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ICOS-deficient regulatory T cells display normal induction of *Il10* but readily downregulate expression of Foxp3

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Abstract

The ICOS pathway has been implicated in the development and functions of regulatory T cells (Treg cells) including those producing IL-10. Treg cell-derived IL-10 is indispensable for the establishment and maintenance of intestinal immune homeostasis. We examined the possible involvement of the ICOS pathway in the accumulation of murine colonic Foxp3- and/or IL-10-expressing cells. We show that, ICOS deficiency does not impair induction of IL-10 by intestinal CD4 T cells, but instead triggers substantial reductions in gut-resident and peripherally-derived Foxp3^{+} Treg cells. ICOS deficiency is associated with reduced demethylation of Foxp3 CNS2 and enhanced loss of Foxp3. This instability significantly limits the ability of ICOS-deficient Treg cells to reverse ongoing inflammation. Collectively, our results identify a novel role for ICOS co-stimulation in imprinting the functional stability of Foxp3 that is required for the retention of full Treg cell function in the periphery.

INTRODUCTION

The B7 family of ligands expressed by antigen-presenting cells (APC) interact with the CD28 family of co-receptors expressed on T cells, delivering unique signals that were historically classified as either co-stimulatory or co-inhibitory. More recently, it has become accepted that these interactions can have mechanistic effects beyond just modulation of T cell activation. For example, signaling via T cell co-receptors including CD28, CTLA-4, herpes virus entry mediator (HVEM), programmed cell death-1 (PD-1) and the inducible-co-stimulator (ICOS) figure prominently in the development and functions of Treg cells - essential mediators of immune homeostasis (1–5).

Compared to wild type mice, *Icos*$^{-/-}$ mice harbor reduced Foxp3^{+} Treg cells in secondary lymphoid tissues (1, 6). Though dispensable for induction of Foxp3^{+}, ICOS labels Treg cells with superior suppressive capacity (5) and promotes resistance of Treg cells to cell death (7). The ICOS pathway has also been implicated in the maintenance and/or functions of CD4^{+} effector cells at homeostasis, following antigenic challenge, and during chronic

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FOOTNOTES

inflammation (6, 8–11). Thus, despite often being used as Treg cell marker, several of the functions of ICOS in T cell lineage maintenance also extend to effector and memory cells. Accordingly, a range of studies in animal models have positioned ICOS as a context-dependent negative or positive regulator, or even a non-factor in T cell-mediated diseases (12–14).

Loss-of-function polymorphisms in the gene encoding the ligand of ICOS (*ICOSL*) (15) confer susceptibility to inflammatory bowel disease but neither *Icos*$^{-/-}$ or *Icosl*$^{-/-}$ mice develop overt intestinal inflammation. This is despite many reported links between ICOSL-ICOS and CD4 T cell production of IL-10 (7, 16–19), which is critical for intestinal immune homeostasis (20, 21). Here, we show that ICOS deficiency actually results in increased induction of *Il10* in colonic CD4 T cells but reduced accumulation of murine large intestinal Foxp3^{+} cells, including microbiota-dependent, peripheral Treg (pTreg) cells. *Icos*$^{-/-}$ Treg cells displayed reduced demethylation of *Foxp3 CNS2* and preferentially down-regulated Foxp3 relative to *Icos*$^{+/+}$ Treg cells. The extinction of Foxp3 rendered *Icos*$^{-/-}$ Treg cells incapable of reversing gut inflammation. Our study identifies ICOS as an important mediator of Foxp3 stability that is dispensable for T cell production of IL-10 in the intestine.

MATERIALS and METHODS

Mice (C57BL/6).

CD45.1, *Rag1*−/−, and *Icos*−/−, and *Icosl*−/−, mice were purchased from Jackson Laboratories. *Foxp3*$^{\text{IRES-GFP}}$, CBir1 TCR transgenic, and *Myd88*$^{-/-}$.*Trif*$^{-/-}$ mice were gifts from Dr. V. Kuchroo, Dr. C. Elson, and Dr. S. Michalek, respectively. 10BiT mice have been previously described (22). All mice were bred and maintained at the University of Alabama at Birmingham in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

Antibodies and flow cytometry.

