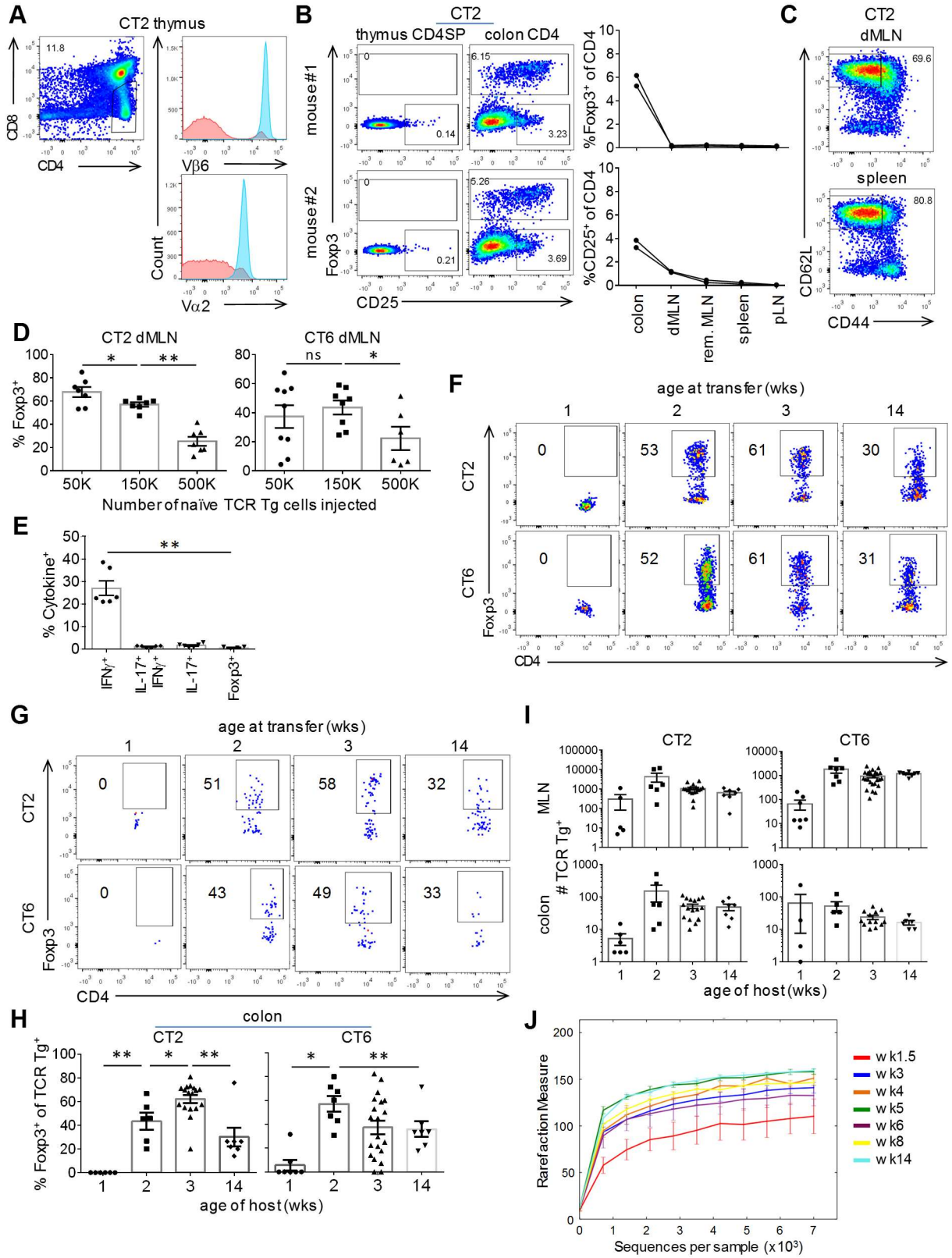

Figure S1, related to Figure 1. CT2 transgenic characterization and peripheral Treg cell selection with host age. (A-C) CT2 transgenic characterization. (A) Representative thymic

CD4 by CD8 FACS plots of *Foxp3*$^{\text{IRES-GFP}}$ *Rag1*$^{-/-}$ CT2 Tg mice. TCR expression is gated on CD4SP. (B) CD25 and Foxp3 expression from two mice of thymic CD4SP ($4.1x10^4$ and $1.1x10^4$ events, respectively), and colon are plotted on the left. Summary of peripheral $CD4^+$ T cells are shown on the right. (C) Expression of CD62L and CD44 in peripheral $CD4^+$ cells from dMLN and spleen are shown. (D) Dose titration of naïve CT2/CT6 cells. $5x10^4$, $1.5x10^5$ or $5x10^5$ cells were injected into 3-4 week old mice. One week later cells were harvested from the dMLN and analyzed for Foxp3 expression (expt=2, n=2-5). (E) Effector cell development in lymphopenic mice. 10^5 naïve CT2 cells were injected into *Rag1*$^{-/-}$ hosts. One week later cells were harvested from the dMLN, stimulated with PMA/Ionomycin, and intracellularly stained for IFNγ and IL-17, and analyzed for Foxp3 expression (expt=2, n=3). (F-I) Data associated with Figure 1A right, peripheral regulatory T cell selection of TCR Tg cells in hosts of different ages. Representative FACS plots of TCR Tg^+ ($CD45.2^+$ $CD45.1^-$ $CD4^+$ $V\alpha2^+$) cells are shown for the MLN (F) and colon (G) for CT2 and CT6 naïve T cell transfer experiments. (H) Summary of colon experiments shown in (G). (I) Total number of transgenic cells seen by flow cytometry is plotted for experiment in right panels of Figure 1A. (J) Bacterial diversity is decreased in 1.5 wk old mice compared to older mice. Fecal DNA was isolated as in Figure 1C and alpha diversity of OTUs assessed by the Chao1 metric. Each dot in plots (B, D, E, H, I) represent data from an individual mouse. Bars indicate mean ± SEM. *$p < .05$, **$p < .005$; Student's t-test.

