Supplementary Materials for

The microbial metabolites, short chain fatty acids, regulate colonic Treg cell homeostasis

Patrick M. Smith[1], Michael R. Howitt[1], Nicolai Panikov[1], Monia Michaud[1], Carey Ann Gallini[1], Mohammad Bohlooly-Y[5], Jonathan N. Glickman[6,7] and Wendy S. Garrett[1-4]

correspondence to: wgarrett@hsph.harvard.edu

This PDF file includes:

Materials and Methods
Figs. S1 to S24
Table S1
References *(26-28)* appear in the SM

Materials and Methods

Mice

Specified pathogen free (SPF) mice were bred and housed in microisolator cages in the barrier facility at the Harvard School of Public Health. Mouse studies and experiments were approved and carried out in accordance with Harvard Medical School's Standing Committee on Animals and the National Institutes of Health guidelines for animal use and care. Foxp3$^{YFP\text{-}Cre}$ mice on a C57BL/6 background were generously provided by Dr. A. Rudensky (Memorial Sloan Kettering Cancer Center) (*26*). *GPR43*$^{-/-}$ mice were produced by Deltagen (CA, USA) and heterozygous embryos back-crossed onto the C57BL/6 background > 10 generations were provided by AstraZeneca Transgenic and Comparative Genomic R&D (Mölndal, Sweden) (*27*). GPR43 heterozygous cTregs express similar levels of GPR43 and Foxp3 as compared to GPR43 WT cTregs by qPCR (fig. S22). GPR43 expression levels were examined in Foxp3-YFP^{-} and Foxp3-YFP^{+} CD4+ T cells isolated from the colon, small intestine, mesenteric lymph node, and spleen by RTqPCR (fig. S22).

Germ-free mouse experiments

Germ-free (GF) mice (BALB/c WT (pre-dominant strain used except where noted) or Swiss Webster as indicated (fig. S16, S23) were bred and maintained in vinyl positive pressure isolators within the Germ Free and Gnotobiotic core facilities at the Harvard Digestive Diseases Center at Brigham and Women's Hospital or Children's Hospital Boston. GF experiments were performed at the Brigham and Women's Hospital Germ Free and Gnotobiotic core facility. Mice were treated for 21 days with either sodium acetate (150mM), sodium propionate (150mM), sodium butyrate (100mM) or a SCFA mix (67.5mM acetate, 40mM Butyrate, 25.9mM Propionate) in the drinking water and water solutions were prepared and changed weekly. We arrived at the concentration of 150 mM for SCFA treatment based on published work (*3)* and small pilot technical experiments wherein we observed no augmented cTreg responses when 50 mM of individual SCFA was given in the drinking water and decreased oral intake when >200 mM SCFA was given in the drinking water. In *in vivo* drinking water treatment experiment, control mice received pH and sodium-matched water. At the conclusion of the experiment, mice were removed from isolators and processed immediately. Data on the stability of the SCFA mix solution are provided in Table S1.

SPF SCFA intervention

For all SPF mouse experiments, mice were treated for 2 weeks with SCFA in the drinking water at the concentrations indicated above and water (pH and sodium-matched) was prepared and changed every three days. To compare oral vs intra-rectal administration of SCFA, mice were intra-rectally instilled with either 200µl of propionic acid (50mM) or pH and sodium matched water, for two weeks, using an umbilical vein catheter 3.5F(fig. S24). For T cell colitis experiments, mice received either sodium propionate (150mM) or the SCFA mix (described above) in the drinking water beginning at day 0.

Antibiotic treatment

Mice were treated with vancomycin (500mg/l; Sigma) in the drinking water for 4 weeks. Fluid intake was monitored and the antibiotic solution was changed every 3 days.

T cell transfer model of colitis

At Day 0, Naive $CD4^+CD25^-CD45RB^{hi}$ splenic T cells were FACS-sorted from BALB/c or C57BL/6 mice and injected i.p. into 6-8 week old BALB/c or C57BL/6 *RAG2*$^{-/-}$ immunodeficient recipients (5×10^5 cells/mouse). At day 10, recipient mice received $CD4^+CD25^+CD45RB^{lo}$ splenic Treg cells (75,000/mouse) isolated from BALB/c or C57BL/6 mice *FFAR2*$^{+/+}$ and *FFAR2*$^{-/-}$ mice where indicated. Mice were monitored weekly for weight loss and morbidity for 6-9 weeks as per our protocol's experimental endpoint guidelines.

Lamina propria (LP) lymphocyte isolation

To isolate LP lymphocytes, small and large intestines were collected and opened longitudinally and washed with PBS to remove fecal contents. To remove epithelial cells, mucus and fat tissue, intestines were incubated 2 times in EDTA (5mM)/DTT (1mM)/Dulbecco's PBS (calcium and magnesium-free) solution (5ml/colon) for 25 min at 37°C. Intestines were then minced and collagenase-digested two times for 45 min at 37°C in RPMI containing 1 mg/ml collagenase type VIII (Sigma), 25μg/ml DNase I (Roche), 50μg/ml Dispase (StemCell Technologies) and 0.01M HEPES (StemCell Technologies). The crude cell suspension was loaded onto a 40%/90% Percoll (GE Healthcare) gradient and centrifuged at 720 X g for 25 min at room temperature with acceleration and brake turned off. LP lymphocytes were collected from the 40%/90% interface.

Flow cytometry staining

Intracellular staining of the transcription factors Foxp3, Helios, and T-bet was performed using the Foxp3 Fix/Perm Buffer Set (Biolegend). For detection of intracellular cytokines, cells were first stimulated for 4 h with 50ng/ml PMA and 1 μg/ml ionomycin in the presence of Brefeldin A (All obtained from Sigma), followed by staining for surface markers. Cells were then fixed and permeabilized using the Foxp3 Fix/Perm Buffer Set (Biolegend) and stained for intracellular cytokines. The following antibodies were used: PE-labeled anti-Foxp3 (FJK-16s, eBioscience), Pacific Blue-labeled anti-Helios (22F6), PE-, FITC- or APC-labeled anti-CD4 (RM4-5), PE-Cy7-labeled anti-CD3 (145-2C11), PE- or APC-labeled anti-IL-10 (JES5-16E3), PerCP-Cy5.5-labeled anti-IL-17 (TC11-18H10.1), APC- or PE-Cy7-labeled anti-IFNγ (XMG1.2), Pacific Blue-labeled anti-TGFβ1 (TW7-16B4), FITC-, PerCP-Cy5.5 or Pacific Blue-labeled anti-CD45 (30-F11), PerCP-Cy5.5 anti-CD45RB (C363-16A), PE- or FITC-labeled anti-CD25 (PC61) and Brilliant Violet 421-labeled anti-CD127 (A7R34), GPR43 rabbit polyclonal (Abcam

Catalog # Ab118449), and anti-rabbit IgG-DyLight649 (Poly4064). All antibodies were obtained from Biolegend unless otherwise noted. Flow cytometry was performed using a BD LSRII (BD Biosciences) and data were exported as FCS 3.0 files using FACSDiva Software 6.1.3 and data were analyzed with FlowJo10.0.5 software (TreeStar, Inc.). Cell sorting was performed using a FACSAriaIIu at the Dana-Farber Cancer Institute Flow Cytometry Core.

