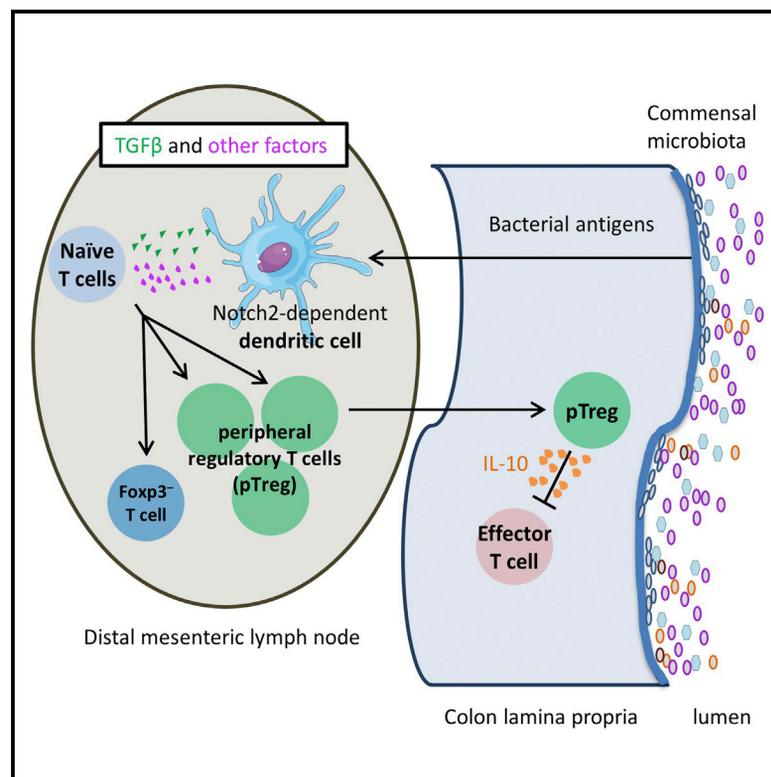


## Rapid and Efficient Generation of Regulatory T Cells to Commensal Antigens in the Periphery

### Graphical Abstract



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### In Brief

The manner in which T cells interact with gut commensal bacteria and become regulatory T cells important for tolerance to these microbes remains incompletely understood. Nutsch et al. use naive transgenic cells specific for commensal antigens to study the kinetics, efficiency, location, and molecular and cellular requirements of peripheral regulatory T cell conversion in the gut.

### Highlights

- Rapid Treg cell selection to commensals occurs in the distal mesenteric lymph node
- TGF-β receptor signaling is not a “master” specifier of Treg versus effector selection
- CNS1 deficiency in Foxp3 delays, but does not abrogate, Treg cell selection
- Notch2-dependent dendritic cells are involved in Treg cell selection



# Rapid and Efficient Generation of Regulatory T Cells to Commensal Antigens in the Periphery

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## SUMMARY

Commensal bacteria shape the colonic regulatory T (Treg) cell population required for intestinal tolerance. However, little is known about this process. Here, we use the transfer of naive commensal-reactive transgenic T cells expressing colonic Treg T cell receptors (TCRs) to study peripheral Treg (pTreg) cell development in normal hosts. We found that T cells were activated primarily in the distal mesenteric lymph node. Treg cell induction was rapid, generating >40% Foxp3<sup>+</sup> cells 1 week after transfer. Contrary to prior reports, Foxp3<sup>+</sup> cells underwent the most cell divisions, demonstrating that pTreg cell generation can be the dominant outcome from naive T cell activation. Moreover, Notch2-dependent, but not Batf3-dependent, dendritic cells were involved in Treg cell selection. Finally, neither deletion of the conserved nucleotide sequence 1 (CNS1) region in *Foxp3* nor blockade of TGF- $\beta$  (transforming growth factor- $\beta$ )-receptor signaling completely abrogated Foxp3 induction. Thus, these data show that pTreg cell selection to commensal bacteria is rapid, is robust, and may be specified by TGF- $\beta$ -independent signals.

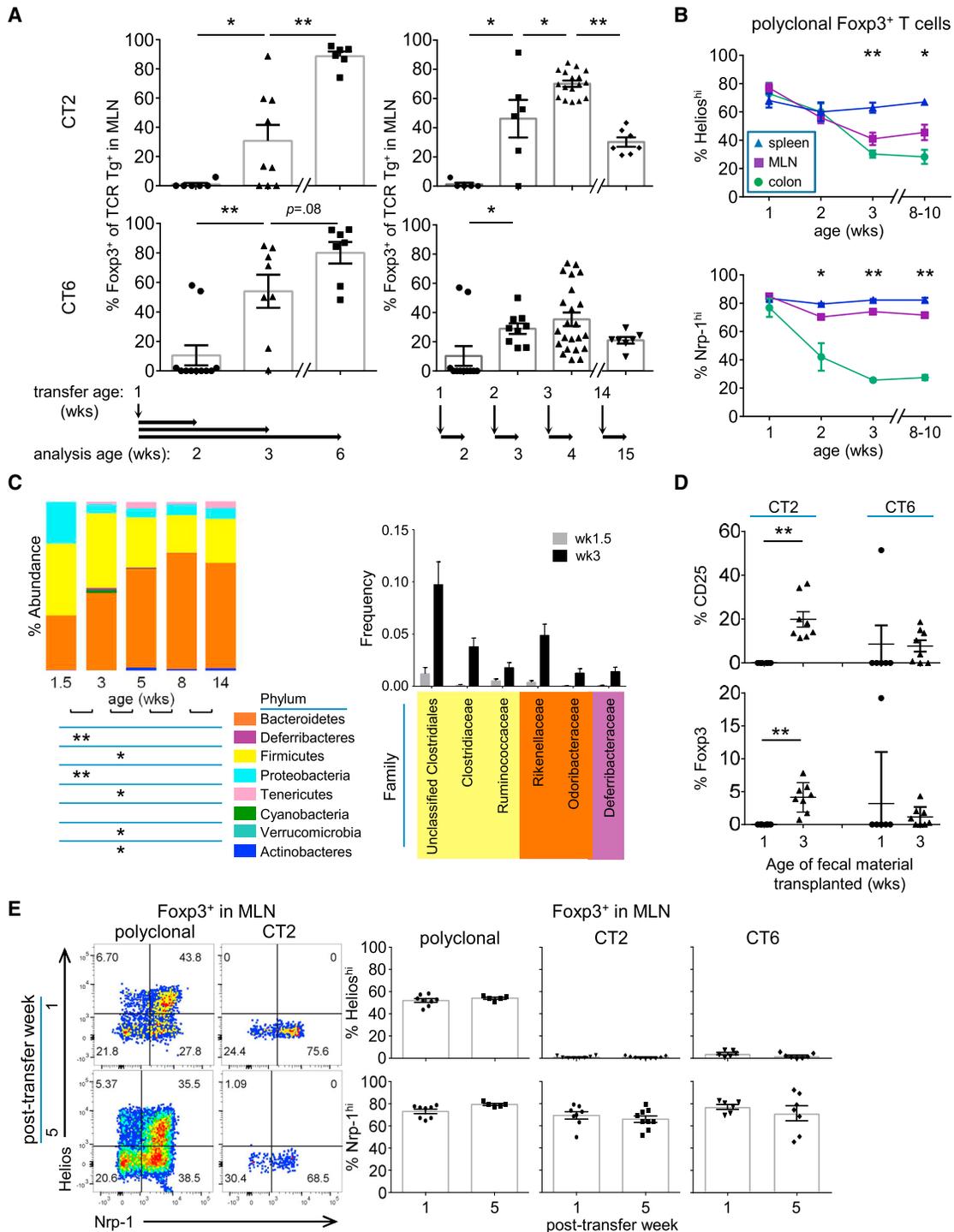
## INTRODUCTION

The trillions of commensal bacteria that live within the intestines play important roles in host health, including metabolism of food and defense against pathogens, but immune responses to commensal bacteria may be harmful and lead to inflammatory bowel disease (Elson and Cong, 2012; Jostins et al., 2012). For example, germ-free mice are highly resistant to murine models of inflammatory bowel disease, suggesting that bacteria are required to drive intestinal inflammation. In normal mice, an essential component of intestinal tolerance to gut bacteria is CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T (Treg) cells (Belkaid and Hand, 2014; Maloy and Powrie, 2011). However, the process by which Treg

cells are generated in response to commensal bacteria is unclear.

One controversy is whether the colonic Treg cell population is primarily generated in the thymus (tTreg) or in the periphery (pTreg) from naive T cells. Some studies have favored pTreg cell differentiation. For example, putative markers of tTreg cells, such as Helios and Nrp-1, are frequent on colonic Treg cells in germ-free, but not conventionally housed, mice (Atarashi et al., 2011; Weiss et al., 2012). Introduction of clostridial or other bacterial species into germ-free mice increased colonic Treg cells and lowered the frequency of Helios<sup>hi</sup> cells, consistent with pTreg cell induction (Atarashi et al., 2011; Geuking et al., 2011). Moreover, deletion of the conserved nucleotide sequence 1 (CNS1) portion of *Foxp3* resulted in a defect in pTreg cell selection, with the loss of Treg cells in the intestines and eventual colitis (Josefowicz et al., 2012; Schlenner et al., 2012; Zheng et al., 2010). Finally, our analysis using a fixed T cell receptor  $\beta$  (TCR $\beta$ ) model suggested that colonic Treg TCRs are unable to facilitate tTreg cell selection (Lathrop et al., 2011). However, a recent study favored tTreg cell generation to commensal bacteria also using a limited TCR repertoire approach (Cebula et al., 2013). As they observed changes in the colonic Treg TCR repertoire after antibiotics, they concluded that commensal bacteria induce the proliferation or retention of tTregs reactive to bacterial antigens. Similarly, bacteria-derived short-chain fatty acids (SCFAs) may act by promoting the expansion of pre-existing Treg cells in the gut (Smith et al., 2013). Thus, the origin of colonic Treg cells is unresolved.