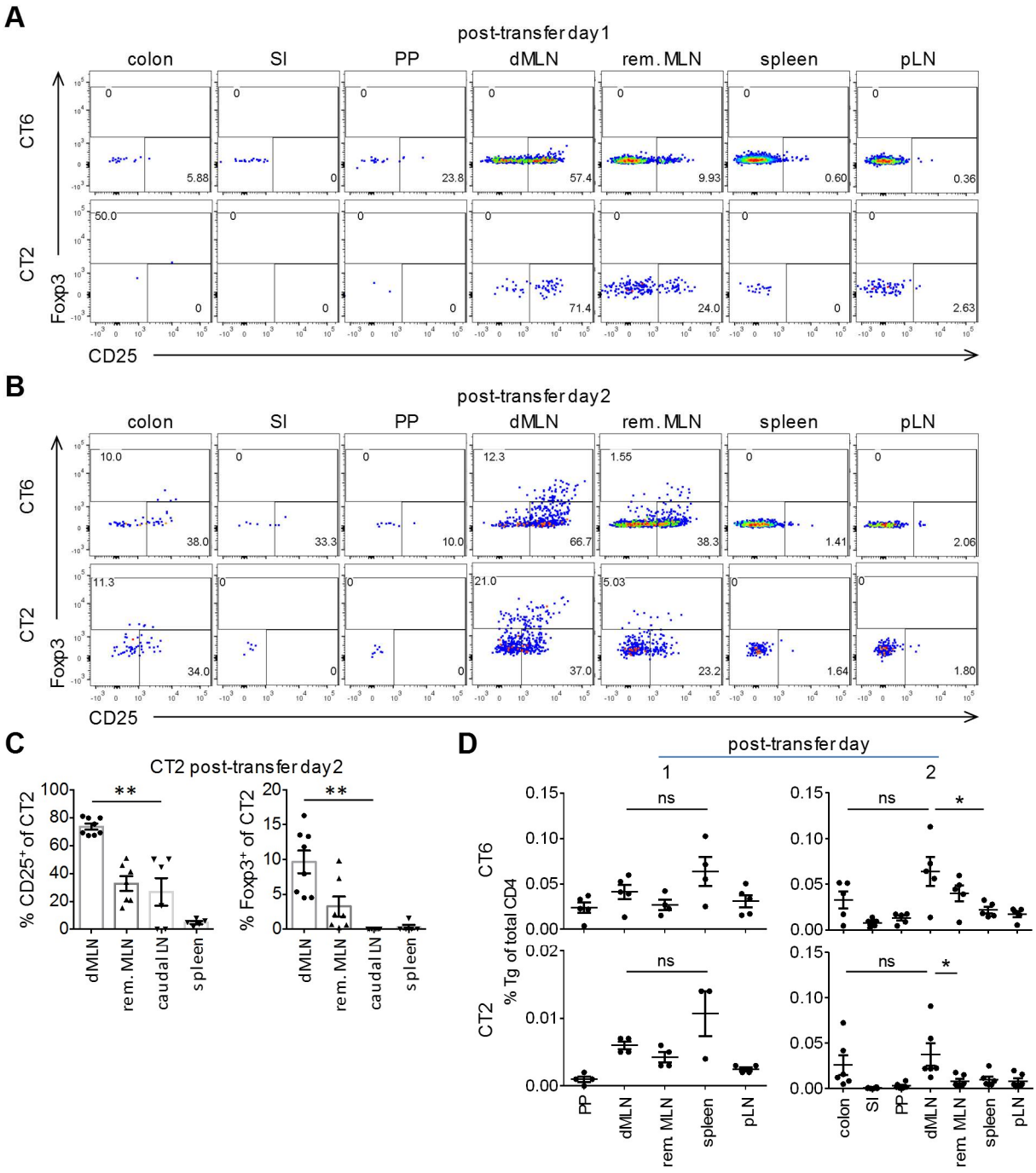

Figure S2, related to Figure 2A. Location of initial T cell activation and Treg cell accumulation. (A-B) Representative FACS plots are shown for experiments summarized in Figure 2A. CD25 and Foxp3 expression on CT6 and CT2 cells (CD45.2$^+$ CD45.1$^-$ Vα2$^+$ CD4$^+$) at d1 (A) or d2 (B) after naïve T cell transfer. dMLN, distal MLN; rem. MLN, remaining MLN; pLN, peripheral LN. (C) Cells in the caudal lymph node at early timepoints are less activated that those in the dMLN. Naïve CT2 Tg cells were intravenously injected into 3-4 week old mice and analyzed after 2 days by flow cytometry for CD25 and Foxp3$^{IRES\text{-}GFP}$ expression (expt=2, n=2-4). (D) The percentage of Tg cells within the whole CD4 T cell population is shown for the

experiments in Figure 2A. Bars indicate mean ± SEM. *$p < .05$, **$p < .005$; ns, not significant; Student's t-test.