The following mouse antibodies were purchased from eBioscience: PE-anti-IL-17A, FITC-anti-Foxp3, APC-anti-Helios, PE-Cy7-anti-CD4 and anti-CD11c, Biotin-anti-ICOSL. The following were purchased from BD Biosciences: PE-anti-CD103, PerCP-anti-CD90.1, and PerCP-Cy5.5-anti-CD45.1. Samples were acquired on an LSRII instrument and data was analyzed using FlowJo software.

Lamina propria cell isolation.

The intestines were removed, stripped of mesenteric fat and luminal contents flushed using sterile HBSS. The epithelial layer was removed by incubating in HBSS with 154 μg/L L-dithioerythreitol (DTT) and 2 μM EDTA. Remaining tissue was digested with 100 U/ml collagenase IV and 20 μg/ml DNase (Sigma) for 30 min at 37°C with gentle stirring. Total lamina propria cells were purified on a 40%/75% Percoll gradient by room temperature centrifugation at 2000 rpm with no brake for 20 min.

Analysis of DNA methylation.

Bisulfite conversion, pyrosequencing and analysis were performed by EpigenDx (Hopkinton, MA). Assays ADS568-FS1 and ADS568-FS2 were used to analyze 9 CpGs of the mouse *Foxp3 CNS2* (−2369 to −2207 from the Foxp3 TSS).

T Cell Transfer Colitis.

CD45RB^{hi} T cells were FACS-purified from B6.CD45.1 splenocytes and 4×10^5 cells were injected into each *Rag1*$^{-/-}$ recipient. CD4$^+$GFP$^+$ cells were FACS sorted from *Icos*$^{+/+}$.*Foxp3*^{gfp} or *Icos*$^{-/-}$.*Foxp3*^{gfp} mice, both on the CD45.2 background. Each recipient received either PBS, 2×10^5 *Icos*$^{+/+}$ GFP$^+$, or 2×10^5 Icos$^{-/-}$ GFP$^+$ cells on either day 0 (prevention) or day 28 (reversal). At necropsy, representative sections of proximal, middle, and distal colon were fixed in formalin, embedded in paraffin, and 5 μm sections were cut and stained with hematoxylin & eosin (H&E). Histological scoring was performed in a blinded fashion. Remaining tissue was processed to isolate lamina propria cells.

Statistical analysis.

Statistical significance was calculated by unpaired Student's t test, Mann-Whitney U or ANOVA as appropriate, using Prism software (GraphPad; San Diego, CA). All p values ≤ 0.05 are considered significant and are referred to as such in the text.

RESULTS and DISCUSSION

Reduced accumulation of Foxp3$^+$ cells in the large intestine of *Icos*$^{-/-}$ mice

Our preliminary analysis of splenic and intestinal CD4 T cells suggested that co-expression of ICOS is not an essential feature of IL-10-competent cells, particularly in the large intestine (Supplemental Fig. 1). To further examine this, we compared the impact of ICOS deficiency on gut Treg cells with that of an extra-intestinal tissue (spleen) and an inductive site (thymus). Consistent with previous findings, there was no difference in Foxp3$^+$ cell frequencies in the thymus of *Icos*$^{+/+}$ and *Icos*$^{-/-}$ mice (Figure 1, A–B) but significant reductions in splenic and large intestine (LI) Foxp3$^+$ Treg cells in absence of ICOS. Importantly, in the LI, where we observed the greatest difference in frequency, we detected similar numbers of total CD4 T cells but significantly reduced numbers of Foxp3$^+$ cells (Figure 1C).

The transcription factor Helios is expressed by the majority of thymic Treg (tTreg) cells but only a minor fraction of colonic lamina propria Treg cells, although the utility of Helios as a marker of tTreg cells remains controversial. As expected (23), in *Icos*$^{+/+}$ mice the majority of Foxp3$^+$ cells in the thymus and spleen co-expressed high levels of Helios, and importantly, the same was true of *Icos*$^{-/-}$ Foxp3$^+$ cells in these 2 compartments (Figure 1, D–E). However, in the colonic lamina propria of *Icos*$^{-/-}$ mice, we detected a significantly increased representation of Helios^{Hi} and a concomitant decrease in Helios$^{Lo/-}$ Foxp3$^+$ cells (Figure 1, D–E). Analysis of T cell receptor excision circles (TREC) (24) indicated that there is a statistically-significant increase in thymic output of Treg cells in *Icos*$^{-/-}$ mice relative to wild type mice (Figure 1F), which may help explain the increase in Helios^{Hi} cells in the lamina propria.