Although transforming growth factor  $\beta$  (TGF- $\beta$ ) is thought to be critical for pTreg cell selection to commensal antigens, this has not been carefully studied in vivo. The importance of TGF- $\beta$  was suggested by the observation that TGF- $\beta$  alone is sufficient for induction of Foxp3 in vitro (Chen et al., 2003). Moreover, transgenic (Tg) expression of a dominant-negative TGF $\beta$ RII (dnTGF $\beta$ RII) blocks both in vitro and in vivo generation of pTreg cells (Kretschmer et al., 2005) and results in the development of spontaneous colitis (Gorelik and Flavell, 2000), consistent with a defect in pTreg cell selection. As TGF- $\beta$  levels are increased in the intestines relative to other tissues, it has been proposed that TGF- $\beta$  is a specification factor that directs naive T cells into the Treg cell lineage in the gut (Konkel and Chen, 2011).



**Figure 1. A TCR Tg Model for pTreg Cell Selection to Commensal Antigens**

(A) Effect of host age on Treg cell selection. Left: sorted naive (CD44<sup>lo</sup>CD62L<sup>hi</sup>CD4<sup>+</sup>) CT2/CT6 Tg cells ( $5 \times 10^4$ ) were injected into 1-week-old congenically marked Foxp3<sup>gfp</sup> lymphoreplete mice. The percentage of transferred cells that become Foxp3<sup>+</sup> in the MLN was analyzed by flow cytometry at the indicated time point. Right: naive Tg cells were injected into 1-, 2-, 3-, or 14-week-old hosts and analyzed 1 week later (experiment [expt] = 2–3, n = 2+).

(B) Changes in putative tTreg cell markers with age in polyclonal Treg cells. Foxp3<sup>gfp</sup> mice were analyzed by Nrp-1 or intracellular staining for Helios at different ages (expt = 2–3, n = 4–6).

(C) Effect of host age on bacterial composition in the gut. 16 s rDNA was analyzed in terminal fecal pellets from 1.5- to 14-week-old mice. Changes in phyla (left) and selected families (right) are shown. Families shown on right are those that increased from 1.5- to 3-week-old mice by  $\geq 1\%$  (5–15 mice per age). Benjamini-Hochberg (BH)-adjusted Mann-Whitney U test p values are used.

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Finally, the role of dendritic cell (DC) subsets in colon Treg cells is not well established. The CD103<sup>+</sup> DC subset has been associated with the induction of Treg cells in the intestine (Coombes et al., 2007) and supported by a recent study using human Langerin-DTA *BATF3*<sup>-/-</sup> mice to deplete this subset in vivo (Welty et al., 2013). However, it is unclear whether the decrease in total Treg cell numbers with DC subset depletion are due to decreased responses to commensal bacteria.

To address these questions regarding pTreg cell selection to commensal bacteria, we generated two TCR Tg lines that express naturally occurring Treg TCRs (Lathrop et al., 2011). Using the adoptive transfer of naive TCR Tg cells into normal lymphoreplete hosts, we analyzed the kinetics and localization of T cell activation, proliferation, and Treg cell selection. We also examined the role of specific factors in pTreg cell generation such as the CNS1 region of Foxp3, DCs, and TGF- $\beta$  signaling.

## RESULTS

### TCR Tg Models for Studying pTreg Cell Selection

The conflict over the source of the colonic Treg cell population may be attributed to the different indirect approaches used to address this question, including TCR repertoire analyses (Cebula et al., 2013), assessments of thymic selection (Lathrop et al., 2011), and the use of putative markers of tTreg cells (Atarashi et al., 2011). We reasoned that a direct analysis using TCR Tg T cells, an approach used previously to study tTreg cell selection (Bautista et al., 2009; Leung et al., 2009), may be useful for understanding the process of colonic Treg cell selection.

We generated TCR Tg lines expressing the microbiota-dependent colonic Treg TCRs CT2 and CT6 (Lathrop et al., 2011). tTreg cells were not detected by routine flow-cytometric analysis of CT2/CT6 TCR Tg mice (Figures S1A and S1B) (Lathrop et al., 2011), consistent with the lack of tTreg cell selection upon retroviral expression of these TCRs in thymocytes (Lathrop et al., 2011). We did observe Treg cells in the periphery of these mice, with increased numbers in the colon (Figure S1B), consistent with the anatomic distribution of these TCRs in the repertoire (Lathrop et al., 2011). However, the majority of T cells in the secondary lymphoid tissues of these TCR Tg mice were phenotypically naive (CD44<sup>lo</sup>CD62L<sup>hi</sup> Foxp3<sup>-</sup>; Figure S1C) and, therefore, suitable for adoptive transfer experiments.

To determine when, during ontogeny, CT2 and CT6 mediate pTreg cell selection, we injected naive TCR Tg cells into congenitally marked 1-week-old lymphoreplete hosts. It took 2 weeks before we observed substantial frequencies of Foxp3<sup>+</sup> CT2 or CT6 T cells in the mesenteric lymph nodes (MLNs) (Figure 1A, left). Treg cell frequency continued to increase by 5 weeks after transfer so that, typically, over 80% of the TCR Tg cells were Foxp3<sup>+</sup>. Thus, transfer of naive CT2/CT6 TCR Tg cells into

normal mice results in robust pTreg cell selection around the age of weaning.

There are several notable characteristics of these TCR Tg models. First, the antigens that drive Treg cell selection are naturally occurring during normal gut physiology, in contrast with a different TCR Tg CBir1 reactive to commensal bacterial flagellin, where T cell activation required intestinal insult (Hand et al., 2012). Second, the injection of large numbers of cells decreased the efficiency of Foxp3 induction (Figure S1D), consistent with intracolonic competition for limiting antigen also seen in the thymus (Bautista et al., 2009; Leung et al., 2009). Studies of TCR Tg mice themselves could, therefore, affect the analysis of pTreg cell generation. Third, there are differences between CT2 and CT6 in the kinetics and efficiency of Foxp3 induction (Figure 1A), which might be predicted, as they do not recognize the same epitope in vitro (Lathrop et al., 2011). While we consider CT2 and CT6 similar in that they represent two examples of colonic Treg TCRs, they should not be considered identical. Last, lymphopenia markedly alters the outcome of T cell selection. Transfer of naive Tg cells into *Rag1*<sup>-/-</sup> hosts resulted in very low frequencies of Treg cells and favored effector cell differentiation (Figure S1E). Thus, the use of lymphopenic hosts to study intestinal tolerance, e.g., the classic Powrie transfer model, may not appropriately permit pTreg cell selection to establish immune homeostasis.

### Age-Dependent Effects on pTreg Cell Selection

The lack of Foxp3<sup>+</sup> Tg cells 1 week after transfer of naive cells into 1-week-old mice could be due to the age of the host. To address this, we transferred cells into mice of different ages and assessed Foxp3 induction after 1 week. Mice that are more than 2 weeks old facilitated pTreg cell selection (Figures 1A, right and S1F–S1I), suggesting that the environment of young mice is unable to support pTreg cell selection of CT2/CT6.

To determine whether the timing of Treg cell selection seen with CT2/CT6 is representative of a polyclonal population, we used Helios and Nrp-1, which have been reported to mark tTreg cells (Weiss et al., 2012; Yadav et al., 2012). In germ-free mice, most colonic Treg cells express tTreg cell markers (Atarashi et al., 2011; Geuking et al., 2011; Lathrop et al., 2011). Similarly, colonic Treg cells 1 week after birth are mostly tTreg cells (Figure 1B). However, the ratio of tTreg/pTreg cells is reversed after 3 weeks of age (Figure 1B), consistent with the CT2/CT6 data suggesting pTreg cell generation at that time (Figure 1A).

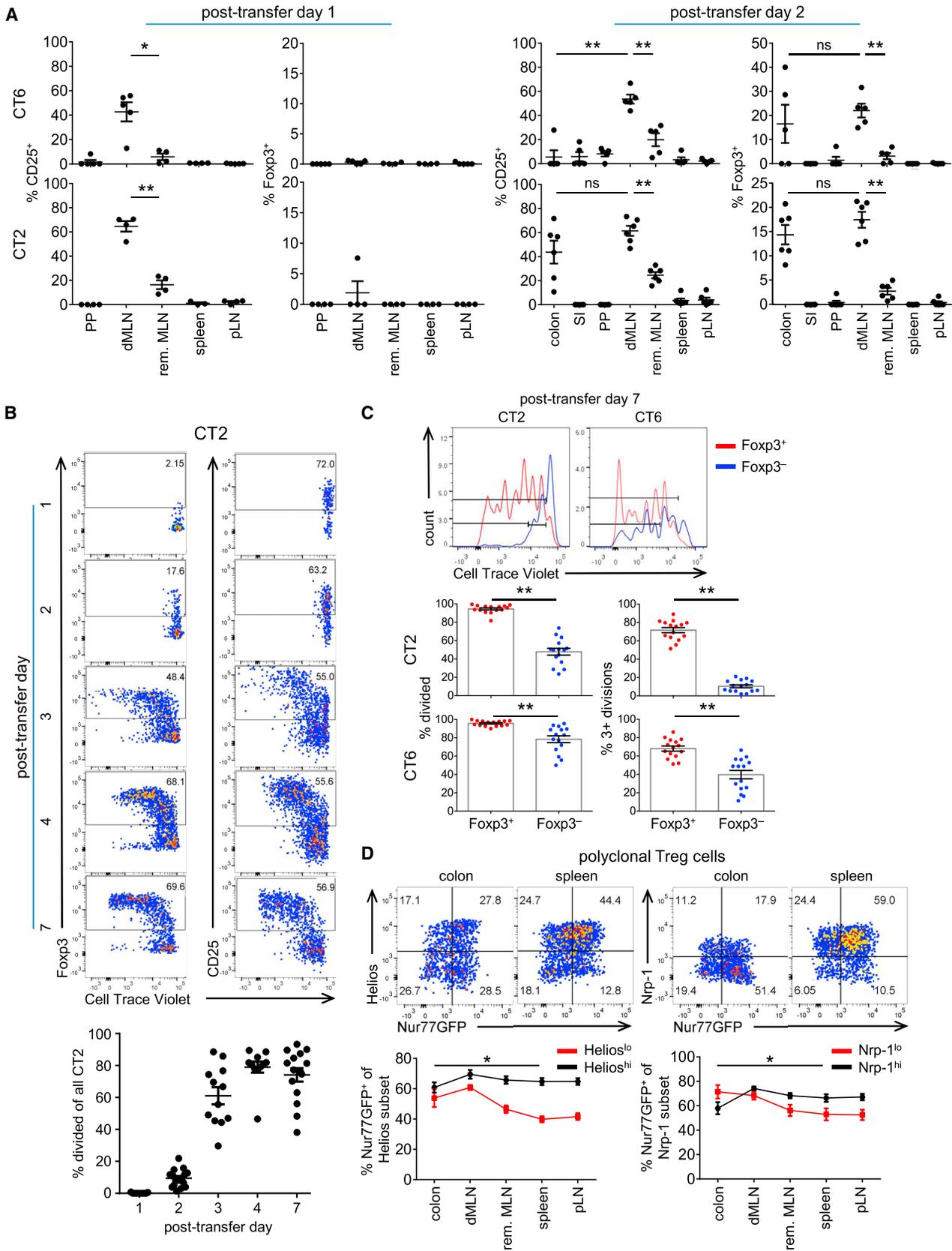
The change of tTreg/pTreg proportions by these markers is associated with a major change in the composition of the gut microbiota around week 3 of life (Pantoja-Feliciano et al., 2013), which we verified by 16S rDNA profiling (Figures 1C and S1J). To test the hypothesis that the absence of adult