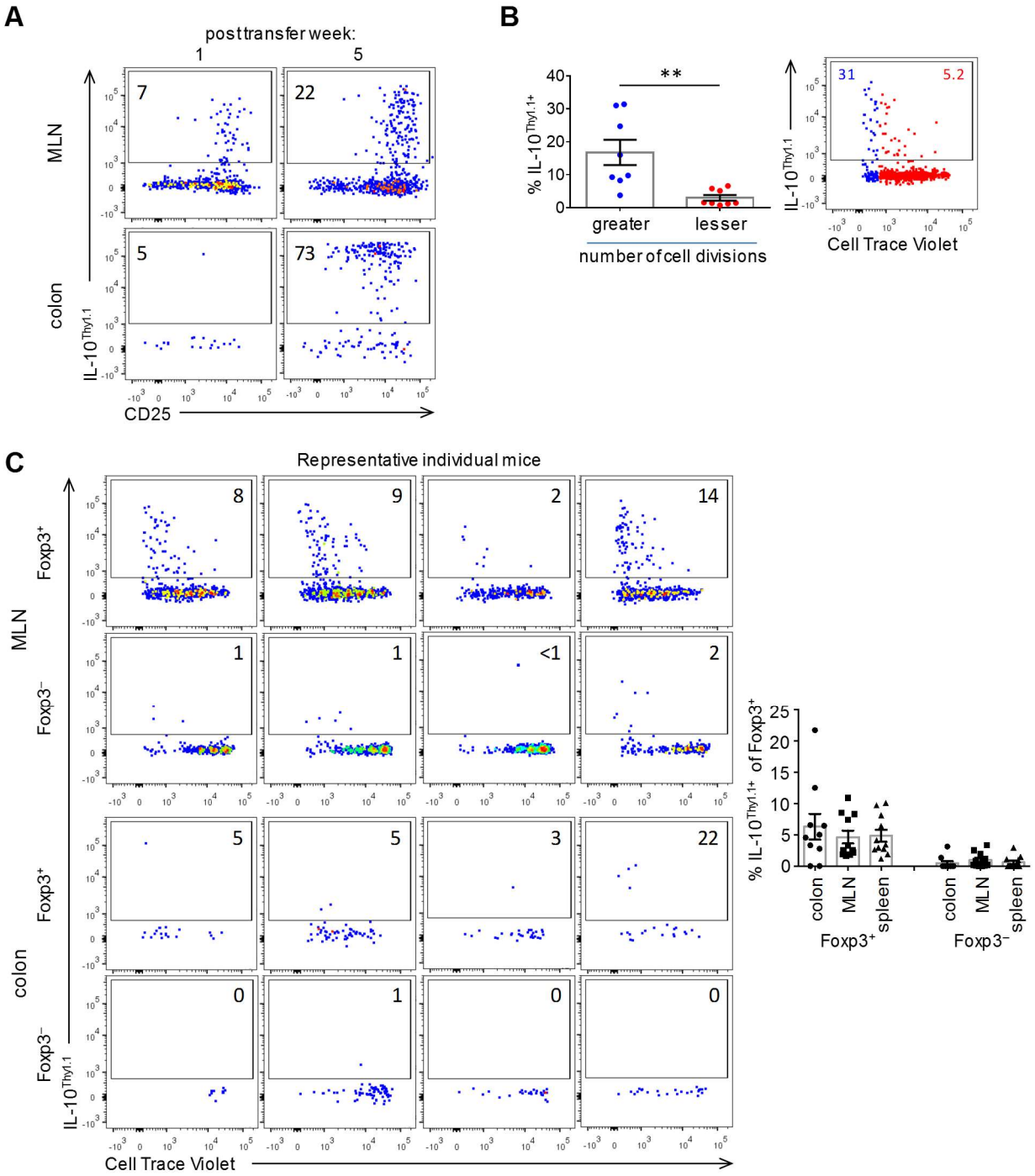

Figure S3, related to Figure 3A. IL-10 expression assessed by the 10BiT reporter. (A) Representative FACS plots are shown of CT2 Foxp3$^+$ Tg$^+$ cells (CD45.1$^-$ CD45.2$^+$ CD4$^+$ Vα2$^+$) generated after the transfer of naïve IL-10$^{Thy1.1-}$ CT2 T cells into 3-4 week old hosts as described in Figure 3A. (B) IL-10$^{Thy1.1+}$ cells are enriched in the most divided population. Cell Trace Violet labelled naïve IL-10$^{Thy1.1-}$ CT2 T cells were injected into 3-4 week old hosts. Cells were harvested one week later and analyzed for the percent of Thy1.1$^+$ cells within the most fully divided versus the rest of the population, indicated by blue/red on the representative FACS

plot (right) and greater/lesser divisions on the bar graph (left), respectively. (C) IL-10$^{Thy1.1+}$ cells are more common in the Foxp3^{+} than Foxp3^{-} populations. Representative FACS plots of wk1 harvest are shown for experiments summarized in Figure 3A and percent IL-10$^{Thy1.1+}$ cells in the Foxp3^{+} or Foxp3^{-} populations are summarized (right). Bars indicate mean ± SEM. $^{**}p < .005$; Student's t-test.