To confirm the impact of ICOS deficiency on definitively colonic pTreg cells and avoid the controversy surrounding the use of Helios as a marker, we employed the CBir1 T cell receptor (TCR) transgenic system (25). This strain expresses a TCR specific for CBir1 flagellin, a microbiota-derived antigen detectable in healthy mice and humans. As with most TCR transgenics, these mice can rearrange a non-transgenic (endogenous) TCR alpha chain meaning they can generate self-antigen reactive Treg cells in the thymus (Figure 1G, left panel). However, by rendering these mice deficient for the recombination-activating gene-1 (*Rag1*), we eliminated thymic Treg cell development (Figure 1G, center and right panels). Therefore, any Treg cell detected in the periphery of these mice is a bona fide pTreg cell. In healthy, 6–8-week-old CBir1.*Rag1*$^{-/-}$ mice, ICOS deficiency resulted in reduced numbers and frequencies of Foxp3+ LI Treg cells, despite an elevated number of total CD4+ T cells (Figure 1, H–I).

Importantly, developing pTreg cells likely receive an ICOSL signal since lamina propria CD11c^{+}CD103^{+} dendritic cells express ICOSL. This expression occurs independent of the microbiota or TLR signaling, suggesting that it is developmentally regulated (Supplemental Fig. 2, A-E). The same signal is likely available to developing tTreg cells since ICOSL is also expressed by MHCII-expressing thymocytes (Supplemental Fig. 2, F-G).

Expression of *Il10* by intestinal CD4 T cells independent of ICOS

To determine whether the impact of ICOS on colonic Foxp3^{+} cells extends to IL-10-producing cells, we utilized IL-10 BAC-In transgenic (10BiT) mice in which induction of *Il10* results in surface expression of Thy-1.1 (CD90.1) (22). In *Icos*$^{+/+}$ mice, the vast majority of colonic IL-10-competent cells co-express Foxp3, with limited numbers of Foxp3-Thy1.1^{+} cells detected. In contrast, in *Icos*$^{-/-}$ mice, we found that the majority of IL-10-competent cells were actually Foxp3^{-} cells (Figure 2A). Overall, ICOS deficiency led to a significant reduction in the proportion of Foxp3^{+}Thy1.1^{+} cells among CD4 T cells as a direct result of reduced Foxp3. (Figure 2, A–B). However, this was counterbalanced by significant increases in Foxp3^{-}IL-10^{+} cells. The net effect was that the total expression of IL-10 by CD4 T cells, as determined by cell frequencies or fluorescence intensity of Thy1.1 expression, was not diminished (Figure 2, A–C).

Consistent with previous results (26), the majority of colonic Foxp3^{+}IL-10^{+} cells in wild type mice were Helios$^{Lo/-}$. However, *Icos*$^{-/-}$ mice displayed a substantial population of Helios-expressing Foxp3^{+}IL-10^{+} cells (Figure 2D). Thus, despite the reduced frequency of Foxp3^{+} cells in *Icos*$^{-/-}$ mice, independent of Helios expression, the proportion that expressed IL-10 and the levels of IL-10 expression remained largely unchanged relative to *Icos*$^{+/+}$ mice (Figure 2, D–F). To eliminate any possible host-intrinsic effects on the foregoing results, we performed adoptive transfer of CD4 single-positive, Foxp3- thymocytes from congenically-marked 10BiT.Foxp3^{gfp}.*Icos*$^{+/+}$ (CD45.1) and 10BiT.Foxp3^{gfp}.*Icos*$^{-/-}$ (CD45.2) mice into the same T cell-deficient (*Tcrβδ*$^{-/-}$) recipients (Figure 2G). After 3 weeks, despite similar frequencies of colonic CD4 T cells derived from each source, the frequency of *Icos*$^{+/+}$ Foxp3^{+} cells was approximately 5 times that of *Icos*$^{-/-}$ and conversely, IL-17 frequencies were elevated in the latter. The frequency of IL-10-competent CD4 T cells was similar in both *Icos*$^{+/+}$ and *Icos*$^{-/-}$ cells (Figure 2, H–I) and

there was a significant increase of Foxp3^{-}IL-10^{+} cells among *Icos*$^{-/-}$ colonic CD4 T cells, collectively mimicking intact mice (Figure 2, J–K).