(D) Transplant of fecal material from older mice can induce CT2/CT6 Treg cell development in neonatal mice. One-week-old mice were gavaged with fecal material from 1- or 3-week-old mice concurrent with intraperitoneal (i.p.) injection of 2.5–5  $\times$  10<sup>4</sup> each of CT2/CT6 Tg cells. TCR Tg cells were analyzed by flow cytometry for CD25 and Foxp3 at 3 days (expt = 2, n = 3).

(E) Expression of Helios and Nrp-1 on Tg cells. Naive Tg cells were injected into 3- to 4-week-old mice and analyzed for Helios and Nrp-1 expression after 1 and 5 weeks. Representative FACS plots and summary graphs show Tg<sup>+</sup> Treg cells versus endogenous polyclonal T cells from the MLN (expt = 2–3, n = 2–5).

Error bars indicate mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.005; Student's t test, except (C), which uses Mann-Whitney U and BH adjusted p values.

See also Figure S1.



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microbiota explains the lack of Foxp3 induction in young mice (Figure 1A), we performed a fecal transplant from 1- or 3-week-old donors into 1-week-old hosts concurrent with intraperitoneal (i.p.) transfer of naive CT2/CT6 cells. After 3 days, a portion of CT2/CT6 cells upregulated CD25 and Foxp3 in mice that received fecal material from 3-, but not 1-week-old mice (Figure 1D), suggesting that young mice have the ability to present commensal antigens but normally do not have the requisite microbiota for CT2/CT6. One mouse receiving 1-week-old fecal material showed substantial CD25 and Foxp3 induction in CT6, which, we speculate, results from the stochastic colonization of young mice with bacteria recognized by CT6 (Figure 1A). Thus, data from both polyclonal and monoclonal TCR Tg studies suggest that there is a dramatic shift in the Treg cell populations in the MLNs and colon around the period of weaning associated with marked changes in microbial composition coinciding with pTreg cell selection.

### Colonic Treg TCR Tg Cells Express Low Levels of Helios but Not Nrp-1

The use of Helios and Nrp-1 in denoting tTreg cells has been controversial (Akimova et al., 2011; Gottschalk et al., 2012; Haribhai et al., 2011). Therefore, we assessed these markers on CT2/CT6 TCR Tg cells after induction of Foxp3. Whereas we observed few Helios<sup>hi</sup> cells at 1 or 5 weeks after transfer, a substantial proportion of Tg Treg cells express Nrp-1 (Figure 1E). This difference is consistent with the higher frequency of Nrp-1<sup>hi</sup> versus Helios<sup>hi</sup> subset in polyclonal MLN Treg cells (Figure 1B). Thus, our data using Tg cells suggest that Helios<sup>lo</sup> is better correlated with colonic pTreg cells than Nrp-1<sup>lo</sup> and supports the notion that Helios<sup>lo</sup> may be a useful, albeit imperfect, surrogate for pTreg cells during homeostasis.

### Initial Naive T Cell Activation Is Rapid and First Seen in the Distal MLN

It is unknown whether the site of initial activation of commensal antigen-specific T cells is the same as that of oral food antigens (Worbs et al., 2006). Therefore, we examined the kinetics of T cell activation and Foxp3 induction of transferred naive CT2/CT6 cells in various gut-associated lymphoid tissues. We first detected T cell activation as evidenced by CD25 upregulation 1 day after transfer primarily in the most distal MLN (dMLN) nearest to the cecum (Figures 2A and S2A), which drains the cecum and descending colon (Mowat and Agace, 2014). Notably, CD25<sup>+</sup> CT2/CT6 cells were not enriched in Peyer's patches, a

site important for immunoglobulin A (IgA) responses to commensal bacteria (Corthesy, 2013).

By 2 days post-transfer, we observed Foxp3<sup>IRES-GFP</sup> in CT2/CT6 cells in the dMLN and colon (Figures 2A, S2B, and S2C). However, the potential for T cell trafficking makes the primary site of pTreg cell differentiation uncertain. The relative frequency of CT2/CT6 cells among total CD4<sup>+</sup> T cells was also increased in the dMLN by day 2 (Figure S2D), suggesting local retention or proliferation. Thus, T cell activation to commensal antigens and Treg cell selection is rapid and easily detected in a specific anatomic location, the dMLN.

### Colonic Treg Cells Appear Chronically Stimulated during Homeostasis

Previous data examining pTreg cell generation to non-commensal antigens showed that Foxp3 induction could occur within 4 days after antigen recognition in vivo (Gottschalk et al., 2010; Kretschmer et al., 2005; Weissler et al., 2015). Notably, Foxp3<sup>+</sup> cells were preferentially found among cells that showed fewer cell divisions in these experiments, suggesting that pTreg cell selection represented an alternative cell fate of naive T cell activation. To address whether this happens in commensal-dependent responses, Cell-Trace-Violet (CTV)-labeled naive CT2 Tg cells were transferred into congenically marked 3- to 4-week-old mice. Tg cell proliferation in the dMLN began on day 2, along with the appearance of Foxp3<sup>+</sup> cells (Figure 2B). However, many Foxp3<sup>+</sup> cells had not diluted CTV, suggesting that cell division is not required for the upregulation of Foxp3. At later time points, a higher percentage of Foxp3<sup>+</sup> than Foxp3<sup>-</sup> cells had divided (Figure 2C), although there were differences in proliferation between CT2 and CT6 cells reflective of the differences in Foxp3<sup>+</sup> frequency (Figure 1A). In contrast with previous studies, our data demonstrate that pTreg cell generation can be the dominant outcome after naive T cell activation in the periphery.

The extensive proliferation (Figures 2B and 2C) and ability of Tg cells to be activated at different host ages (Figure 1A) imply that intestinal Treg cells are constantly exposed to commensal antigens during immune homeostasis. To assess whether colonic Treg cells are activated in a polyclonal population, we utilized a Nur77GFP BAC (bacterial artificial chromosome)-Tg line as an in vivo marker for TCR activation (Moran et al., 2011). In the colon and dMLN, ~55%–60% of Helios<sup>lo</sup> Treg cells are Nur77GFP<sup>hi</sup>, whereas this is seen in only ~40% of Helios<sup>lo</sup> Treg cells in non-gut-associated tissues such as the spleen