SCFA measurement

Cecal or small intestinal contents were collect immediately after animals were sacrificed and flash frozen in N_2(l). Samples were mashed in 500µl HPLC grade water and centrifuged at > 14,000 X g, the resulting supernatant was then passed through a 0.22µm syringe filter to remove bacterial cells and debris. Samples were then acidified with 1/10 volume of 0.01M H_2SO_4, heated, and passed through a condenser to isolate volatile compounds within a sample. SCFA analyses were performed using an Agilent 1200 series HPLC and a Poroshell 120 SB C18 column (2.7µm, 3.0x100mm) with guard column (Agilent Technologies). Sulfuric acid (0.01M) was used as the mobile phase. SCFA were identified by comparing sample peak retention times to a standard volatile acid mix (Matreya 1075) and concentrations were determined by external standard calibration method using Agilent Chemstation 3D for LC systems Rev. B. 04.02 SP1 (Agilent Technologies). All samples and standards were prepared as described above. SCFA concentrations were weight corrected.

Single colonies were inoculated into chopped meat glucose media (Anaerobe Systems) and cultured in an anaerobic hood for 48 hrs at 37 ° C. Tubes were vortexed, 1 ml of media was transferred to an eppendorf tube, and in parallel serial dilutions were grown on pre-reduced Brucella agar with 5% sheep blood to determine the colony forming units per ml of the liquid cultures. Eppendorf tubes were immediately removed from the anaerobic hood upon collection, spun at 14,000 X g for 5 minutes, supernatant was transferred to a fresh tube, and samples were flash frozen until they were processed as described above. A non-inoculated chopped meat glucose media tube was used as a control and processed in the same manner as the experimental samples. No propionate, acetate, or butyrate were detected in the non-inoculated media tube. Butyrate was not detected in any of the culture supernatants and these data are consistent with the short chain fatty profiles published for these species in the Virginia Polytechnic Institute Anaerobe Laboratory Manual Fourth Edition.

RNA isolation

Tregs were isolated from the colon, small intestine, mesenteric lymph node or spleen of C57BL/6 Foxp3$^{YFP\text{-}Cre}$ mice and FACS-sorted based on expression of YFP. RNA samples were prepared using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the Bio-Rad iScript cDNA Synthesis Kit. Real-time (RT)-qPCR was performed using the KAPA SYBR FAST Universal qPCR Kit (KAPA Biosystems) and a Stratagene MX3005P (Agilent Technologies). The following primer sequences were used: Foxp3, 5' - GGCAATAGTTCCTTCCCAGAGTT-3' and 5' - GGGTCGCATATTGTGGTACTTG-

3'; IL-10, 5' - TTTGAATTCCCTGGGTGAGAA-3' and 5' - GGAGAAATCGATGACAGCGC-3'; TGFβ1, 5' - CCGCAACAACGCCATCTATG-3' and 5' - CCCGAATGTCTGACGTATTGAAG-3'; GPR43, 5' - AATTTCCTGGTGTGCTTTGG-3' and 5' - ACCAGACCAACTTCTGGGTG-3'; HDAC1, 5' - CCAAGTACCACAGCGATGAC-3' and 5' - TGGACAGTCCTCACCACG-3'; HDAC2, 5' - TGAAGGAGAAGGAGGTCGAA-3' and 5' - GGATTTATCTTCTTCCTTAACGTCTG-3'; HDAC7, 5' - CTCGGCTGAGGACCTAGAGA-3' and 5' - CAGAGAAATGGAGCCTCTGC-3'; HDAC3, 5' - CACCATGCCAAGAAGTTTGA-3' and 5' - CCCGAGGGTGGTACTTGAG-3'; HDAC6, 5' - CTGCATGGCATCGCTGGTA-3' and 5' - GCATCAAAGCCAGTGAGATC-3'; HDAC9, 5' - GCGGTCCAGGTTAAAACAGAA-3' and 5' - GCCACCTCAAACACTCGCTT-3'; GPR15, 5' - GGAGGACTGGCTCTTTCCTG - 3' and 5' - AAGGCTGGGTGCATGATAGC - 3'.

In vitro T cell suppression assay

$CD4^+CD25^-$ T effector cells (Teff) were sorted from spleens of SPF mice and labeled with CFSE (Invitrogen) or Cell Trace Violet (Invitrogen) as per manufacturers instructions. Teff cells were plated at 5 x 10^4 cells/well in 96-well round-bottom plates with 5 x 10^4, CD4 depleted, irradiated (3000rad) splenocytes, 1 μg/ml anti-CD3 antibody and various numbers of $CD4^+CD25^+$ Tregs from the colonic lamina propria of SPF mice. Where *indicated* 0.1mM sodium acetate, sodium propionate or sodium butyrate was added to the culture. After 96 h, cells were collected and analyzed by flow cytometry.

In vitro Treg proliferation

Colonic lamina propria Tregs were isolated from C57BL/6 $Foxp3^{YFP\text{-}Cre}$ mice as described above and FACS-sorted based on expression of YFP. Isolated Tregs were cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco), 4mM L-glutamine, 80 U/ml penicillin, 80 μg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES and 1x nonessential amino acids (all obtained from Cellgro). Tregs were stimulated with 1 μg/ml anti-CD3 antibody and with or without 0.1 mM sodium propionate for 24 h at 37°C. The division index is calculated by dividing the total number of cell divisions by the number of cells added at the start of the culture and represents the average number of divisions a cell from the original population has undergone and includes the undivided fraction in the calculation.

Western Blot

Colonic lamina propria Tregs were isolated and whole cell lysates generated using RIPA buffer in the presence of protease inhibitors. Protein lysates were resolved using SDS-PAGE and transferred to PVDF membrane using a Bio-Rad wet transfer apparatus. Blots were probed with antibodies directed against acetyl-histone H3 (K9) and histone H3 (All from Cell Signaling Technologies). After incubation with the appropriate HRP-

conjugated antibody, ECL was used for developing. Densitometry analysis of western blots was performed using Image-J software (http://rsbweb.nih.gov/ij/)

ELISAs

Cytokines were measured in culture supernatants using standard cytokines, antibodies and protocols. IL-10 was measured using the BD OptEIA ELISA Kit (BD Biosciences) and TGFβ1 levels were measured using the mouse TGF-beta 1 DuoSet (R&D Systems).

Histology and colitis scores

Colons were excised and cleaned with DPBS prior to fixation in 4% PFA and then processed by routine paraffin embedding, sectioning and H&E staining. Colitis scores were determined by J.N.G., who was blinded to the experimental parameters. Each of 4 histologic parameters were scored as absent (0), mild (1), moderate (2), or severe (3): mononuclear cell infiltration, polymorphonuclear cell infiltration, epithelial hyperplasia, and epithelial injury. The scores for the parameters were summed to generate the cumulative histologic colitis score as previously described (*28*).

Statistical analyses

GraphPad Prism® Software was used for the calculation of statistical measures, including mean values, standard errors, students' *t* test, Mann-Whitney test and Kruskal-Wallis test.