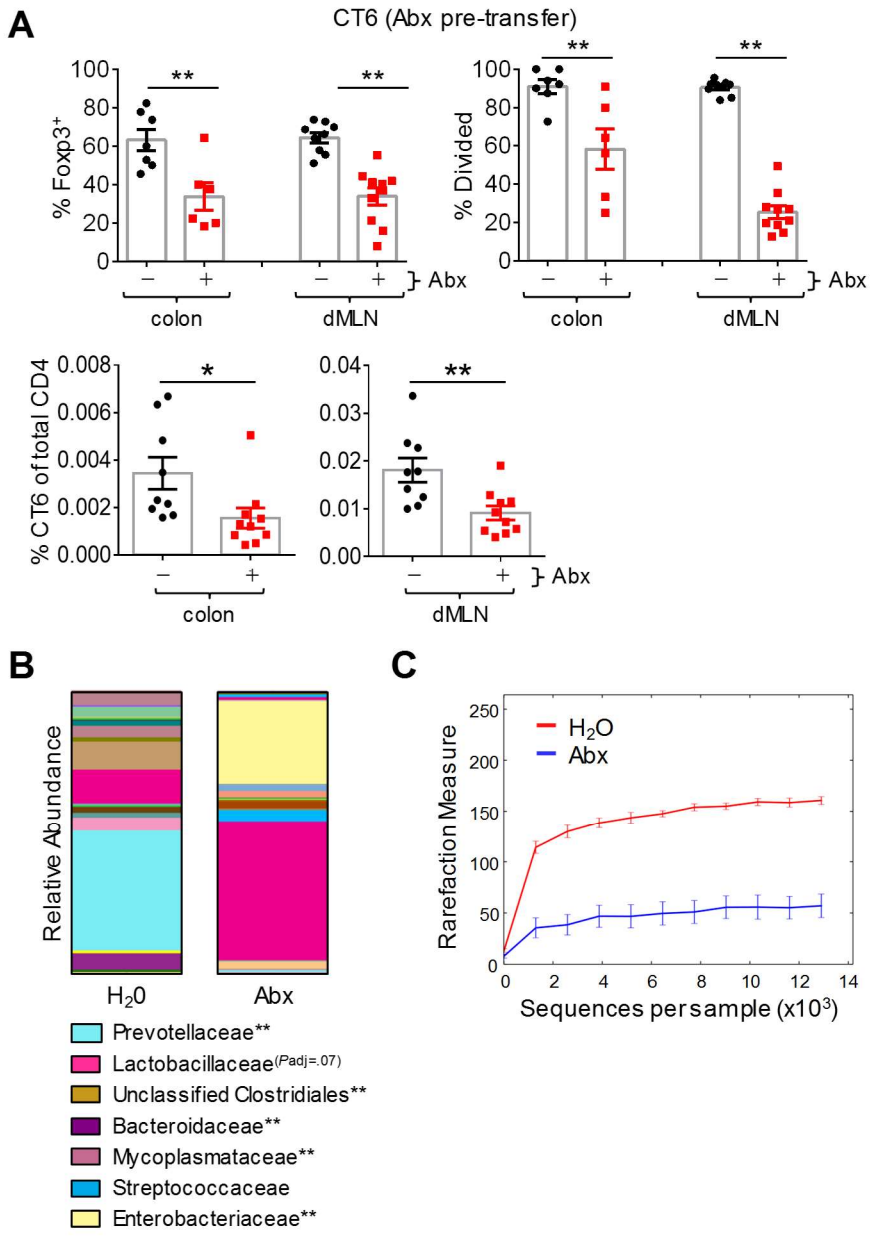

Figure S4, related to Figure 4. Effect of antibiotics on Treg cell generation and expansion. (A) Effects of antibiotics on peripheral Treg cell selection of CT6. Naïve Cell Trace Violet labelled CT6 cells ($5x10^4$) were injected into 4 week old mice treated with clindamycin/streptomycin for 6 days. Transferred cells were analyzed one week later for Foxp3 expression, the percentage of divided CT6 cells, and the percentage of CT6 cells within the CD4 population (expt=2, n=3-5). (B, C) Antibiotic treatment affects CT2, related to Figure 4B. (B) Effect of antibiotics on microbiota. Fecal DNA was isolated from the terminal pellets of mice treated for two weeks with water or antibiotics. DNA was sequenced using the Illumina Miseq platform and analyzed for the relative proportion of bacterial familes (B) (asterix indicate *P*adj, Benjamini-Hochberg false discovery rate corrected Mann-Whitney U), and alpha diversity of OTUs using the Chao1 metric, p≤.05 for all points (C). n=6-7 mice per group. Bars indicate mean ± SEM. **p* < .05, ***p* < .005; Student's t-test.