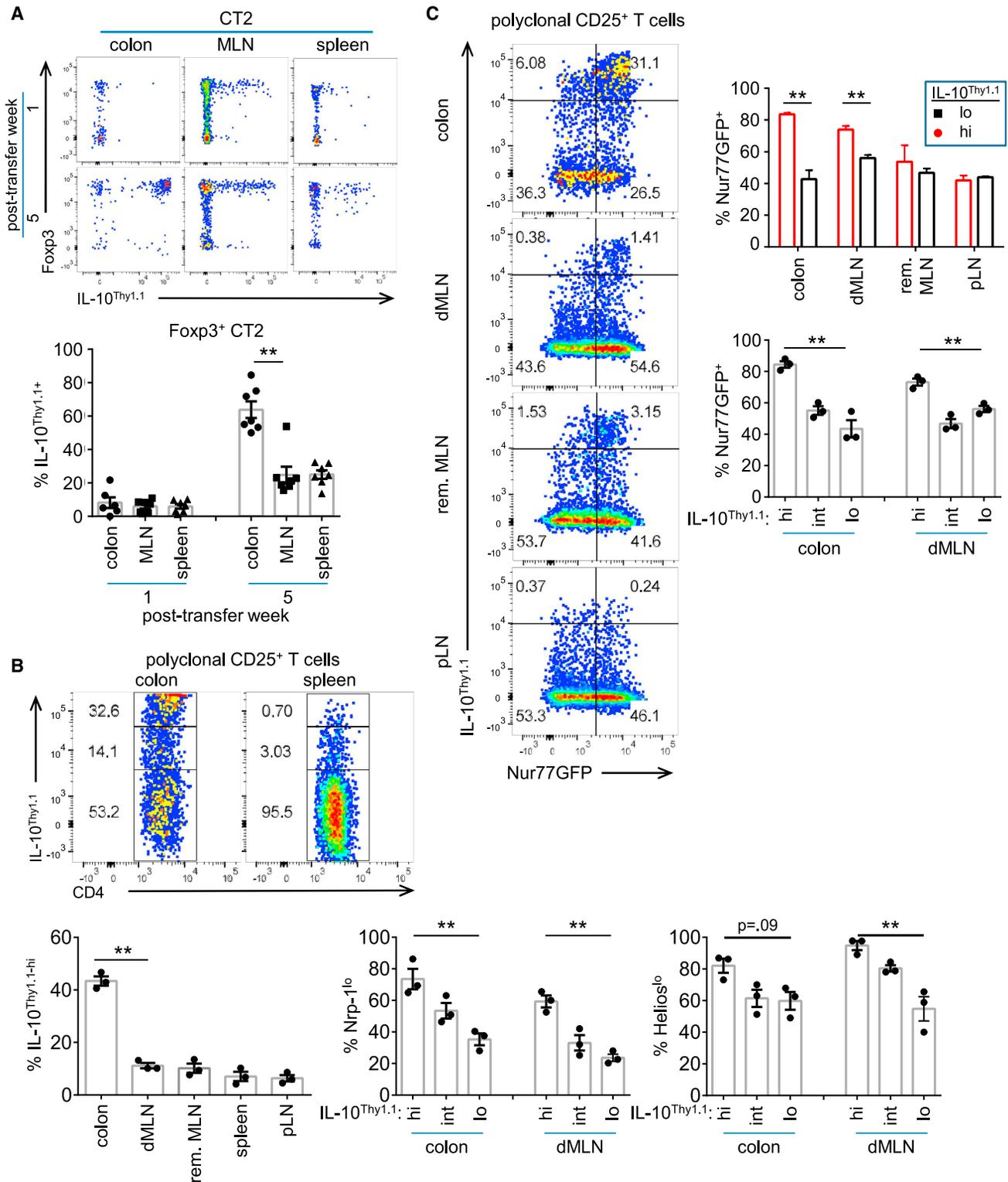
## Figure 2. Kinetics and Biogeography of T Cell Activation of CT2 and CT6

(A) Analysis of early time points after transfer of naive CT6/CT2 T cells. Naive Tg cells were injected into 3- to 4-week-old mice and analyzed after 1–2 days by flow cytometry for CD25 or Foxp3<sup>IRES-GFP</sup> (experiment [expt] = 2, n = 2–4). Tissues: SI, small intestine; PP, Peyer's patch; dMLN, distal MLN; rem. MLN, remaining MLN; pLN, peripheral LN (pooled axial, brachial, and inguinal). Error bars indicate mean ± SEM. \*p < 0.05, \*\*p < 0.005; Student's t test. ns, not significant.

(B and C) Proliferation of CT2/CT6 cells after transfer. CTV-labeled naive CT2/CT6 cells were injected into 3- to 4-week-old mice. Foxp3 expression and cell division were assessed by flow cytometry of dMLN at the indicated times. Representative FACS plots of CT2 (expt = 2–4, n = 2–5) (B) and of CT2/CT6 at day 7 after transfer (expt = 4) (C) are shown. Data include points from Figure 1A. In (C), the percentage of cells that have undergone any (left) or 3+ divisions (right) by CTV are shown. Error bars indicate mean ± SEM. \*\*p < 0.005, Student's t test.

(D) Increased TCR activation in colonic Treg cells during homeostasis. T cells were analyzed from 6- to 8-week-old mice Tg for Nur77-GFP, a reporter for recent TCR activation. Nrp-1<sup>hi</sup> and Helios<sup>hi</sup> were used as markers for tTreg cells. Numbers in representative FACS plots of Foxp3<sup>+</sup> cells indicate frequency in the quadrant. Summary data are the percentages of Nur77GFP by Helios or Nrp-1 subset. Error bars indicate mean ± SEM. Asterisk indicates significance in Nrp1<sup>lo</sup> or Helios<sup>lo</sup> colon versus spleen (expt = 2, n = 1–2).

Error bars indicate mean ± SEM. \*\*p < .005; Student's t test. See also Figure S2.



**Figure 3. IL-10 Reporter Is Induced in the Colon Post-Foxp3 Induction**

(A) Induction of IL-10 reporter on CT2 cells. Naive 10BIT CT2 Tg cells were injected into 3- to 4-week-old mice and analyzed at the indicated time points. Representative FACS plots and summary of 10BIT in Foxp3<sup>+</sup> cells are shown (experiment [expt] = 2, n = 2–4).

(B and C) Analysis of polyclonal CD25<sup>+</sup> Treg cells for IL-10 reporter (B) and correlation with TCR activation (C). 2- to 3-month-old 10BIT Nur77GFP mice were analyzed by flow cytometry (expt = 2, n = 1–2). Representative FACS plots and summary plots are shown. The frequency of Helios<sup>lo</sup>/Nrp-1<sup>lo</sup> by IL-10<sup>Thy1.1</sup>

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(Figure 2D). By contrast, Helios<sup>hi</sup> Treg cells show only minor changes in the frequency of Nur77GFP<sup>hi</sup> cells with location. A similar pattern is seen in Nrp-1<sup>lo</sup> and Nrp-1<sup>hi</sup> cells. Together with the TCR Tg studies (Figure 2A), these data suggest that pTreg cells are enriched in the colon due to chronic activation by commensal antigens.

### Induction of IL-10 Reporter Occurs Post-Treg Cell Selection

An important effector molecule produced by intestinal Treg cells is interleukin (IL)-10. We used the 10BiT reporter, in which Thy1.1 expression reflects current and historic IL-10 expression (Maynard et al., 2007). We observed that IL-10 reporter expression substantially lagged behind Foxp3 expression in CT2 cells (Figures 3A and S3A–S3C). In preliminary experiments, similar results were observed with CT6 in the MLN (data not shown), but IL-10<sup>+</sup> frequencies in the colon were difficult to assess due to lower cell recoveries (Figure S11). These data suggest that the potential for IL-10 expression is induced post-Treg cell selection and that Foxp3<sup>−</sup> IL-10-expressing Tr1 cells are not commonly generated from naive CT2 cells.

At 5 weeks, we observed that the frequency of IL-10<sup>+</sup> cells among CT2 Treg cells was increased in the colon and that the level of IL-10 reporter expression was also much higher (Figures 3A and S3A). Consistent with the CT2 data, we found that IL-10 reporter expressing CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in polyclonal populations is enriched in the colon (Figure 3B). Moreover, we observed that the IL-10<sup>+</sup> population is enriched in the polyclonal pTreg cell subset using Helios<sup>lo</sup> or Nrp-1<sup>lo</sup> markers (Figure 3B), in agreement with previous data (Atarashi et al., 2011). Finally, we found that IL-10<sup>hi</sup> Treg cells in the colon are mostly Nur77GFP<sup>hi</sup>, compared to cells in peripheral lymph nodes (Figure 3C). The CT2 and polyclonal data are, therefore, consistent with the hypothesis that many of the IL-10-producing CD4<sup>+</sup> T cells in the colon are pTreg cells that recognize antigens presented during homeostasis.

### Tuning of pTreg Cell Numbers to Commensals

In our previous study, we showed several lines of evidence supporting the colonic commensal antigen reactivity of CT2/CT6, including in vitro reactivity to an unclassified *Clostridium* species for CT6 (Lathrop et al., 2011). Therefore, we tested whether CT2/CT6 cells could respond in vivo to a consortium of Clostridia species maintained in gnotobiotic mice (Stefka et al., 2014). However, we did not observe CD25, CD44, or Foxp3 upregulation in CT2/CT6 cells 1 week after naive cell transfer into either germ-free or Clostridia-colonized mice (four to five mice per group; data not shown).

We then asked whether microbial depletion with antibiotics would affect the development of CT2/CT6. Three-week-old mice started on vancomycin, ampicillin, metronidazole, and neomycin (VAMN) ad libitum (Rakoff-Nahoum et al., 2004) showed a partial inhibition of Treg cell selection and expansion

of transferred naive CT2 cells (Figure 4A). For CT6, we found that clindamycin and streptomycin ad libitum were more effective at blocking Treg conversion and proliferation (Figure S4A) than VAMN in preliminary studies (data not shown), consistent with the notion that CT2 and CT6 recognize different bacteria. No individual antibiotic was responsible for the activity in preliminary experiments (data not shown). The incomplete blockade of Treg induction suggests that the antigens were only partially eliminated, as seen in the incomplete blockade of Th17 development with vancomycin (Ivanov et al., 2009). Thus, the activation and Treg selection of CT2/CT6 can be, at least partially, blocked by antibiotics.