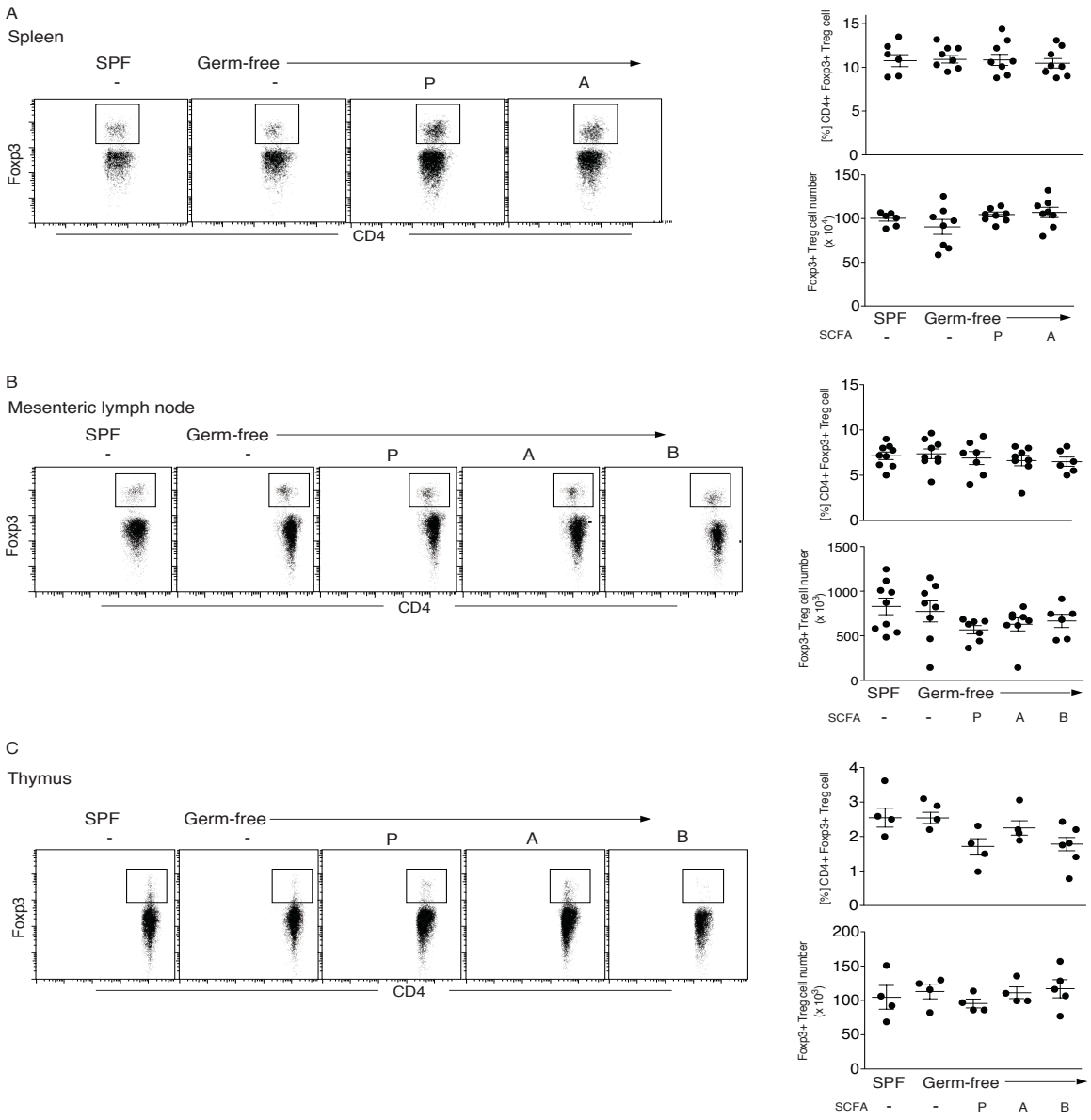

Fig. S1. SCFA do not affect splenic, mesenteric lymph node or thymic Foxp3$^+$ Treg populations in germ-free mice.

Lymphocytes were isolated from the **(A)** spleen, **(B)** MLN or **(C)** thymus and stained for CD4 and Foxp3. Upper left panel: Representative flowgrams. Upper right panel: Percentage of and Lower right panel: Number of CD4$^+$Foxp3$^+$ within the CD45$^+$CD3$^+$ population from SPF, GF, and GF mice treated with P, A or B in the drinking water per mouse colon as indicated. Each symbol represents data from an individual mouse and data reflect 3-5 independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines show the mean and error bars the SD.

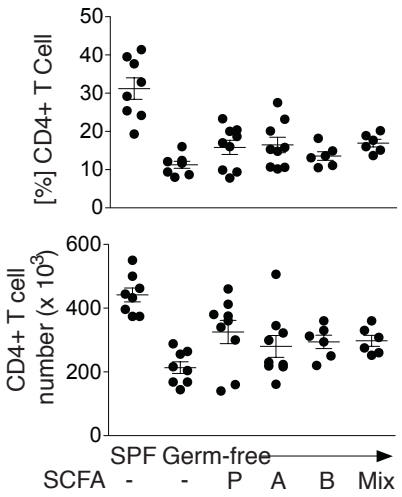

Fig. S2. SCFA affect total colonic $CD4^+$ T cells.

Lymphocytes were isolated from the colon and stained for CD4 and Foxp3. Upper panel: Percentage of and Lower panel: Number of $CD4^+$ within the $CD45^+CD3^+$ population from SPF, GF, and GF mice treated with P, A, B, and SCFA mix per mouse colon. Each symbol represents data from an individual mouse and the data reflect 3-5 independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines show the mean and error bars the SD.

