4 and IFN-γ expression was determined by intracellular staining. Representative results of three experiments are shown. (B) Relative mRNA amounts of *IL-4*, *IL-5*, *IL-10*, *IL-13* and *TGF-β* in sorted YFP^+ T_{reg} cells from the spleens were determined by RT-PCR. (C) FACS-sorted $CD4^+YFP^-RFP^+$ ex-T_{reg} cells from the spleen of *Itch*$^{fl/fl}$*Foxp3*^{Cre} and *Itch*$^{+/+}$*Foxp3*^{Cre} mice were stimulated with PMA and ionomycin in the presence of Golgi-stop for 4 hr, and stained for IL-4 and IFN-γ. A representative of two experiments is shown (mean ± SD of triplicates done in one experiment).