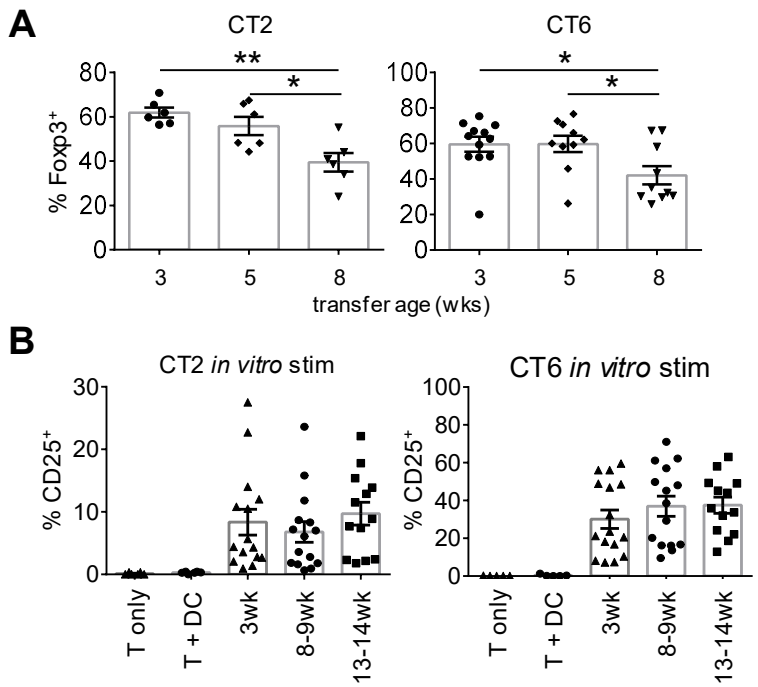

Figure S5, related to Figure 5. Effect of host age on Treg cell induction of CT2 and CT6. (A) Host age and peripheral Treg cell induction. $5x10^4$ CT2 or CT6 cells were injected into 3, 5, or 8 week old mice. One week later cells were harvested and analyzed for Foxp3 expression (expt=2, n=2-5). (B) Ability of luminal contents to stimulate CT2 or CT6 does not decrease with age. Terminal fecal pellets were harvested from 3wk, 8-9wk and 13-14wk old mice, homogenized, autoclaved, and added *in vitro* to wells containing $2.5x10^4$ naïve Tg T cells and $3x10^4$ $CD11c^+$ dendritic cells. Two days later, T cells were assessed for CD25 expression. Each dot represents an individual well/pellet sample from 2 independent experiments. Bars indicate mean ± SEM. *$p < .05$, **$p < .005$; Student's t-test.