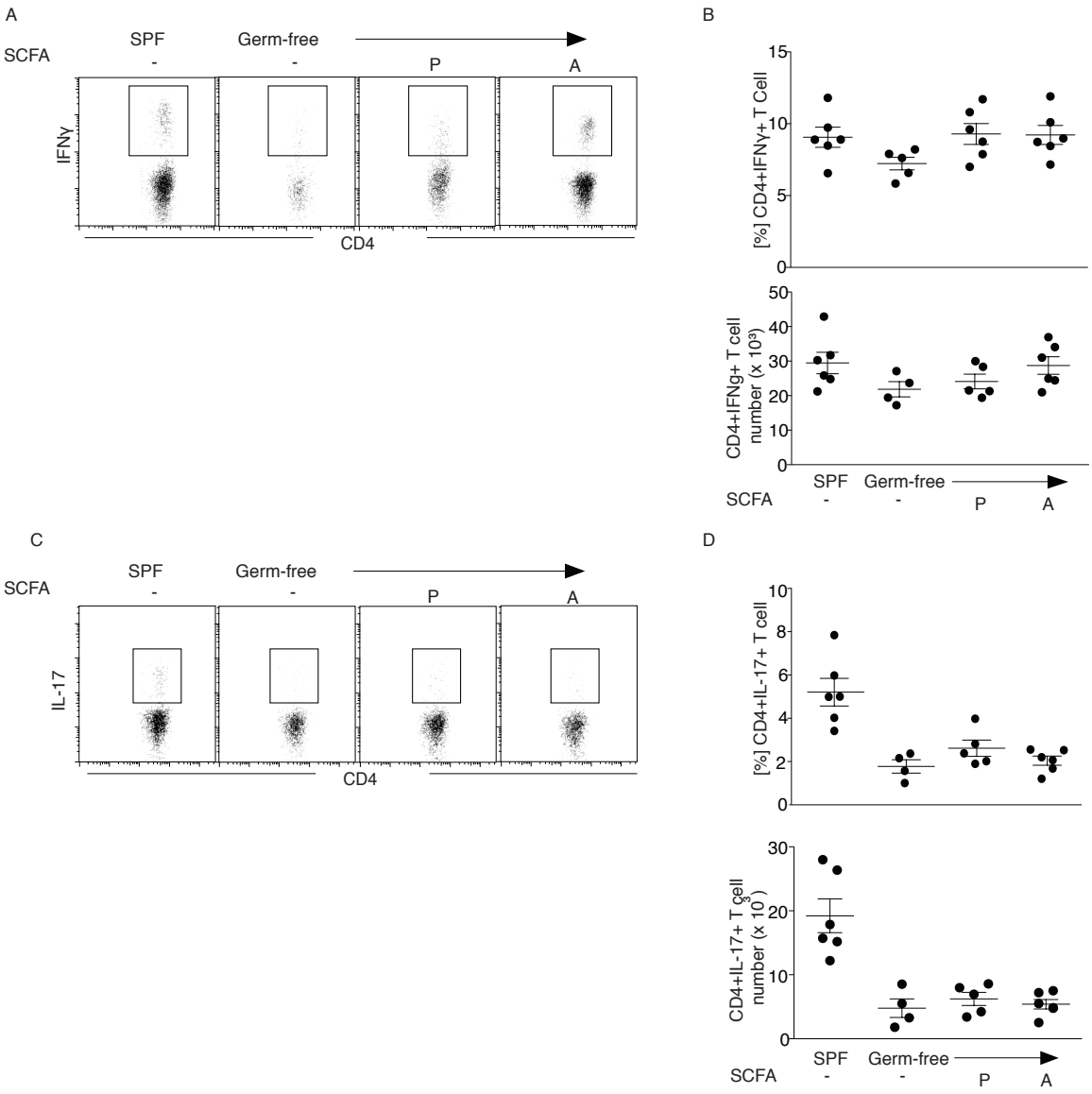

Fig. S3. SCFA do not affect colonic Th1 and Th17 cells in GF mice.

Lymphocytes were isolated from the colon of SPF, GF and GF mice treated with P or A in the drinking water for three weeks and stained for CD45, CD3, CD4, IFNγ, and IL-17. (A) Representative flowgrams of $CD45^+CD3^+CD4^+IFN\gamma^+$populations. (B) Upper right panel: Percentage of and Lower right panel: Number of $CD4^+IFN\gamma^+$ cells within the $CD45^+CD3^+$ population. (**C**) Representative flowgrams of $CD45^+CD3^+CD4^+IL\text{-}17^+$ populations. (D) Upper right panel: Percentage of and Lower right panel: Number of $CD4^+IL\text{-}17^+$ cells within the $CD45^+CD3^+$ population. Each symbol represents data from an individual mouse and data reflect 3 independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines show the mean and error bars the SD.

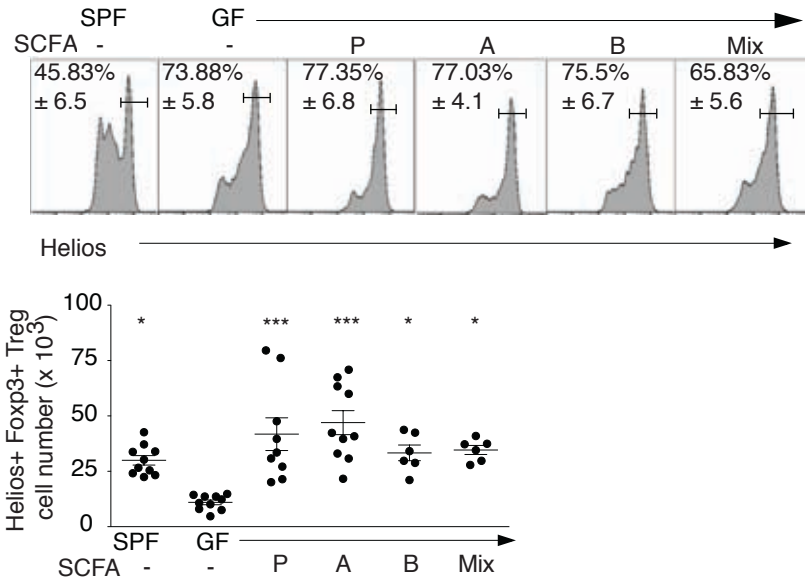

Fig. S4. SCFA expand Foxp3$^+$Helios$^+$ Tregs in the colon of GF free mice.

Colonic LP lymphocytes were isolated from SPF, GF, and GF mice treated with P, A, B, or the SCFA mix in the drinking water for three weeks and stained for CD45, CD4, Helios, and Foxp3. Upper panel: Representative flowgrams and percentage of Foxp3$^+$CD4$^+$ population with Helios staining. Lower panel: Numbers of Helios$^+$Foxp3$^+$ Tregs for the upper panel. Each symbol represents data from an individual mouse and data reflect 3 independent experiments. Kruskal-Wallis with Dunn's post-hoc was performed. *** $P < 0.001$, * $P < 0.05$. Horizontal lines represent the mean and error bars the SD.

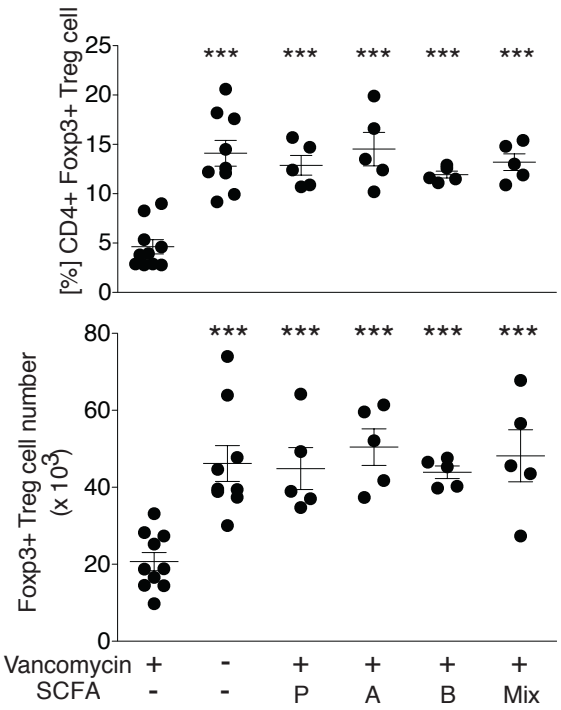

Fig. S5. SCFA restore colonic Foxp3$^+$ Treg levels following antibiotic treatment.

Mice were treated orally with vancomycin for 4 wks and also given P, A, B, or the SCFA mix in the drinking water starting in week 2. After 4 wks, colon LP lymphocytes were isolated and stained for CD45, CD4 and Foxp3. The percentage and number of CD4$^+$Foxp3$^+$ within the CD45$^+$ population from SPF mice or vancomycin-treated SPF mice exposed to water alone (-), P, A, B, or the SCFA mix are shown. Each symbol represents data from an individual mouse and data reflect 3 independent experiments. Kruskal-Wallis with Dunn's post-hoc was performed. *** $P < 0.001$. Horizontal lines represent the mean and error bars the SD.