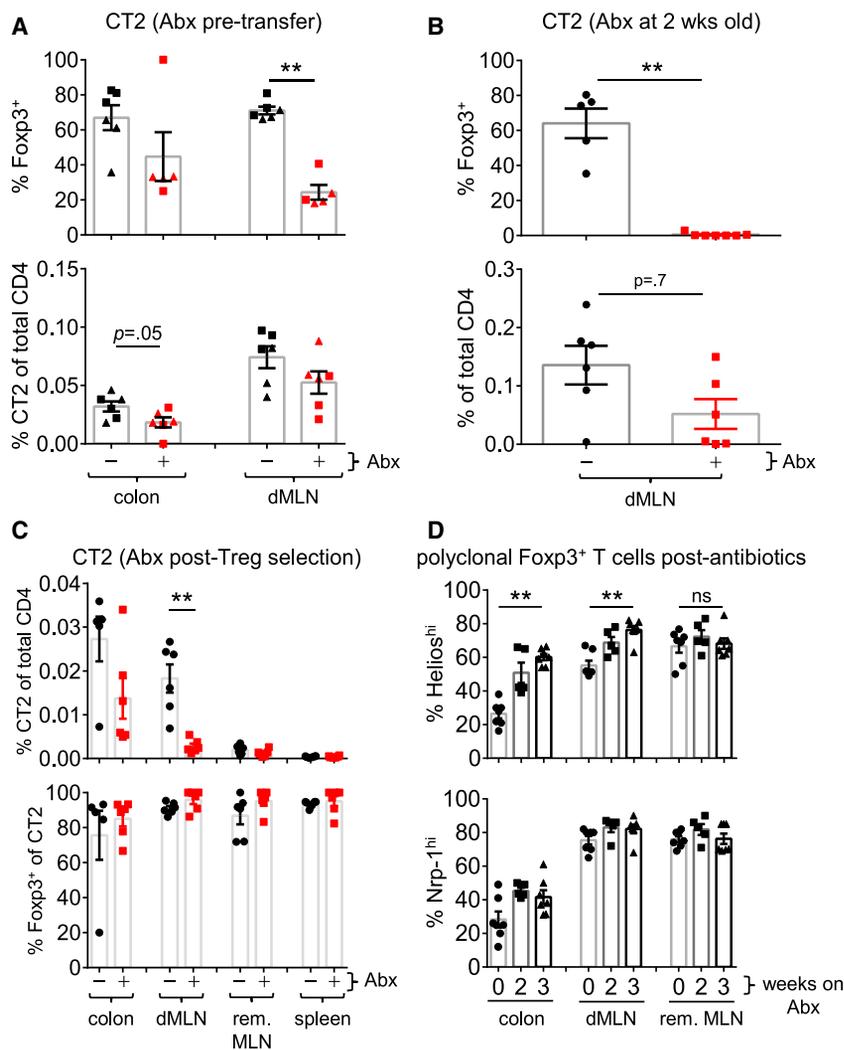
Antibiotic gavage of pre-weaning mice has been reported to result in more complete bacterial elimination (Stefka et al., 2014). We tested this protocol on CT2, as (1) it had higher frequencies in the colon (Figures 4A and S4A), and (2) an oral antibiotic cocktail similar to VAMN had been used at 2 weeks of life (Stefka et al., 2014). We found that treatment of younger mice blocked the induction of Foxp3<sup>+</sup> cells (Figure 4B) as well as decreased the percentage of CT2 cells within the total CD4 population, suggestive of poor T cell expansion (Figure 4B). Moreover, it drastically altered microbial composition and diversity, as assessed by 16S rDNA sequencing (Figures S4B and S4C). Thus, these data show that it is possible, with the appropriate protocol, to abrogate Treg cell selection of CT2 with antibiotics.

Since antibiotics are commonly used in humans, we asked whether antibiotics could affect the existing pTreg cell population. We injected naive CT2 Tg cells into 3-week-old mice, allowed them to develop into pTreg cells, and then treated the mice with antibiotics for 3 weeks. Because of the length of the experiment, the ad libitum protocol was used. Although the frequency of Foxp3<sup>+</sup> cells among the TCR Tg population did not decrease (Figure 4C), the frequency of total CT2 Tg cells was reduced, which was indicative of decreased commensal antigen stimulation of CT2 (Figure 4C). The polyclonal T cell population also showed decreases in colonic Nrp-1<sup>lo</sup> and Helios<sup>lo</sup> pTreg cell fraction after treatment with antibiotics, consistent with the overall decrease in CT2 cells (Figure 4D). Thus, these data suggest that the gut Treg cell population is maintained during homeostasis by continuous commensal bacterial antigen stimulation.

### pTreg Cell Selection in Older Mice Is Associated with Lower Foxp3 and CD25 Expression

pTreg selection efficiency increased from 1- to 3-week-old mice (Figure 1A) but decreased in 8- to 14-week-old adult mice (Figures 1A, right and S5A). CT2 cells also proliferated less in older mice (Figure 5A) and had a relative increase of Foxp3<sup>int</sup> CD25<sup>lo</sup> cells among the CT2 Foxp3<sup>+</sup> population (Figures 5B and 5C). However, CT2 cells become mostly Foxp3<sup>+</sup> by 5 weeks after transfer (Figure 5C), suggesting that older mice are kinetically slower in their ability to support pTreg cell selection.

expression is shown in (B). The frequency of Nur77GFP<sup>hi</sup> by IL-10<sup>Thy1.1</sup> expression in the CD25<sup>+</sup> subset is shown in (C). dMLN, distal MLN; rem. MLN, remaining MLN; pLN, peripheral LN (pooled axial, brachial, and inguinal). Error bars indicate mean ± SEM. \*\*p < 0.005, Student's t test. See also Figure S3.



**Figure 4. Antibiotics Affect pTreg Cell Induction of CT2 but Not Stability of Treg Cell Percentage**

(A and B) Effect of antibiotics (Abx) on CT2 pTreg cell selection at weaning (A) or before (B). In (A), weaned mice were treated with VAMN in drinking water for 5 (squares) or 14 days (triangles) prior to the transfer of naive CT2 Tg cells. One week later, CT2 cells were analyzed by flow cytometry for Fcpx3 expression and the percentage of CT2 cells within the CD4 population. In (B), 2-week-old mice were gavaged with VAMN for 1 week prior to transfer of CT2.

(C) Effect of antibiotics on differentiated CT2 pTreg cells. Naive CT2 cells were transferred into 3-week-old mice and allowed to develop into Treg cells for 2 weeks. Mice were then treated with VAMN in drinking water for 3 weeks and then analyzed by flow cytometry. Data shown are the percentage of CT2 cells among the entire CD4 population and the frequency of Fcpx3<sup>+</sup> cells within CT2 cells (experiment [expt] = 2, n = 2+). rem. MLN, remaining MLN.

(D) Effect of antibiotics on polyclonal Treg cells. Fcpx3<sup>9p</sup> mice were treated with VAMN in drinking water for 2–3 weeks before assessing the expression of Nrp-1 and Helios in Fcpx3<sup>+</sup> cells (expt = 3, n = 2–3). Error bars indicate mean ± SEM. \*\*p < 0.005; Student's t test. ns, not significant.

See also Figure S4.

### TGF-β Is Not a “Master” Specifying Factor for pTreg Cell Generation

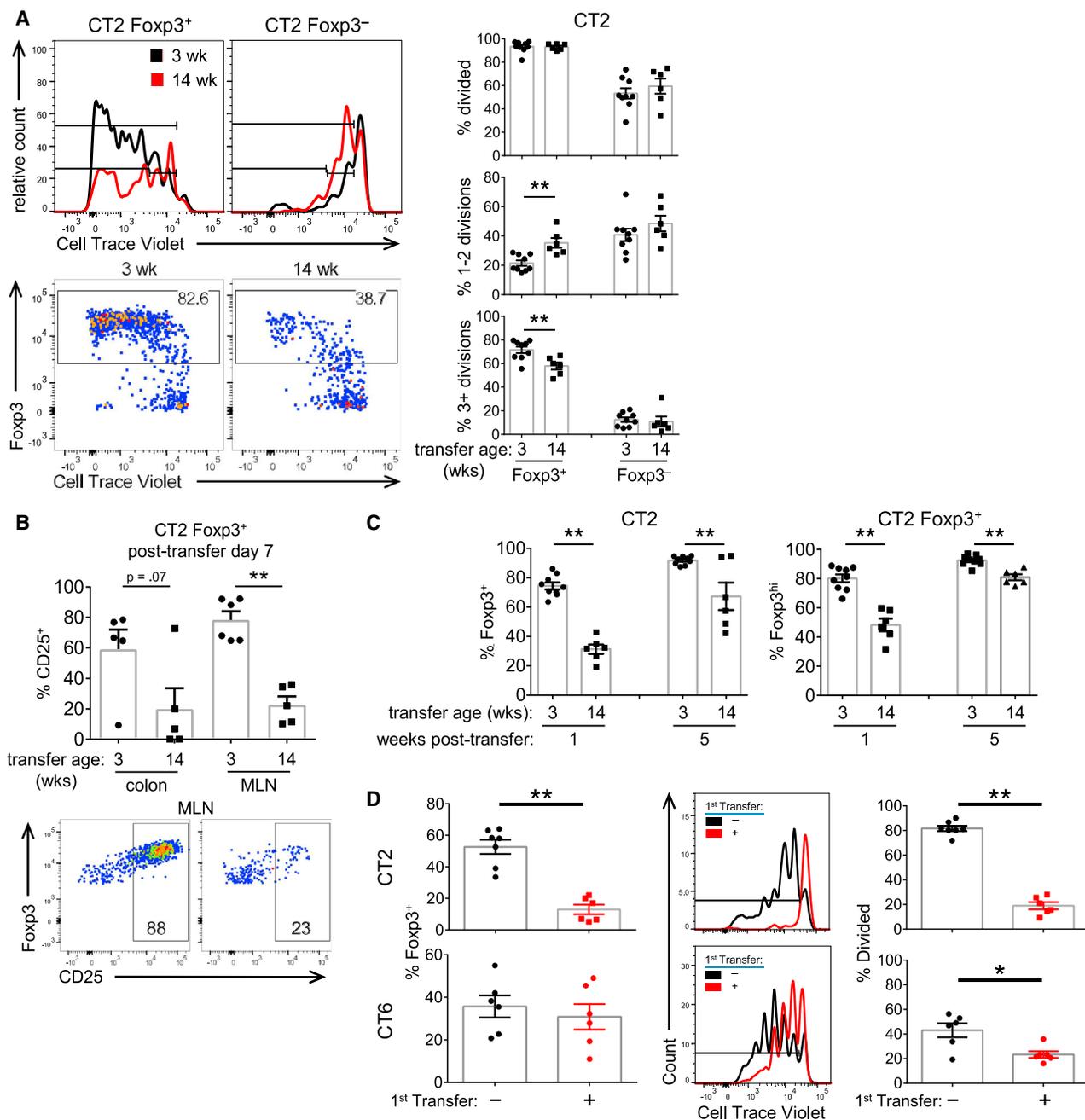
TGF-β is generally accepted as a critical signal for pTreg cell selection in the gut (Konkel and Chen, 2011). However, in vivo studies analyzing the role of TGF-β in Treg cell development to intestinal commensal antigens have not been performed. To address this, we bred the CT6 TCR Tg line to CD4-cre *Tgfb2<sup>fl/fl</sup>* mice and observed a