Interestingly, among lamina propria CD4 T cells, the frequencies of IL-10-competent cells actually increased with aging (Supplemental Fig. 3, A-B), suggesting a potentially compensatory role for IL-10 in the face of the colonic Treg cell deficits in *Icos*$^{-/-}$ mice. Collectively, these results argue that ICOS is dispensable for induction of *Il10* in intestinal Treg cells.

ICOS-deficient Treg cells display robust methylation of *Foxp3 CNS2* and preferentially downregulate Foxp3 *ex vivo* and *in vivo*.

The intensity of Thy1.1 expression by Foxp3^{-} cells in the LI lamina propria of *Icos*$^{-/-}$ mice strongly resembled that of LI Foxp3^{+}Thy1.1^{+} (Figure 2A), raising the possibility that some of the Icos−/− Foxp3-Thy1.1+ cells were ‘ex-Foxp3’ cells. To determine whether ICOS impacts the stability of Foxp3, we conducted pyrosequencing analysis of conserved non-coding sequence 2 (CNS2) of the *Foxp3* locus, which is demethylated in Treg cells that stably express Foxp3 (27). In *Icos*$^{-/-}$ Treg cells, there was significant methylation of *Foxp3 CNS2* relative to *Icos*$^{+/+}$ cells. In fact, the methylation levels closely resembled that of naïve T cells (Figure 3A). To confirm the instability of *Icos*$^{-/-}$ Foxp3^{+} cells, we FACS-sorted CD4^{+}GFP^{+} cells from wild type (CD45.1) and *Icos*$^{-/-}$ (CD45.2) Foxp3^{gfp} reporter mice (Figure 3B, left panel) and co-cultured equal numbers in the presence of IL-2, anti-CD3 and anti-CD28. On day 3, the majority of CD45.2^{+} cells were Foxp3^{-}, in contrast to cells of wild type origin that were still mostly Foxp3^{+} (Figure 3B, right panel). Neither a cocktail of pro-inflammatory cytokines IL-1β, IL-6, IL-12, and IL-23 (stim + cytokines) nor blocking antibodies targeting IL-6R, IL-12/23p40, and IL-21R (stim + blockade) had any major impact on the Foxp3 loss by *Icos*$^{-/-}$ T cells (Figure 3B). Altogether, these data argue that ICOS helps to imprint stable expression of Foxp3 mainly by promoting demethylation of *Foxp3* CNS2. The highly methylated CNS2 leading to the rapid downregulation of Foxp3 may help to explain why the elevated thymic output of Foxp3^{+} cells (Figure 1F) is incapable of restoring to wild type levels the numbers of Foxp3^{+} cells in the spleen and especially the large intestine.

We then decided to definitively examine the functional consequence of this instability *in vivo*, first under homeostatic conditions. We employed the T cell co-transfer model of colitis and injected equal numbers of CD45.2^{+} wild type or *Icos*$^{-/-}$ Foxp3^{+} cells into *Rag1*$^{-/-}$ recipients that simultaneously received CD45.1^{+} naïve CD45RB^{hi} T cells. As expected, naïve cells alone induced severe weight loss and colonic inflammation (Figure 3, C–E). However, as previously shown (28), both *Icos*$^{+/+}$ and *Icos*$^{-/-}$ Treg cells prevented the development of colitis. Despite similarly-sized donor CD45.2 fractions of *Icos*$^{+/+}$ and *Icos*$^{-/-}$ origin, we detected significantly reduced frequencies of the latter that still expressed Foxp3 (Figure 3, F–H). Thus, *Icos*$^{-/-}$ Treg cells were able to inhibit the development of colitis, despite the enhanced loss of Foxp3. In longer term analyses under similarly homeostatic conditions, we examined the ability of *Icos*$^{-/-}$ Treg cells to prevent the spontaneous autoimmunity and premature death experienced by *Foxp3*$^{-/-}$ mice. Our results showed that *Icos*$^{-/-}$ Treg cells could temporarily rescue *Foxp3*$^{-/-}$ mice but preferentially lost

expression of Foxp3 and ultimately failed to promote long-term survival, in contrast to *Icos*$^{+/+}$ Treg cells (Supplemental Fig. 4).

ICOS-deficient Treg cells are unable to reverse ongoing colitis.