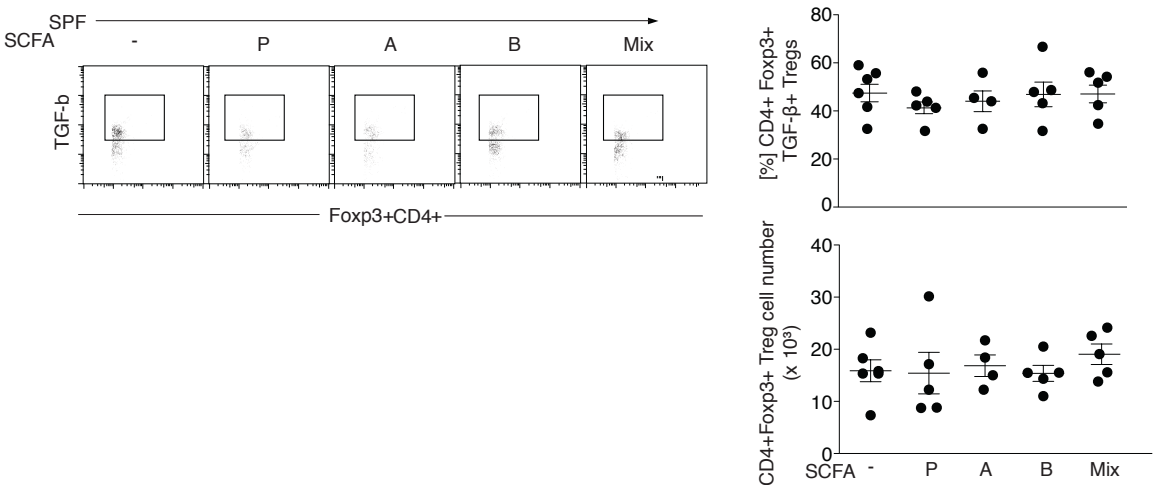

Fig. S6. SCFA do not affect TGFβ levels of colonic Foxp3$^+$ Treg populations in SPF mice.

BALB/c SPF mice were treated with pH and sodium-matched water alone (-), P, A, B or the SCFA mix in the drinking water for two weeks. Colonic LP lymphocytes were isolated and stained for CD45, CD4, Foxp3, and TGF-β. Upper left panel: Representative flowgrams. Upper right panel: Percentage of and Lower right panel: Number of CD4$^+$Foxp3$^+$ Tregs expressing TGFβ within the CD45$^+$ population from all of the treatment groups. Each symbol represents data from an individual mouse and the data reflect 3 independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines represent the mean and error bars the SD.

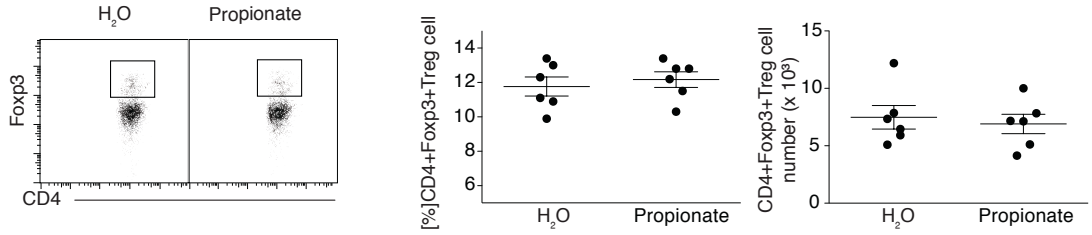

Fig. S7 Propionate does not affect small intestinal Foxp3$^+$ Treg populations in SPF mice.

LP lymphocytes were isolated from the distal 10 cm of the ileum and stained for CD45, CD4, and Foxp3. Left panel: Representative flowgrams. Middle panel: Percentage of and Right panel: Number of CD4$^+$Foxp3$^+$ within the CD45$^+$ population from SPF mice treated with propionate or pH and sodium-matched water per mouse distal ileum. Each symbol represents data from an individual mouse and data reflect two independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines represent the mean and error bars the SD.

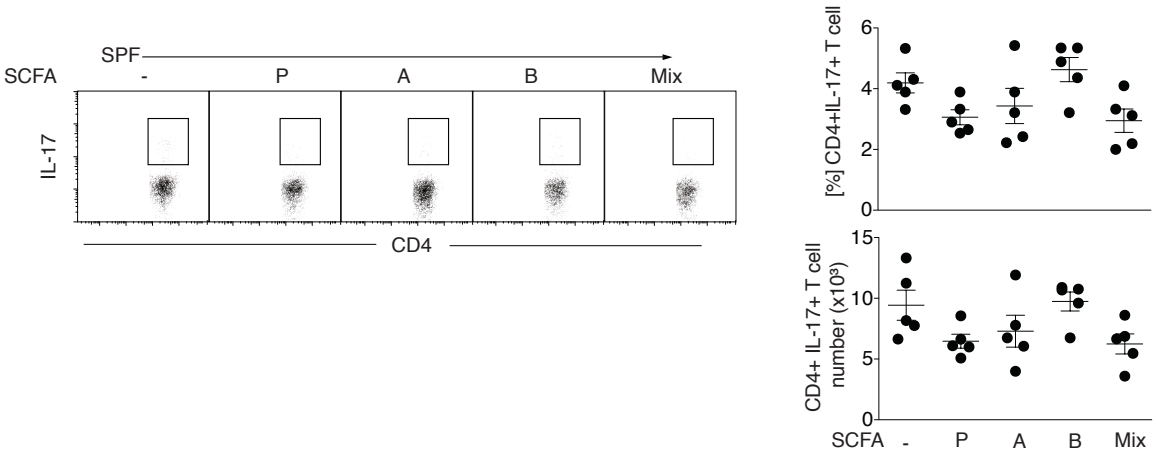

Fig. S8. SCFA do not affect colonic Th17 populations in SPF mice.

BALB/c SPF mice were treated with pH and sodium-matched water alone (-), P, A, B or the SCFA mix. Colonic LP lymphocytes were isolated and stained for CD45, CD4, Foxp3, and IL-17. Upper left panel: Representative flowgrams. Upper right panel: Percentage of and Lower right panel: Number of $Foxp3^{-}CD4^{+}$ T cells expressing IL-17 per mouse colon. Symbols represent data from individual mice. Data reflect three-five independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines represent the mean and error bars the SD.

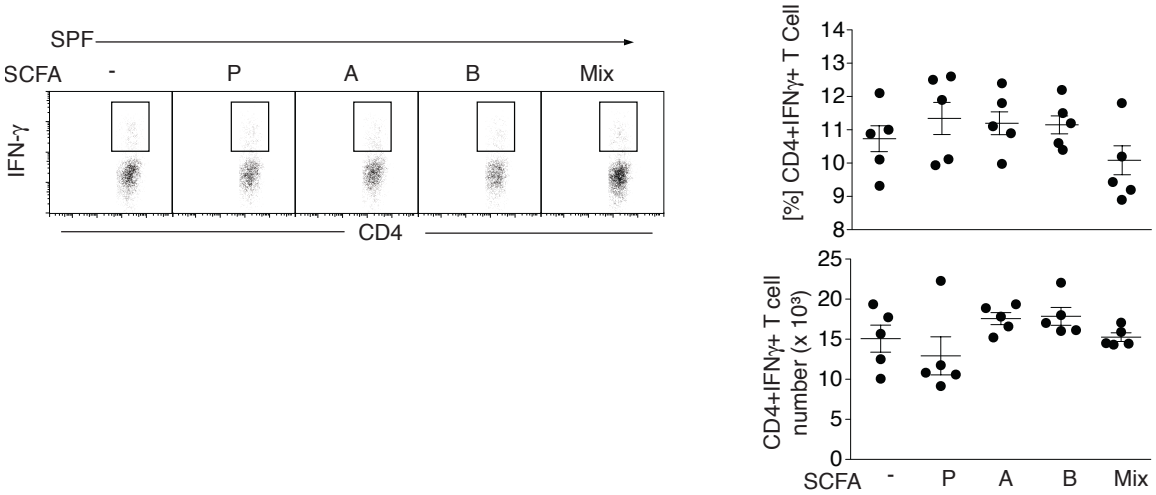

Fig. S9. SCFA do not affect colonic Th1 populations in SPF mice.