partial decrease in Treg cell selection in vivo (Figure S6A). Although we observed the reported lethality in the non-TCR Tg littermates around 3–4 weeks of age (Marie et al., 2005), substantial TGF-β-dependent Fcpx3 induction still occurred in vitro (Figure S6B), suggesting that CD4-cre *Tgfb2<sup>fl/fl</sup>* incompletely abrogated TGF-β signaling or affected TGF-β signaling differently than dnTGFβRII (Ishigame et al., 2013). Therefore, we bred CT2 and CT6 to the dnTGFβRII Tg line, which diminishes in vivo pTreg cell generation by 20-fold (Kretschmer et al., 2005). We confirmed that dnTGFβRII inhibits by 100- to 1,000-fold the ability of exogenous TGF-β to induce Fcpx3 in vitro (Figure S6C) and blocked the in vitro induction of TGF-β-responsive genes (Figure 6A) (Feuerer et al., 2010; Hill et al., 2008). Consistent with the notion that gut T cells receive TGF-β signals in vivo, we found that a subset of TGF-β-responsive genes were upregulated in CT6 cells (Figure 6A). Contrary to the in vitro inhibition of Fcpx3 (Figure S6C), dnTGFβRII only led to a relatively modest 50% reduction of CT6 Fcpx3<sup>+</sup> cells in vivo at 3 or 7 days post-transfer, in comparison to wild-type (WT) CT6 cells (Figure 6B, S6D). We did not find

One possible explanation is decreased bacteria in older mice. However, in vitro stimulation with fecal antigens from 3- to 14-week-old mice caused equivalent CD25 upregulation (Figure S5B), suggesting similar antigen concentration in the lumen. Another possibility is that the Treg niche is already filled with endogenous cells, leading to decreased conversion of naive cells. Consistent with this hypothesis, we found that pre-existing CT2 cells decrease the efficiency of Treg cell selection after a second transfer of naive CT2 cells (Figure 5D). Pre-existing CT6 cells decreased proliferation, but not Fcpx3 induction, of newly transferred naive CT6 cells (Figure 5D), which is consistent with the milder effect of older age on CT6 pTreg generation (Figure 1A, right). Although these data do not exclude other possibilities, such as changes in the consortia resulting in antigen-independent effects (e.g., SCFAs or other metabolites), they suggest that inhibition by pre-existing antigen-specific Treg cells is one possible mechanism for the decreased Fcpx3 induction and proliferation seen in older, 14-week-old mice (Figures 1A and 5D).

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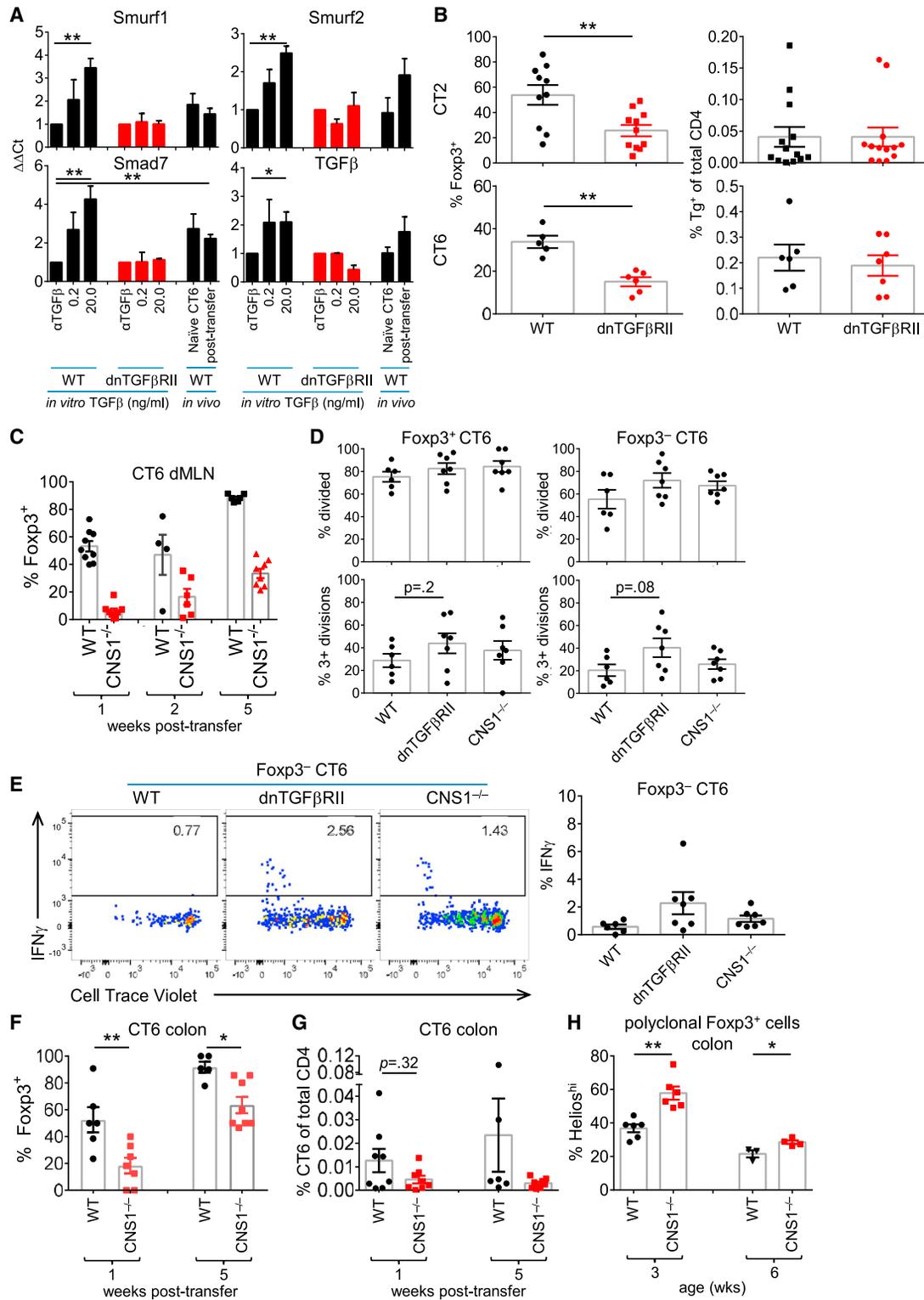
**Figure 5. Effects of Host Age on Foxp3 Induction and Maintenance**

(A–C) Treg cells generated in older mice show lower proliferation, Foxp3 expression, and CD25 expression. Naive CTV-labeled CT2 Tg cells were injected into 3- or 14-week-old mice. CT2 dMLN cells were assessed for Foxp3 expression and cell division at 1 week (A), with representative FACS plots on the left, and cell divisions summarized on the right (experiment [expt] = 2+; this includes data in Figures 1A and 2C). CD25 expression is shown in (B). Foxp3 percentage and expression level at 1 and 5 weeks after transfer are summarized in (C) (expt = 2+, n ≥ 2).

(D) Pre-existing cells can inhibit subsequent pTreg selection. 3- to 4-week-old littermates were injected with PBS or naive CT2 or CT6 cells. One week later, congenically marked naive CTV-labeled CT2/CT6 cells were injected into all hosts. 4 days later, cells from the second transfer were analyzed for Foxp3 expression and CTV dilution (expt = 2, n = 2–4).

Error bars indicate mean ± SEM. \*p < 0.05, \*\*p < 0.005; Student's t test.

See also Figure S5.



**Figure 6. Partial Reduction of Treg Cell Selection with Impaired TGF- $\beta$  Receptor Signaling and CNS1 Deficiency**

(A) Analysis of TGF- $\beta$ -dependent genes. For in vitro experiments (expts), sorted naive polyclonal T cells were stimulated with anti-CD3/CD28 with the indicated TGF- $\beta$  condition. After 2 days, TGF- $\beta$ -responsive genes (Table S1) were analyzed by qPCR. For in vivo expts,  $1.5 \times 10^5$  naive CT6 cells were injected into WT mice. After 2 days, CD25<sup>+</sup>Foxp3<sup>-</sup> cells were sorted, and gene expression was assessed by qPCR. Data represent fold change over in vitro  $\alpha$ TGF $\beta$  condition (in vitro expt = 2, n = 1; in vivo expt = 5, n = 1 for naive and n = 1–2 post-transfer of four to five pooled mice).

(legend continued on next page)

evidence that hyper-expansion of a small fraction of Foxp3<sup>+</sup> dnTGFβRII cells was responsible for the higher-than-expected Foxp3<sup>+</sup> frequency (Figure S6E). As the dnTGFβRII is a hypomorph (Figure S6C), we cannot exclude the possibility that low levels of TGF-β signals are sufficient for Foxp3 induction in vivo. However, our data suggest that blockade of TGF-β signals by >100-fold has a disproportionately small effect on pTreg generation in vivo, compared with in vitro, arguing that TGF-β is unlikely to be the singular, or “master,” signal that specifies a naive T cell to upregulate Foxp3 in vivo.

### CNS1 Is Important for Initial, but Not Late, pTreg Cell Selection

TGF-β mediates its effects via the activation of SMAD transcription factors, which can bind to the promoter and CNS1 enhancer region in the Foxp3 locus (Schlenner et al., 2012; Zheng et al., 2010). Therefore, we asked whether CNS1 deficiency would phenocopy the inhibition of TGF-β receptor signaling on Treg cell selection. However, CNS1 deletion had a much greater effect on Treg cell selection of naive CT6 cells (Figure 6C), consistent with prior reports (Josefowicz et al., 2012; Zheng et al., 2010).