To determine the potential consequences of *Icos*$^{-/-}$ Treg cell instability during inflammation, we first induced colitis in recipient mice then transferred equal numbers of wild type or *Icos*$^{-/-}$ Treg cells. In this setting, *Icos*$^{-/-}$ Treg cells failed to prevent the wasting disease characteristic of mice that did not receive a secondary Treg cell transfer (Figure 4A). In contrast, recipients of *Icos*$^{+/+}$ Treg cells were rescued from disease, as further confirmed by histological analysis (Figure 4, B–C). Furthermore, the failure of *Icos*$^{-/-}$ Treg cells to mitigate the ongoing inflammation in recipient mice correlated with their almost complete loss of Foxp3 expression, reflected in reduced frequencies and numbers of *Icos*$^{-/-}$ Foxp3^{+} cells (Figure 4, D–E), despite similar numbers of cells of CD45.2 origin (Figure 4F). These results provide compelling evidence that ICOS imprints Treg stability that is particularly important for Treg cell function during inflammation.

In this study we identified a novel role for ICOS signaling in imprinting the epigenetic stability of Foxp3^{+} Treg cells, with no impairment in induction of *Il10* in gut CD4 T cells. Despite this novel role, *Icos*$^{-/-}$ mice retain a sizeable pool of Treg cells and under specific pathogen-free housing conditions, do not succumb to the spontaneous autoimmunity characteristic Foxp3-deficient mice. This may be explained by (1) the thymic output of Foxp3^{+} cells even in aged mice, and (2) the increase in IL-10-producing cells throughout life. However, during inflammation, this instability of Foxp3 produces detrimental consequences for the host. Our data, together with recent findings of a role for PD-1 signaling in stabilizing induced Treg cells (4) identify yet another contribution of T cell co-receptors in imprinting the long-term fate of Treg cells. Ultimately, these discoveries will present unique opportunities to target these pathways in cell-based treatment of chronic inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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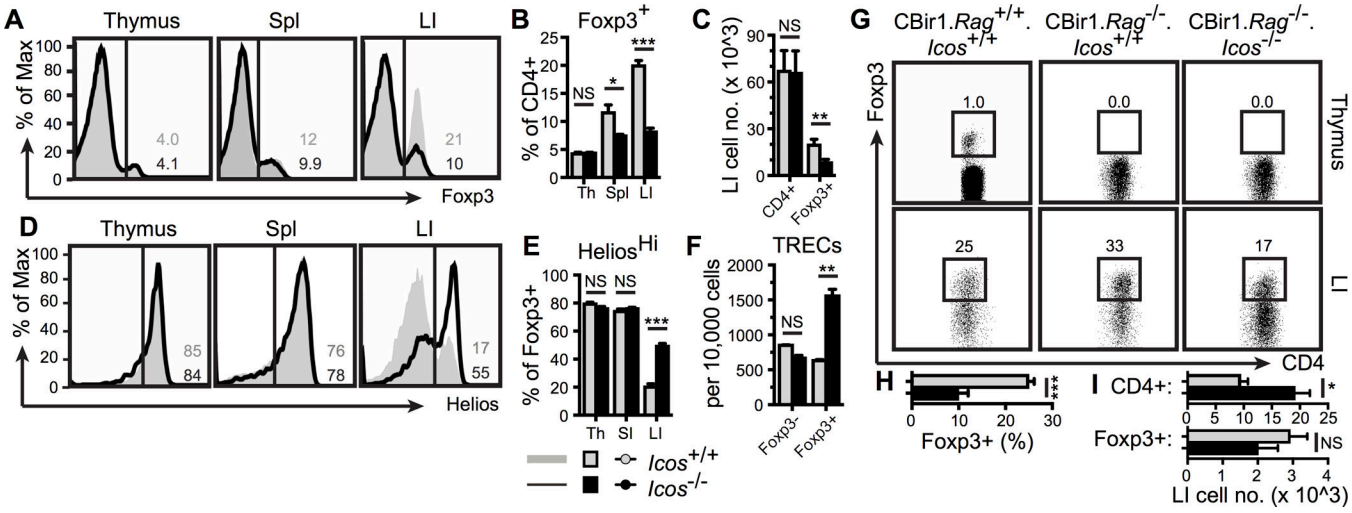

Figure 1: Altered distribution of colonic Foxp3$^+$ subsets in absence of ICOS.