BALB/c SPF mice were treated with pH and sodium-matched water alone (-), P, A, B or the SCFA mix. Colonic LP lymphocytes were isolated and stained for CD45, CD4, and IFNγ. Upper left panel: Representative flowgrams. Upper right panel: Percentage of and Lower right panel: Number of $CD4^+$ T cells expressing IFNγ within the $CD45^+$ population from all of the treatment groups. Symbols represent data from individual mice and reflect data from three independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines represent the mean and error bars the SD.

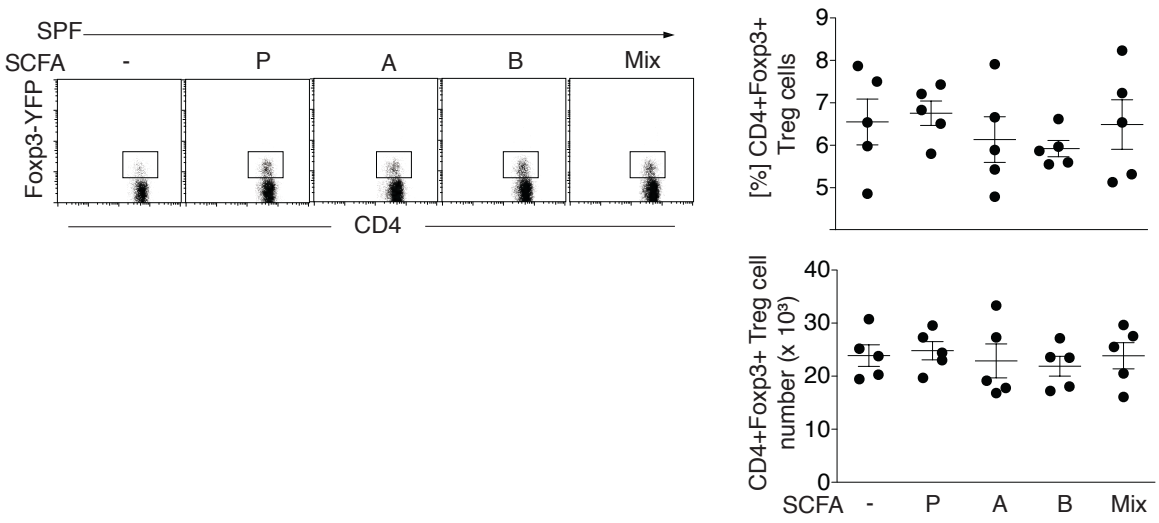

Fig. S10. SCFA do not affect mesenteric lymph node Foxp3$^+$ Treg populations in SPF mice.

Lymphocytes were isolated from the MLNs of BALB/c SPF mice treated with pH and sodium-matched water alone (-), P, A, B or the SCFA mix and stained for CD45, CD4, and Foxp3. Upper left panel: Representative flowgrams. Upper right panel: Percentage of and Lower right panel: Number of CD4$^+$Foxp3$^+$ within the CD45$^+$ population. Symbols represent data from individual mice and reflect data from three independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines represent the mean and error bars the SD.

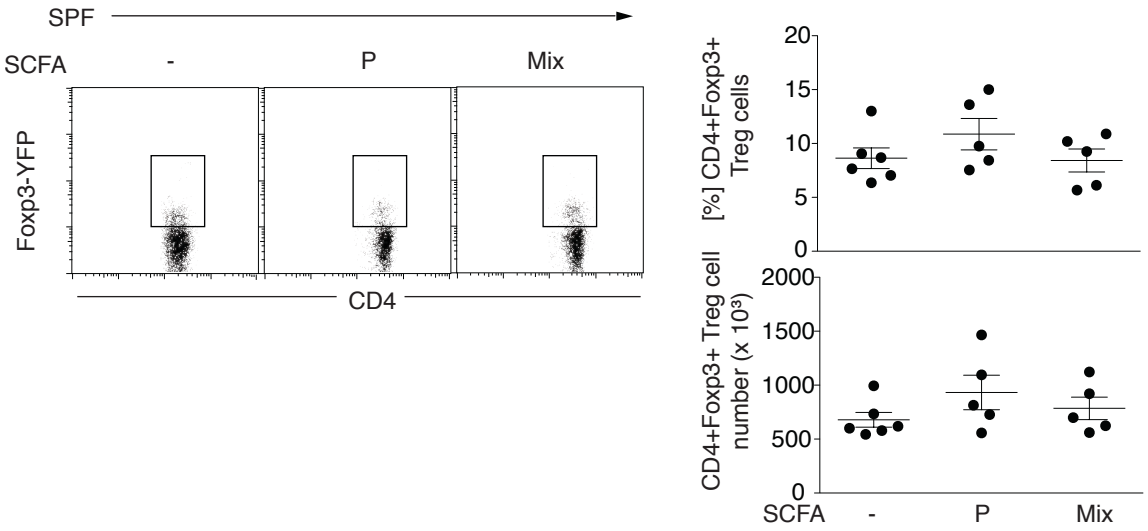

Fig. S11. SCFA do not affect splenic Foxp3+ Treg populations in SPF mice.

Lymphocytes were isolated from the spleen of BALB/c SPF mice treated with pH and sodium-matched water alone (-), P, or the SCFA mix and stained for CD45, CD4, and Foxp3. Upper left panel: Representative flowgrams. Upper right panel: Percentage of and Lower right panel: Number of $CD4^{+}Foxp3^{+}$ within the $CD45^{+}$ population. Symbols represent data from individual mice and reflect data from two independent experiments. *P*-values are not shown as differences with SCFA treatment were not statistically significant. Horizontal lines represent the mean and error bars the SD.

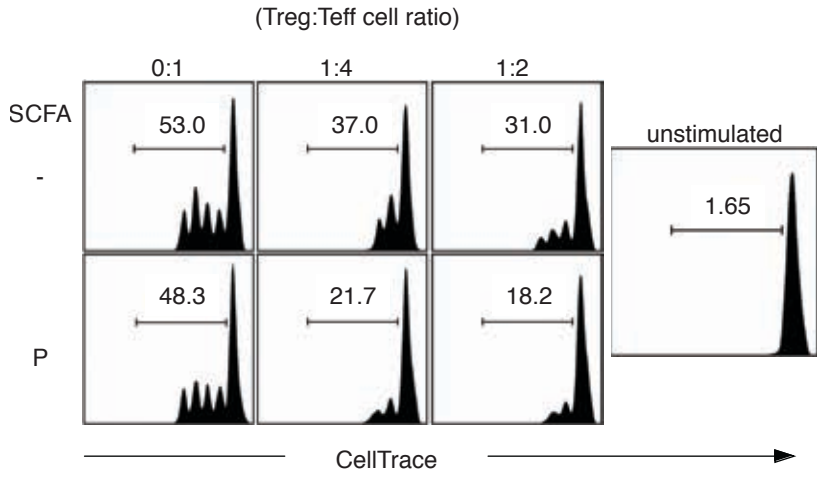

Fig. S12. SCFA increase colonic Treg suppressive capacity.