The reduction of Foxp3<sup>+</sup> cells was not due to effects on T cell activation, as there were no differences in proliferation among the Foxp3<sup>+</sup> or Foxp3<sup>-</sup> cells lacking CNS1 (Figure 6D). There was a trend toward increased proliferation of both Foxp3<sup>+</sup> and Foxp3<sup>-</sup> dnTGFβRII cells (Figure 6D). Despite this, few of the Foxp3<sup>-</sup> dnTGFβRII or CNS1<sup>-/-</sup> CT6 cells developed the ability to produce interferon γ (IFNγ) or IL-17 in 1 week (Figures 6E and S7A). None of the Foxp3<sup>+</sup> cells expressed IFNγ, and the frequency of CT6 Tg cells remained the same (Figure S7A). We did note a decreased frequency of CNS1<sup>-/-</sup> CT6 cells in the colon, suggesting that the block in Treg cell selection may result in decreased cell survival or trafficking (Figures 6F and 6G). Thus, these data suggest that CNS1 is essential for the rapid induction of Foxp3 in response to intestinal commensal antigens.

Unexpectedly, a substantial proportion of CNS1<sup>-/-</sup> CT6 cells expressed Foxp3 by 2 weeks, increasing by 5 weeks post-transfer (Figures 6C and 6F), suggesting that CNS1 is not absolutely required for pTreg selection. Consistent with the CT6 data, polyclonal T cells in CNS1<sup>-/-</sup> mice showed increased Helios<sup>hi</sup> tTreg cell frequencies at 3, but not 6, weeks of age (Figures 6H and S7B). Thus, it appears CNS1 primarily affects the kinetics of Foxp3 induction without completely altering cell fate.

### Notch2-Dependent DCs Are Necessary for Optimal pTreg Cell Generation

To determine whether DCs are primarily responsible for activating commensal-specific T cells, we used bone marrow chimeras with Zbtb46-DTR to deplete all conventional DCs (Meredith et al., 2012). We tested CT2 cells, as they show more efficient Treg selection (Figure 1A), and found that conversion and proliferation of CT2 were markedly blocked with diphtheria toxin (DT) depletion of DCs (Figure 7A).

In the gut, CD103<sup>+</sup> DCs are reported to be the primary antigen-presenting cell (APC) subset that induces pTreg cell selection in the MLNs (Coombes et al., 2007; Sun et al., 2007). However, their role in pTreg generation to commensal bacterial antigens has not been tested in vivo. To address the role of CD103<sup>+</sup> DCs, we used *BATF3*<sup>-/-</sup> mice to ablate CD103<sup>+</sup>CD11b<sup>-</sup> DCs (Hildner et al., 2008) and CD11c-cre *Notch2*<sup>fl/fl</sup> (*Notch2*<sup>CKO</sup>) mice to ablate CD103<sup>+</sup>CD11b<sup>+</sup> DCs (Satpathy et al., 2013). Whereas the frequency of CT2/CT6 Foxp3<sup>+</sup> cells appeared unaffected in *BATF3*<sup>-/-</sup> mice, we observed significant decreases in Treg cell selection in *Notch2*<sup>CKO</sup> hosts (Figures 7B and 7C). In *Notch2*<sup>CKO</sup> hosts, the frequency of Foxp3<sup>-</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> effector Tg cells and proliferation of Foxp3<sup>-</sup> Tg cells were increased (Figure 7D). We interpret this to indicate that *Notch2* deficiency in DCs does not affect the overall antigen presentation and T cell activation of commensal-antigen reactive T cells but rather skews the balance of differentiation from the Treg cell subset.

## DISCUSSION

Treg-cell-mediated immune tolerance to commensal antigens is crucial for intestinal homeostasis. Using Tg lines that express TCRs derived from colonic Treg cells, we have characterized the kinetics, anatomy, and molecular mechanisms of Treg cell selection to commensal antigens during homeostasis in lymphoreplete animals. We made the following observations: (1) generation of Treg cells can be the dominant outcome of naive T cell activation to commensal antigens; (2) this process is first seen in the dMLN; (3) the acquisition of effector cytokines such as IL-10 occurs post-Foxp3 expression; (4) TGF-β does not appear to be the “master” specifying factor for Treg versus effector cell differentiation; (5) the CNS1 region in *Foxp3* affects the kinetics of pTreg cell selection but does not abrogate it; and (6) *Notch2* deficiency in DCs skews T cell differentiation from Treg to effector cell subsets.

(B) Treg cell selection with impaired TGF-β receptor signaling. Naive CT2 ( $5 \times 10^4$ ) or CT6 ( $2 \times 10^5$ ) cells on a WT or dnTGFβRII background were injected into 3- to 4-week-old mice and analyzed 1 week later. Data show frequency of Foxp3<sup>+</sup> cells among CT2/CT6 Tg cells in the dMLN (left) and the percentage of Tg cells in the total CD4<sup>+</sup> population (right).

(C) Impaired Treg cell selection in CNS1-deficient CT6 cells. Naive CT6 Tg cells (WT or CNS1<sup>-/-</sup>) were injected into 3- to 4-week-old mice. Cells from the dMLN were analyzed at the indicated time point (experiment [expt] = 2–3, n = 2–4).

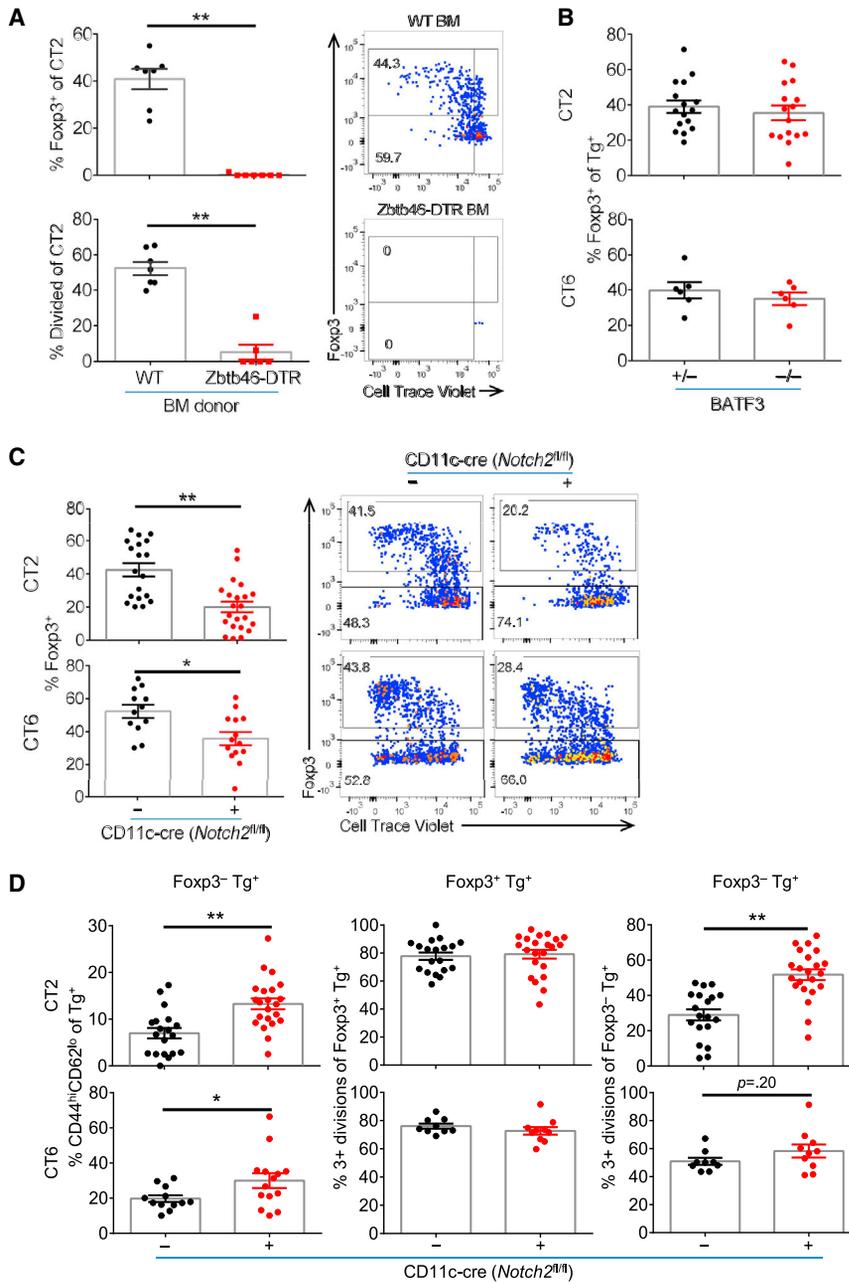
(D) Analysis of proliferation. The percentage of divided cells with the indicated mutations is shown (expt = 2, n = 2–4 pools of two mice).

(E) Assessment of IFNγ expression. Naive CT6 Tg cells ( $2 \times 10^5$ ) (WT, dnTGFβRII, or CNS1<sup>-/-</sup>) were stained with CTV and injected into 3- to 4-week-old hosts. One week later, cells were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for intracellular staining. Representative plots are shown (left) and summarized (right) (expt = 2, n = 2–4 pools of two mice).

(F and G) CNS1-independent Treg cell selection of CT6 cells. (F) Naive WT or CNS1<sup>-/-</sup> CT6 Tg cells were injected into 3- to 4-week-old mice, and the colons were analyzed 1 or 5 weeks later for the frequency of Foxp3<sup>+</sup> CT6 cells (F) and of CT6 cells among the total CD4<sup>+</sup> T cell population (G) (expt = 3, n = 1–3).

(H) Helios expression in polyclonal colonic CNS1<sup>-/-</sup> Treg cells with age. CNS1<sup>-/-</sup> mice were cross-fostered to limit variability in microbiota (expt = 2–3, n = 1–3). Error bars indicate mean ± SEM. \*p < 0.05, \*\*p < 0.005; Student's t test.

See also Figures S6 and S7 and Table S1.