(**A**) Total CD4 single-positive T cells from the thymus, spleen (Spl), and large intestine (LI) of co-housed wild type (grey fill) and *Icos*$^{-/-}$ (black line) mice were examined for expression of Foxp3. (**B**) Graph summarizing the frequencies of Foxp3$^+$ cells in wild type and *Icos*$^{-/-}$ mice analyzed as in A. (**C**) Graph displaying actual numbers of LI CD4$^+$ and Foxp3$^+$ cells. (**D**) Helios expression by Foxp3-gated cells. (**E**) Graph summarizing the frequencies of Helios$^+$ cells among Foxp3$^+$ cells. (**F**) TREC counts among purified splenic CD4 T cells from *Icos*$^{+/+}$ and *Icos*$^{-/-}$ mice. (**G**) Analysis of thymic and lamina propria CD4 T cells from 6-week-old CBir1 transgenic mice. Graphs summarize frequencies of LI Foxp3$^+$ cells (**H**) and numbers of colonic CD4$^+$ and Foxp3$^+$ cells (**I**) from *Icos*$^{+/+}$ and Icos $^{-/-}$ CBir1.*Rag*$^{-/-}$ mice analyzed as in G. Graphs represent data from 2 (C, E) or 3 (G) similar experiments each with 3–5 mice per group. Graphs show mean +/− SEM. *p<0.05, ** p<0.01, ***p<0.001, NS=not significant.

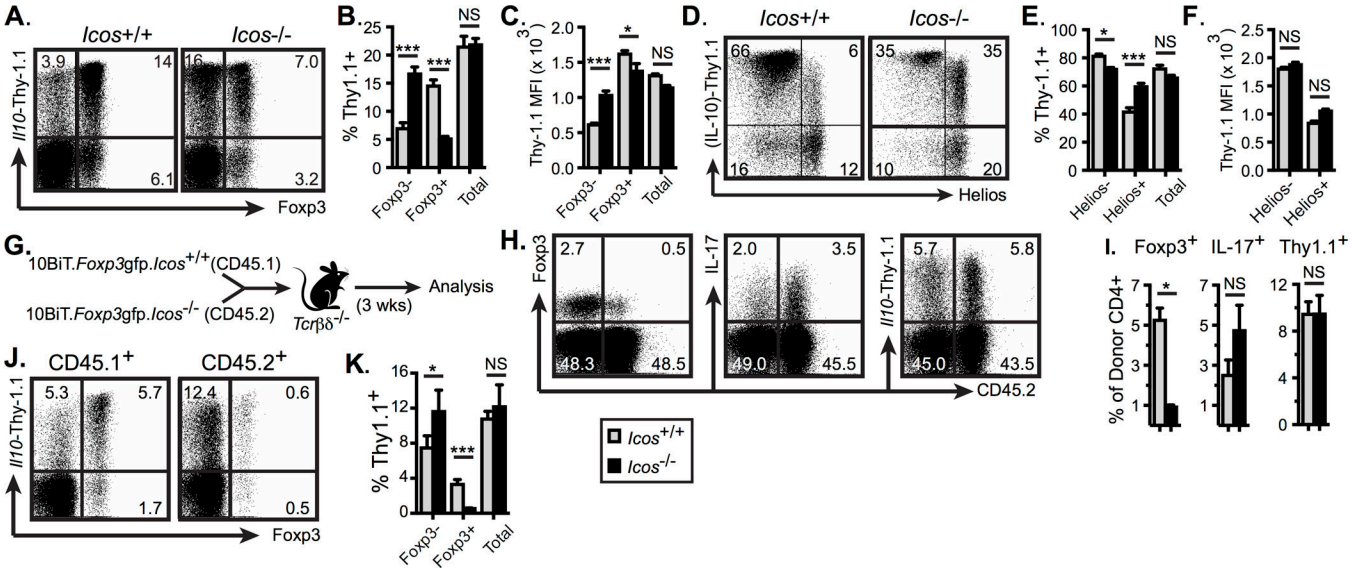

Figure 2: Induction of *Il10* in intestinal CD4 T cells independent of ICOS.