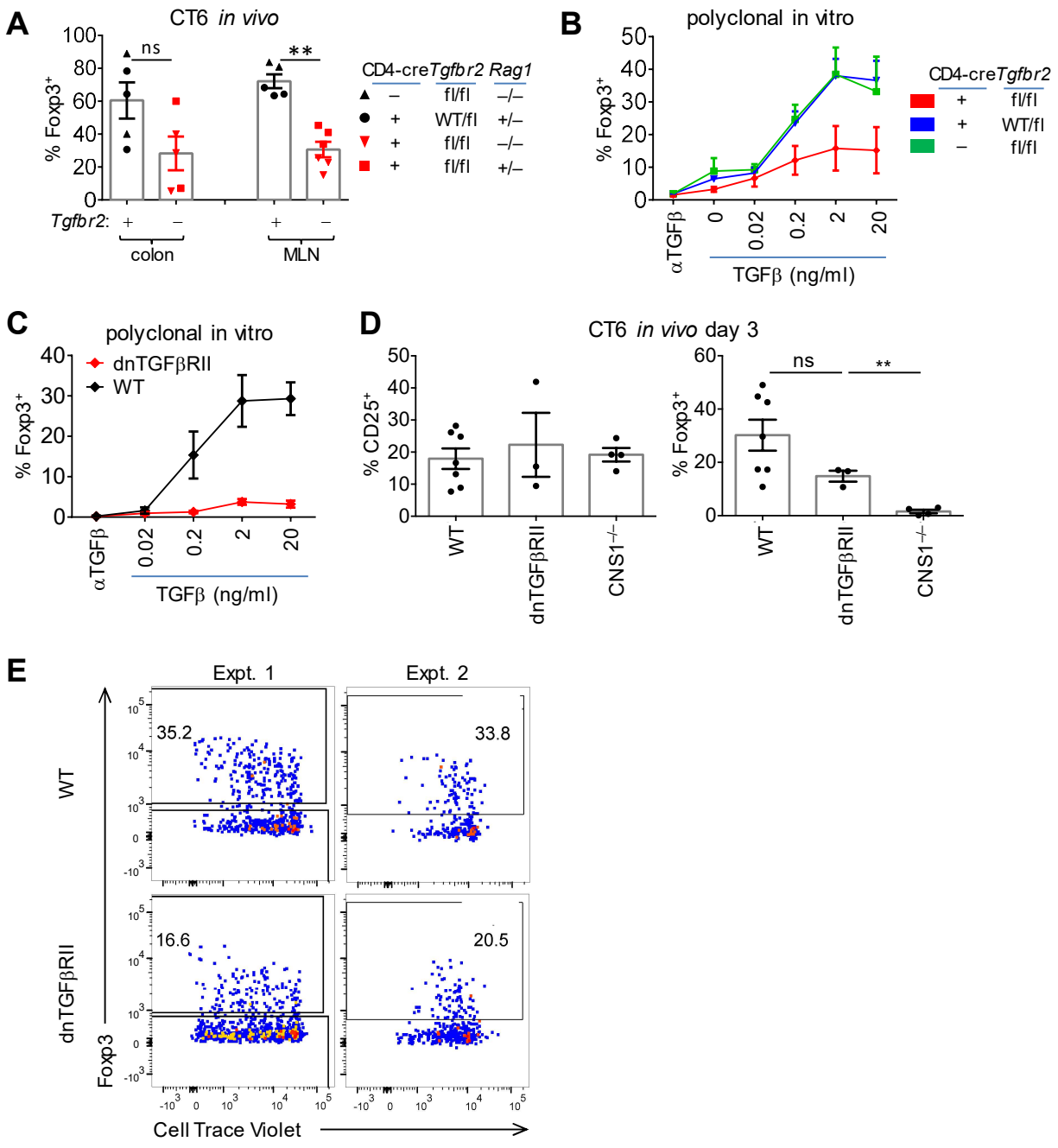

Figure S6, related to Figure 6. Characterization of dnTGFβRII and CNS1$^{-/-}$ on Treg cell induction.

(A-B) Naïve CT6 CD4-cre *Tgfbr2*$^{fl/fl}$ cells are still responsive to TGFβ. (A) Naïve CT6 cells of the indicated genotypes were injected into 3-4 week old mice and analyzed one week later for Foxp3 induction (expt=2, n=2-3). (B) Naïve CT6 Tg cells were isolated and stimulated *in vitro* as described in Figure 6A. At d2 Foxp3$^{IRES-GFP}$ expression was assessed (expt=2, n=1-2). (C) Blockade of TGFβ induced Foxp3 by dnTGFβRII *in vitro*. Naïve wildtype or dnTGFβRII-expressing cells sorted and stimulated as in Figure 6A. At d2 cells were analyzed for Foxp3 expression via intracellular staining. (D) Effect of dnTGFβRII and CNS1 on early Foxp3

induction. $2x10^5$ naïve CT6 Tg cells (WT, dnTGFβRII, or CNS1$^{-/-}$) were injected into 3-4 week old mice and cells in the dMLN analyzed after 3 days (expt=7 WT, 3 dnTGFβRII, 4 CNS1$^{-/-}$, n=1-2). (E) Selective expansion of small Treg cell subset is not seen with dnTGFβRII. Representative FACS plots are shown for experiments summarized in Figure 6D. Bars indicate mean ± SEM. ***p* < .005; ns, not significant; Student's t-test.