**Figure 7. Role of Notch2-Dependent DCs in Foxp3 Induction to Commensal Antigens**

(A) Conventional DCs cells are necessary for activation of CT2. Bone marrow (BM) chimeras with WT or Zbtb46-DTR donors were generated as described in [Supplemental Information](#). CTV-labeled naive CT2 cells were injected into DC-depleted mice and analyzed after 1 week (experiment [expt] = 2–3, n = 2–5).

(B) Loss of CD103<sup>+</sup>CD11b<sup>-</sup> DCs does not decrease pTreg cells. Naive Tg cells were injected into BATF3<sup>+/-</sup> or BATF3<sup>-/-</sup> littermates and analyzed at 1 week for Foxp3 (expt = 2–3, n = 1–3). (C and D) Notch2-dependent DCs are involved in pTreg cell generation. Naive Tg cells were stained with CTV and injected into Notch2<sup>CKO</sup> mice. Foxp3 expression and representative FACS plots (C) and the summary of effector cell frequency (CD44<sup>hi</sup>CD62L<sup>lo</sup>) and cell divisions (D) are shown for dMLN cells 1 week after transfer (expt = 4, n = 2–5).

Error bars indicate mean ± SEM. \*p < 0.05, \*\*p < 0.005; Student's t test.

See also [Supplemental Information](#).

for pTreg cell generation ([Gottschalk et al., 2010](#)). Though the response to food antigens may differ, a previous study also observed the proliferation of Treg cells in OTII TCR Tg cells, though with a lower Foxp3<sup>+</sup> frequency, in response to oral ovalbumin (OVA) ([Hadis et al., 2011](#)). Thus, our data show that naive T cell responses can be dominated by the generation of pTreg cells.

We found that the efficiency of Treg cell selection has a monophasic dependence on age, with a peak around weaning, consistent with literature showing that early host-commensal interactions are important for immune homeostasis in the gut ([Keeney et al., 2014](#); [Stefka et al., 2014](#)). The low, if any, pTreg development in young mice appeared related to the microbiota, which undergoes marked changes around the time of weaning ([Pantoja-Feliciano et al., 2013](#)). Another

We found that pTreg differentiation from naive T cells in response to commensal antigens was unexpectedly rapid and efficient, in contrast with previous studies ([Gottschalk et al., 2010](#); [Kretschmer et al., 2005](#); [Weissler et al., 2015](#)), which found pTreg cells in the least divided population, suggesting that they were a byproduct of the immune response. This difference may arise from the models used. First, we used activation in a mucosal tissue that may favor Treg cell selection ([Sun et al., 2007](#)). Second, our model utilizes endogenous antigen presentation of commensal antigens rather than transient exogenous administration. Finally, the TCR affinity/avidity of CT2/CT6 for their antigens is unknown and may fall within a range favorable

possibility that our studies do not address is that changes in the consortia of commensal species also affect pTreg cell generation via factors such as SCFAs that are independent of TCR specificity ([Arpaia et al., 2013](#); [Furusawa et al., 2013](#); [Smith et al., 2013](#)). Additionally, it is possible that, in older mice, pre-existing adaptive responses to commensal bacteria may limit antigen access to the immune system ([Peterson et al., 2007](#)). We also observed that pre-existing T cell responses can limit naive CT2/CT6 cell activation and CT2 Treg cell generation. In addition to these non-mutually exclusive possibilities, Treg cell selection may be affected by other changes in microbial, mucosal, or immune physiology in very young or adult mice.

Analysis of early time points after T cell transfer allowed the identification of the primary site of T cell activation for CT2/CT6 to the dMLN. Prior mapping studies revealed that this lymph node drains the cecum and proximal colon (Mowat and Agace, 2014). Thus, these data suggest a pathway for commensal antigen uptake in the colon, perhaps via bacteria outer membrane vesicles (Hickey et al., 2015) or colonic goblet-cell-associated passages (Knoop et al., 2015).

The TCR Tg transfer model also allows the study of specific molecular and cellular events affecting commensal bacteria-specific T cells within a normal intestinal environment. For example, it has been postulated that TGF- $\beta$  is a crucial cytokine for pTreg cell selection in the gut (Konkel and Chen, 2011). We were, therefore, surprised that dnTGF $\beta$ R1I showed a relatively modest (50%) effect on early Treg cell selection, as it was previously reported to markedly (90%) inhibit Treg cell selection in a different TCR Tg model (Kretschmer et al., 2005). Although dnTGF $\beta$ R1I is a hypomorph, our data showing a >100-fold blockade of TGF- $\beta$  effects on Foxp3 induction in in vitro assays, versus a 2-fold reduction in Foxp3<sup>+</sup> cells in vivo, suggest either that (1) TGF- $\beta$  levels in vivo are well over 100-fold greater than that necessary for Treg cell specification or that (2) TGF- $\beta$  is not a singular “master” specifying factor for Treg cell selection in vivo. However, the first scenario, that TGF- $\beta$  levels are excessively high in vivo, seems unlikely, based on our analysis of TGF- $\beta$ -dependent gene expression as well as previous transcriptome analysis showing that the signature of TGF- $\beta$ -responsive genes was not enriched in Treg cells from the lamina propria versus other peripheral organs (Feuerer et al., 2010). Thus, our data support the hypothesis that TGF- $\beta$  may not be the singular “master” specifying factor for pTreg cell selection in the gut and suggest the involvement of additional factors.

One target downstream of TGF- $\beta$ -dependent transcription factors is the CNS1 region in the *Foxp3* locus (Zheng et al., 2010). Mice deficient in this region develop spontaneous colitis (Josefowicz et al., 2012), and those lacking the SMAD-binding site in this region show decreased Treg cells in the gut (Schlenner et al., 2012). However, the colitis develops only in older mice and was not initially observed on a mixed genetic background. Consistent with these data, we found a striking defect in initial, but not late, Treg cell selection, which may explain why CNS1 mice show less pathology than might be predicted if pTreg cell selection was completely abrogated (Haribhai et al., 2011).

Finally, our studies using *BATF3*<sup>-/-</sup> and Notch<sup>ckO</sup> mice suggest that CD103<sup>+</sup> CD11b<sup>+</sup> DCs play a role in Treg/Teffector cell selection in the gut, contrary to reports that removal of this DC subset in several models (IRF4, human Langerin-DTA, and Notch2) does not affect total Treg cell numbers but does lower Th17 cell numbers in the intestines (Lewis et al., 2011; Persson et al., 2013; Satpathy et al., 2013; Welty et al., 2013). The discrepancy could be due to the analysis of steady-state Treg cell numbers, which may be less sensitive than a kinetic analysis of pTreg generation. Future studies are needed to investigate which DC subsets present to commensal-specific T cells, as well as the mechanisms by which APCs influence the effector-regulatory cell-fate decision.

In summary, the use of TCR Tg lines expressing Treg TCRs has allowed us to evaluate the process of pTreg cell selection to commensal antigens, providing insight regarding the kinetics, efficiency, geography, and molecular and cellular requirements. Future studies will be required to understand the molecular mechanism determining Treg versus effector cell differentiation, which may facilitate the development of treatments for diseases of disordered gut tolerance, such as inflammatory bowel disease.

## EXPERIMENTAL PROCEDURES

### Mice

CT6 (Lathrop et al., 2011) and CT2 Tg mice were generated as described previously (Bautista et al., 2009), with microinjection into B6  $\times$  129 fertilized eggs and backcrossed more than five generations to B6 background *Foxp3*<sup>IRE5-GFP</sup> (Lin et al., 2007) and *Rag1*<sup>-/-</sup> mice obtained from Jackson Laboratories. Additional mice are described in the Supplemental Experimental Procedures. Animal experiments were performed in a specific pathogen-free facility in accordance with the guidelines of the Institutional Animal Care and Use Committee at Washington University.

### T Cell Transfers into Normal Hosts

Naive (CD44<sup>lo</sup> or CD25<sup>-</sup>CD44<sup>lo</sup>CD62L<sup>hi</sup>) CD4<sup>+</sup>Foxp3<sup>-</sup> T cells were FACS (fluorescence-activated cell sorting) purified from the MLN and spleen from CD45.2 *Foxp3*<sup>IRE5-GFP</sup> *Rag1*<sup>-/-</sup> TCR Tg mice.  $5 \times 10^4$  (typical, up to  $5 \times 10^5$ ) cells were injected intraperitoneally (1 or 2 weeks old) or retro-orbitally (older hosts) into congenic CD45.1 *Foxp3*<sup>9D</sup> mice. The entire colon lamina propria, small intestine lamina propria, Peyer's patches, half of the spleen, and mesenteric and peripheral lymph nodes were harvested at various times and analyzed by flow cytometry. Transferred cells were identified as CD4<sup>+</sup>CD45.2<sup>+</sup>CD45.1<sup>-</sup>V $\alpha$ 2<sup>+</sup>. Data from colon samples with ten or more TCR Tg cells were used, except in cases where the experimental condition typically resulted in fewer than ten cells (e.g., Figures 2A, 7A, and S1G). In order to recover enough cells for qPCR or intracellular cytokine staining, mice in Figures 6A, 6D, 6E, and S7A were injected with additional cells ( $1.5\text{--}2 \times 10^5$ ).

### Statistical Analysis

GraphPad Prism v6 was used for statistical and graphical analysis. Student's t test was used for between-subjects analyses. Benjamini-Hochberg false discovery rate correction (adjusted p value [padj]) was used on Mann-Whitney U calculations for phyla and class comparisons (padj, R v3.2). Unless otherwise indicated, each dot represents data from an individual host; error bars indicate mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.005, Student's t test. Experiments are performed independently with n replicates per experiment.

Additional procedures are given in the Supplemental Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.08.092>.

## AUTHOR CONTRIBUTIONS

K.N., T.F., C.R.N., and C.-S.H. conceived of the project and designed the experiments; K.N., J.N.C., T.L.A., T.F., and E.R.-G. performed the experiments; and K.N. and C.-S.H. wrote the manuscript.

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