(**A**) LI lamina propria CD4 T cells from 10BiT and 10BiT.*Icos*$^{-/-}$ mice were examined for co-expression of Thy1.1 and Foxp3. (**B-C**) Graphs summarizing frequencies and MFI respectively of Thy1.1^{+} cells among Foxp3^{-}, Foxp3^{+}, and total CD4 T cells as shown in A. (**D**) LI lamina propria Foxp3-gated CD4 T cells from 10BiT and 10BiT.*Icos*$^{-/-}$ mice were examined for co-expression of Helios and Thy1.1. (**E-F**) Graphs summarizing frequencies and MFI of Thy1.1^{+} cells among Helios^{-}, Helios^{+}, and total Foxp3^{+} T cells. (**G**) Schematic overview of thymocyte transfer experiment. CD4 single-positive Foxp3^{-} thymocytes were FACS-sorted from congenically-marked WT (CD45.1) and *Icos*$^{-/-}$ (CD45.2) 10BiT.Foxp3 mice and transferred to *Tcrβδ*$^{-/-}$ recipients. After 3 weeks, donor CD4^{+}TCRβ^{+} cells from the LI lamina propria were analyzed by FACS. (**H**) Analysis of Foxp3, IL-17 and Thy1.1 expression by donor T cells 3 weeks after transfer. (**I**) Graphs summarizing frequencies of the various cell populations from all mice analyzed as in H. (**J**) Analysis of Foxp3 and Thy1.1 expression by wild type and *Icos*$^{-/-}$ T cells recovered from the LI lamina propria. (**K**) Graphs summarizing frequencies of the various cell populations from mice analyzed as in J. Graphs represent data pooled from 1 of 2 similar experiments with 3–5 mice per group and display mean + SEM. *p<0.05, ***p<0.001, NS=not significant.

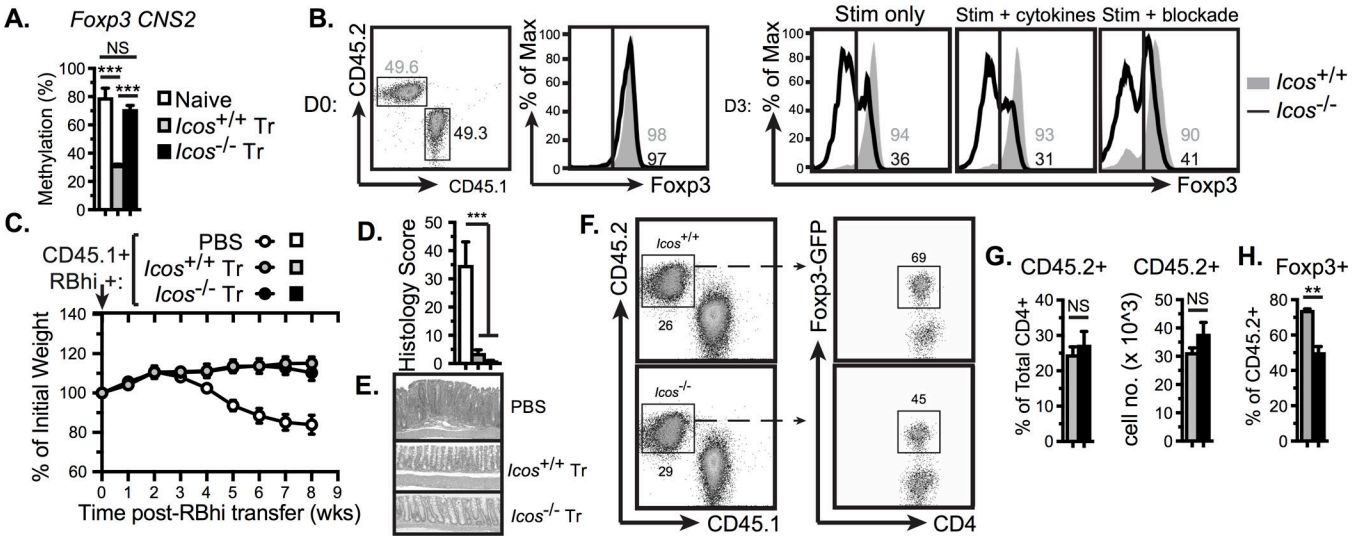

Figure 3: ICOS-deficient Treg cells display robust methylation of Foxp3 CNS2 and readily downregulate Foxp3 *ex vivo* and *in vivo*.