Colonic LP Tregs and splenic effector T cells were isolated by FACS sorting. Splenic effector T cells were first labeled with CellTrace cell violet dye (5μM) and cultured alone or co-cultured with cTregs. Cultures were either unstimulated or stimulated with 1μg/ml plate bound anti-CD3, 1μg/ml soluble CD28 and P or pH and sodium matched media for 96 hrs. Flowgrams depicted are representative of three independent experiments. Significance is shown in Figure 2D. Horizontal lines represent the mean and error bars the SD.

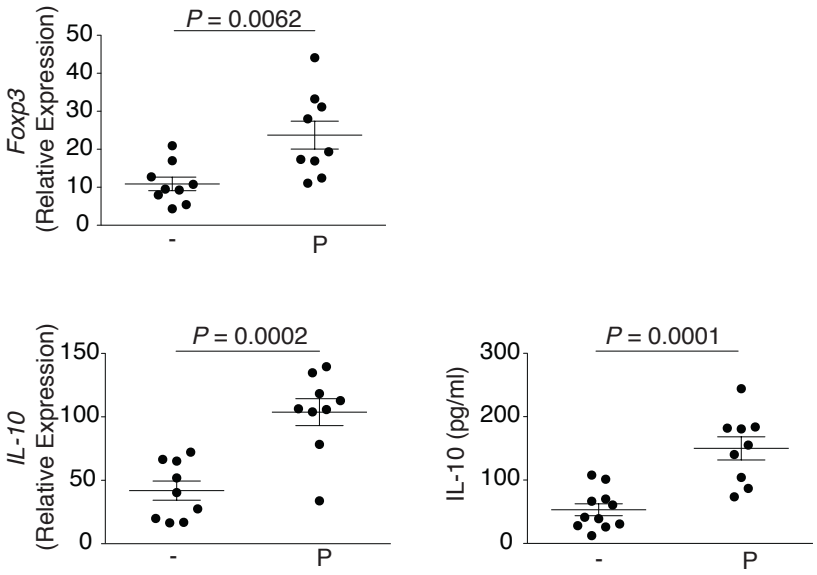

Fig. S13. *In vitro* treatment of colonic Tregs with propionate increases *Foxp3* and *IL-10* expression, as well as IL-10 protein production.

Colonic LP Tregs were isolated from SPF Foxp3$^{YFP\text{-}Cre}$ mice, cultured for 24 hrs in the presence or absence of 0.1 mM propionate, and examined for *in vitro* expression of Foxp3, and IL-10 by RTqPCR or secretion of IL-10 by ELISA. Upper left panel: expression of Foxp3. Lower left panel: expression of IL-10. Lower right panel: IL-10 protein production. Each symbol represents pooled colonic Tregs from 3-4 mice and reflects data from at least 4 independent experiments. Mann-Whitney U test was performed to determine statistical significance. Horizontal lines represent the mean and error bars the SD.

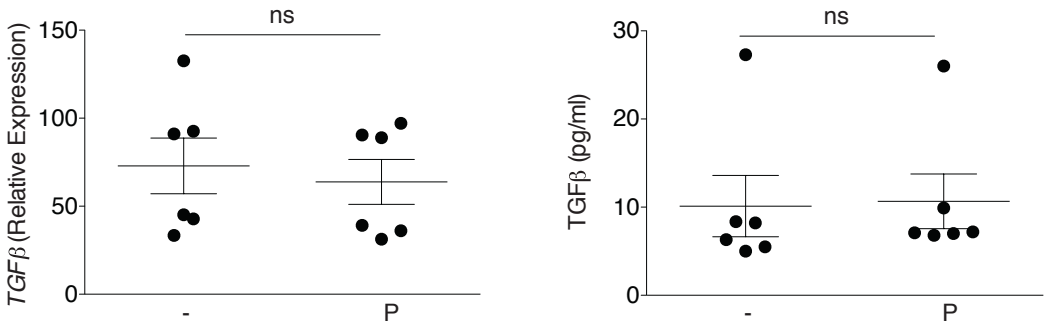

Fig. S14. *In vitro* treatment of colonic Tregs with propionate does not affect TGFβ expression or production.

Colonic LP Tregs were isolated from SPF Foxp3$^{YFP\text{-}Cre}$ mice, cultured for 24 hrs in the presence or absence of 0.1 mM propionate, and examined for *in vitro* expression of *TGFβ* by RTqPCR or secretion of TGFβ by ELISA. Left panel: expression of *TGFβ*. Right panel: TGFβ protein production. Each symbol represents pooled colonic Tregs from 3-5 mice and reflects data from 3 independent experiments. Mann Whitney U test was performed to determine statistical significance. Horizontal lines represent the mean and error bars the SD.

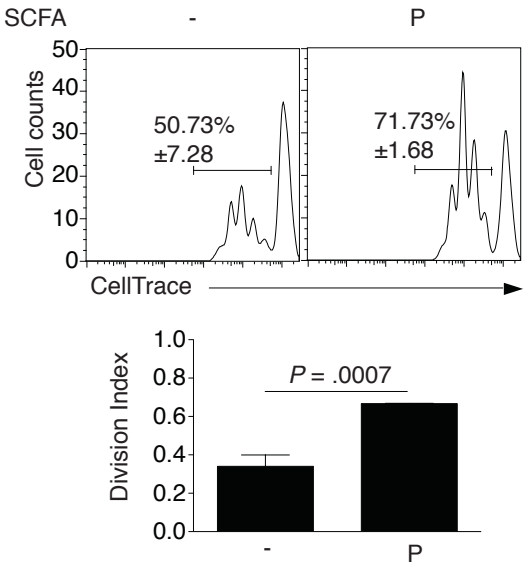

Fig. S15. Treatment with propionate enhances proliferation of colonic Tregs.
$CD4^+$ Foxp3-YFP^+ colonic Tregs were FACS sorted, labeled with CellTrace cell violet (5μM) and cultured with 1 μg/ml anti-CD3 and 500ng/ml IL-2 in the presence or absence of 0.1mM propionate. Histograms showing cell division are shown in the upper panel with average percentage of divided population ± SD on the histogram. Division index is plotted for the experimental conditions in the lower panel. Data are representative of two independent experiments. Student's t-test was performed to determine statistical significance. Horizontal lines represent the mean and error bars the SD.