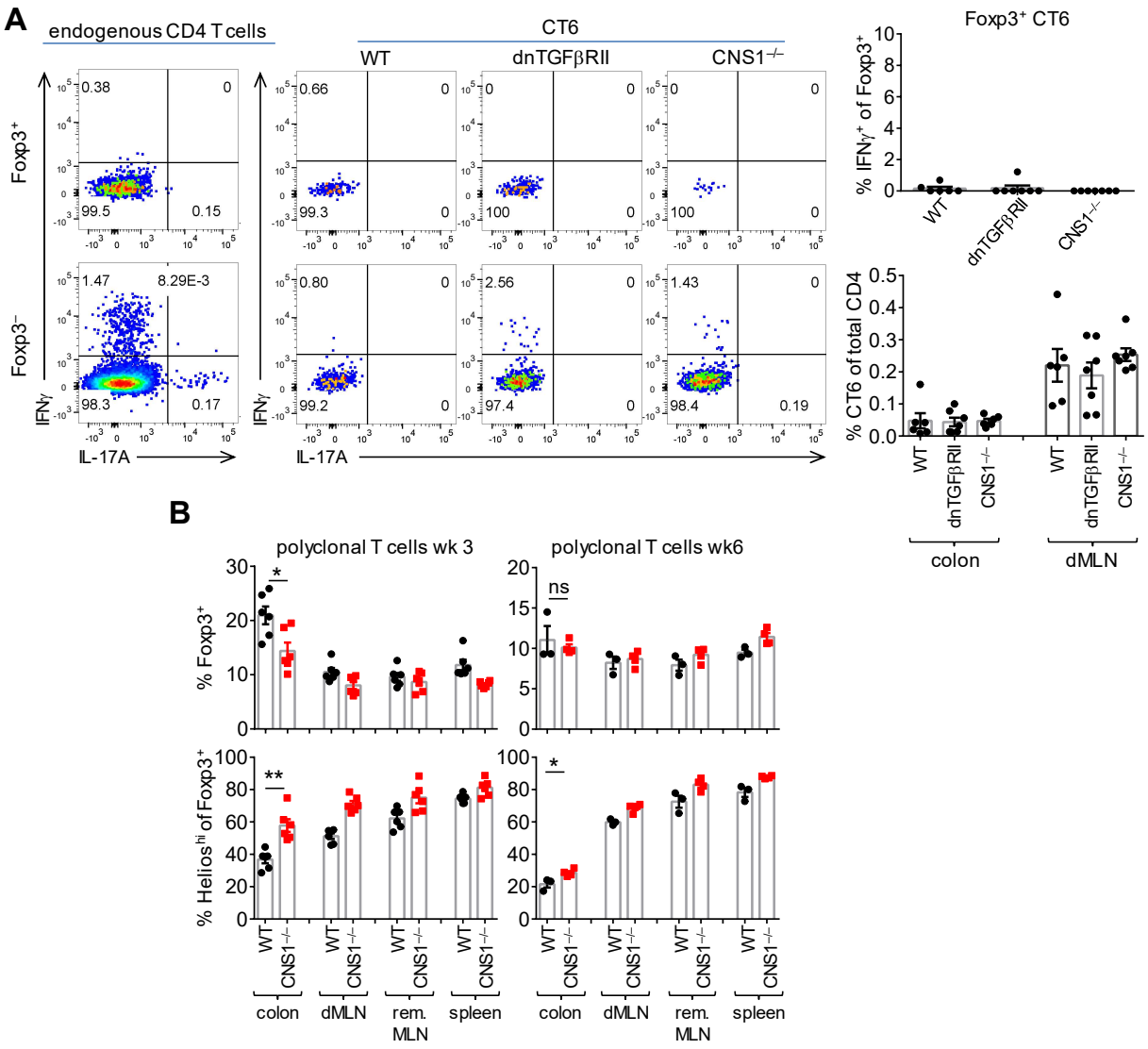

Figure S7, related to Figure 6. Effect of CNS1 on pTreg cell generation.

(A) Cytokine expression of CT6 Treg cells. As per Figure 6E, 2x10^5 naïve CT6 TCR Tg cells on the WT, dnTGFβRII transgenic, or CNS1$^{-/-}$ background were injected into 3 week old hosts. One week later, cells from the dMLN of 2 mice were pooled, stimulated with PMA/Ionomycin, and stained for intracellular IFNγ and IL-17A. The % of Foxp3$^+$ CT6 cells that stain for IFNγ and the % of CT6 cells (CD45.2$^+$ CD45.1$^-$ CD4$^+$ Vα2$^+$) within the total CD4$^+$ population are summarized to the right. IL-17A and IFNγ expression in the endogenous polyclonal CD4$^+$ T cell population is shown as a staining control (left) (expt=2, n=2-4 pools of 2 mice). (B) Age-dependent effects on Helios and Foxp3 expression in polyclonal CNS1-deficient Treg cells. Data shown are the percentage of Foxp3$^+$ cells (left plots) as well as Helios^{hi+} of Foxp3$^+$ CD4$^+$ cells (right plots) from 3 or 6 week old littermates on a CNS1$^{-/-}$ or wildtype background. CNS1-deficient mice were cross-fostered to control variability in microbiota (expt=2-3, n=1-3). Bars indicate mean ± SEM. *p < .05, **p < .005; ns, not significant; Student's t-test.

Gene	Forward Primer	Reverse Primer	Reference
Smurf1	5'-AGTTCGTGGCCAAATAGTGG-3'	5'-GTTCCTTCGTTCTCCAGCAG-3'	(Kaneki et al., 2006)
Smurf2	5'-ATGAAGTCATTCCCCAGCAC-3'	5'-AACCGTGCTCGTCTCTCTTC-3'	(Ramkumar et al., 2012)
TGFβ1	5'-GCTACCATGCCAACTTCTGT-3'	5'-CGTAGTAGACGATGGGCAGT-3'	(Kuczma et al., 2009)
Smad7	5'-AAAGTGAGGAGCAAGATCGGCTGT-3'	5'-AGCCTTGATGGAGAAACCAGGGAA-3'	(Zhou et al., 2014)
GAPDH	5'-ACAAGATGGTGAAGGTCGGTGTGA-3'	5'-AGCTTCCCATTCTCAGCCTTGACT-3'	(Zhou et al., 2014)

Table S1. Forward and reverse primer sequences for qPCR

Forward and reverse primer sequences used in Figure 6A. Previously published sources for the primer sequences are also shown.