(**A**). Graph shows average methylation of 9 CpG sites of *Foxp3 CNS2* as determined by pyrosequencing of purified $CD4^+GFP^+$ cells from $Icos^{+/+}$ and $Icos^{-/-}$ $Foxp3^{gfp}$ mice. (**B**) Purified $CD4^+GFP^+$ cells from the WT (CD45.1) and $Icos^{-/-}$ (CD45.2) $Foxp3^{gfp}$ mice were co-cultured with anti-CD3 and anti-CD28 plus IL-2 and IL-7. IL-1β, IL-6, IL-12, and IL-23 (stim + cytokines), or anti-IL-6R, anti-IL-12/23p40, and anti-IL-21R (stim + blockade) were added to select wells. Expression of Foxp3 was examined on Day 3. (**C**) $Rag1^{-/-}$ mice received naïve $CD4^+$ $CD45RB^{hi}$ T cells purified from B6.CD45.1 mice. Mice in each cage were then randomly assigned to 1 of 3 groups and received either vehicle (PBS), or identical doses of wild type or $Icos^{-/-}$ Treg cells, each on the CD45.2 background. Mice were weighed weekly until they were euthanized at week 8. (**D**-**E**) Histology scores and representative photomicrographs of H&E-stained colonic tissues from mice in the 3 recipient groups, 10X magnification. (**F**) FACS analysis of LI $CD4^+$ cells (left panel) and of Foxp3+ cells among $CD45.2^+$ cells (right panel). Graphs summarize relative frequencies and numbers of LI $CD45.2^+$ cells (**G**) and frequencies of Foxp3+ among CD45.2+ analyzed as in F (**H**). Bar graphs display mean + SEM and represent data from 1 of 2 (A) or 1 of 3 (D, G, H) similar experiments. **p<0.01, ***p<0.001, NS, not significant.

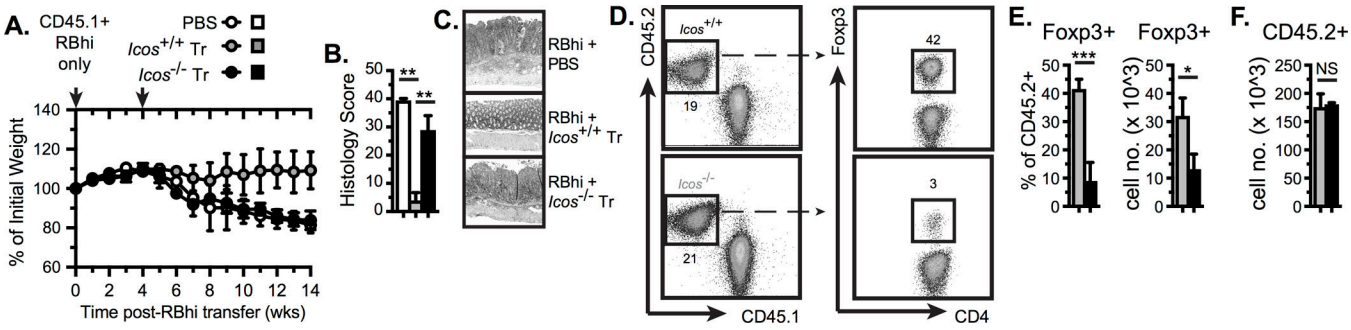

Figure 4: Downregulation of Foxp3 correlates with the inability of ICOS-deficient Treg cells to reverse ongoing inflammation.

(**A**) *Rag1*$^{-/-}$ mice were injected with CD45.1^{+} CD45RB^{hi} T cells as in Figure 3 and monitored for 4 weeks. At week 4, mice were randomly assigned to 1 of 3 groups that received PBS, or identical numbers of CD45.2^{+} of wild type or *Icos*$^{-/-}$ Treg cells. Mice were weighed weekly until week 14. (**B and C**) Histology scores and representative photomicrographs of H&E-stained colonic tissues from mice in the 3 recipient groups, 10X magnification. (**D**) Representative plots depicting relative frequencies of CD4^{+} cells of Treg origin (CD45.2^{+}) remaining at the end of the experiment (left panel) and the percentage of CD45.2^{+} cells that still expressed Foxp3 (right panel). (**E**) Bar graphs displaying relative Foxp3+ cell frequencies and numbers of Foxp3+ cells from all mice analyzed as in D. (**F**) Graph shows numbers of cells of CD45.2 origin recovered from the LI or recipient mice. All data are from 1 of 2 replicate experiments each with 5 recipients per group. Graphs show mean +/− SEM. ** p<0.01, ***p<0.001, NS=not significant.