Supplemental Experimental Procedures

Mice

CT2 and CT6 transgenic mice were bred onto a *Rag1*-deficient background to exclude secondary TCR alpha rearrangements that can facilitate thymic Treg cell selection (Itoh et al., 1999; Olivares-Villagomez et al., 1998) as well as a Foxp3$^{IRES\text{-}GFP}$ reporter (Lin et al., 2007). *Rag1*$^{-/-}$, dnTGFβRII (Gorelik and Flavell, 2000), CD4-Cre, and floxed *Tgfbr2* (Leveen et al., 2002) mice were obtained from Jackson. Nur77-GFP (Moran et al., 2011) mice were kindly provided by Kris Hogquist (U of MN). *Foxp3*^{gfp}, *Foxp3* CNS1$^{-/-}$ (Zheng et al., 2010) and *Foxp3*$^{Thy1.1}$ (Liston et al., 2008) mice were kindly provided by A. Rudensky (MSK); CD11c-cre Notch2$^{fl/fl}$ (McCright et al., 2006) and BATF3$^{-/-}$ (Hildner et al., 2008) mice by Ken Murphy (Wash U); and Zbtb46-DTR (Meredith et al., 2012) by M. Nussensweig (Rockefeller). Host *Foxp*^{gfp} CD45.1 mice are housed together and interbred to maintain microbial integrity as assessed by CT2/CT6 transfer experiments. Littermate host mice were used for all comparisons between wild-type and altered transgenic cells and antibiotic experiments.

Cell isolation from colonic and small intestinal lamina propria

Colonic or small intestinal segments were treated with RPMI medium containing 3% FBS and 20 mM Hepes (HyClone) with DTT and EDTA for 20 min at 37°C with constant stirring. Tissue was further digested with 28.3 μg/ml liberase TL (Roche) and 200 μg/ml DNase I (Roche), with continuous stirring at 37°C for 30 min. Digested tissue was forced through a Cellector tissue sieve (Bellco Glass) and passed through a 40-μm cell strainer.

Reagents, antibodies, and flow cytometry

Fluorescently conjugated antibodies were purchased from Biolegend, eBioscience, and Becton Dickinson. Intracellular Foxp3 staining was done as directed using the Foxp3 staining kit from ebioscience. Samples were analyzed using a FACSAria (Becton Dickinson) and data were processed with FlowJo (Treestar).

qPCR

Cells were injected and harvested from host mice as described above or cultured *in vitro* with 1 μg/ml plate-bound αCD3, 1μg/ml soluble αCD28 and 50 U/ml IL-2 with 5 μg/ml αTGFβ antibody or .02-20 ng/ml hTGFβ1. Cells were sorted into lysis buffer with TCEP and frozen at -20ºC. RNA was extracted with RNeasy XS kits (Macherey-Nagel) according to instructions and reverse

transcribed with qScript supermix (Quanta Bio). Luminaris Color HiGreen qPCR Master Mix (Thermofisher) or Bullseye EvaGreen qPCR mix (MidSci) and a LightCycler 480 (Roche) were used for real-time quantitative RT-PCR. Quantities of transcripts were normalized to that of GADPH. All samples were run at 94 ºC melting temperature for 10", 60 ºC annealing temp for 20", 72 ºC elongation temp for 20". Forward and reverse primers are listed on Table S1.

Fecal pellet stimulation *in vitro*

Fecal pellets were harvested and frozen at -80ºC. Pellets were thawed and weighed. PBS was added at 30ul/mg and pellets were homogenized. Slurry was autoclaved and added to 96-well experimental plates at a 1:200 dilution with $1.5x10^4$ naïve Tg T cells and $3x10^4$ flt3-ligand-elicited dendritic cells.

Bone marrow chimeras and DT treatment

Bone marrow was obtained by flushing donor humerus, tibia, and femur of Zbtb46-DTR or wildtype $Foxp3^{gfp}$ donor mice. After RBC lysis, $2.5\text{-}5x10^6$ cells were injected into 950 rad lethally irradiated C57BL/6 host mice from NCI. At 8 weeks after BM-injection mice were gavaged with oral antibiotics followed by two days of gavage of fecal contents from our colony. Two weeks after fecal gavage, mice were injected with 20 ng/g DT on day -2 and day -1 prior to T cell injection, and 5 ng/g DT every 2-3 days thereafter to maintain depletion.

Fecal DNA 16S rDNA Sequencing

Fecal material from the last half of the colon of 1.5wk-old mice or the terminal fecal pellet of 3-14wk-old was collected and stored at -80 degrees. Fecal DNA was isolated using the ISOLATE fecal DNA kit (Bioline). PCR was performed using barcoded 16S primers (Caporaso et al., 2011) and sequenced using the MiSeq platform (Illumina). 16S analysis was performed using UPARSE for OTU calling (Edgar, 2013) and phylogeny ascertained using uclust in the QIIME package (Caporaso et al., 2010).

Antibiotic treatment

When antibiotic administration was begun at 2 weeks of age, 100-200 μl antibiotics in water were administered by oral gavage every day (Stefka et al., 2014). When antibiotic administration was begun at 3 or 5 weeks of age, littermates were divided into two groups and treated ad libitum with water alone or water with antibiotics continuously for the time indicated. Antibiotic (VAMN) concentrations were as described (Rakoff-Nahoum et al., 2004): vancomycin (.5mg/ml),

ampicillin (1mg/ml), metronidazole (1mg/ml) and neomycin (1mg/ml). For CT6, clindamycin (.1g/L) and streptomycin (.5g/L) were used.

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