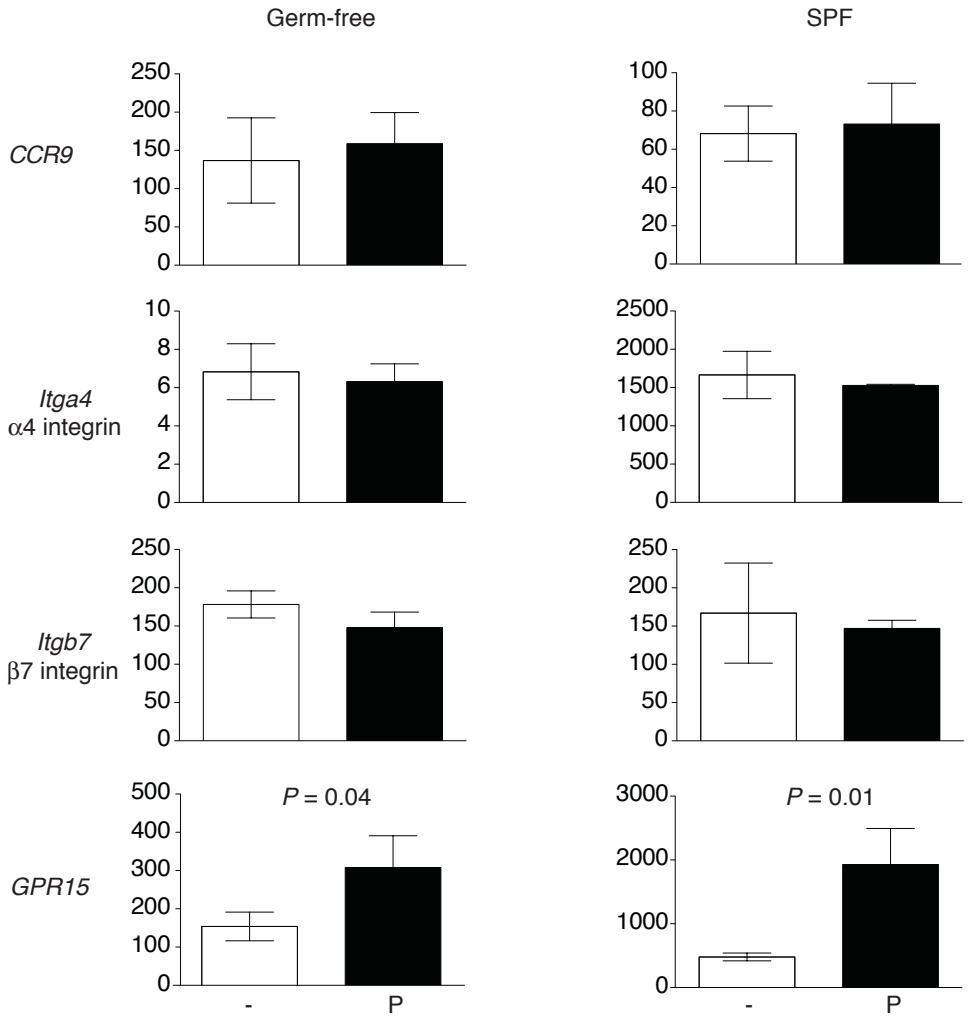

Fig. S16. Chemokine receptor expression in colonic Tregs from water vs propionate treated GF and SPF mice.

Colonic LP Tregs were isolated from water or *in vivo* propionate treated Swiss Webster GF (left panels) or SPF Foxp3$^{YFP\text{-}Cre}$ (right panels) mice and the *ex vivo* expression of the chemokine receptors CCR9, α4β7 and GPR15 was measured by RTqPCR. Data consist of 5 pooled mice per group and are representative of two independent experiments. Student's t-test was performed to determine significance. Error bars represent SD.

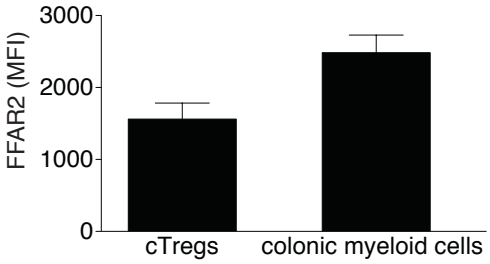

Fig. S17. *Ffar2* receptor is highly expressed in colonic Tregs and myeloid cells.
Colonic LP lymphocytes were isolated from SPF *Ffar2*$^{+/+}$ and *Ffar2*$^{-/-}$ mice, stained for CD45, CD4, Foxp3 and CD11b and the mean fluorescent intensity (MFI) was determined by flow cytometry. The MFI was calculated by averaging the geometric means for Ffar2 expression of individual mice. The bar graph shown is the average of 5 individual mice per group and is representative of 3 independent experiments. Horizontal lines represent the mean and error bars the SD.

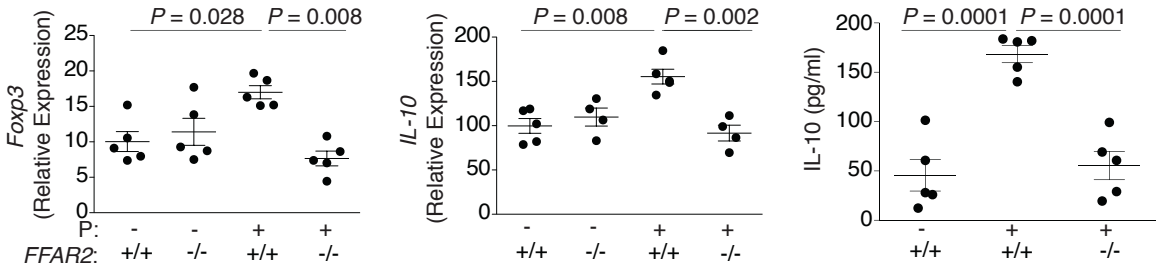

Fig. S18. *Ffar2* is required for propionate-mediated increase of Foxp3 and IL-10 levels in colonic tregs from SPF mice.

Colonic LP Tregs were isolated from *Ffar2*$^{-/-}$ and littermate *Ffar2*$^{+/+}$ mice, purified as in 1D, cultured in the presence of 0.1 mM propionate for 24hrs and examined for expression of *Foxp3* and *IL-10* by RTqPCR and secretion of IL-10 by ELISA. Each symbol represents pooled data from 3-5 mice and data are representative of 2 independent experiments. Student's t-test was performed to determine significance. Horizontal lines represent the mean and error bars the SD.

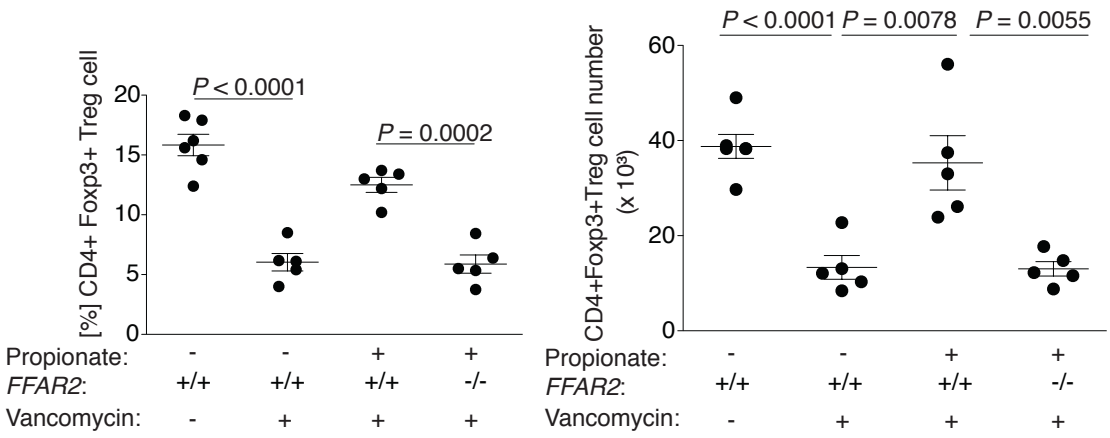

Fig. S19. *Ffar2* is required for the propionate-mediated restoration of colonic Treg levels in SPF antibiotic treated mice.

Ffar2$^{-/-}$ and littermate *Ffar2*$^{+/+}$ mice were treated orally with vancomycin for 4 wks and also given propionate in the drinking water starting in week 2. After 4 wks, colon LP lymphocytes were isolated and stained for CD45, CD4 and Foxp3. The percentage and number of CD4^{+}Foxp3^{+} cells within the CD45^{+} population are shown. Each symbol represents data from an individual mouse and data represent 2 independent experiments. Student's t-test was performed to determine significance. Horizontal lines represent the mean and error bars the SD.

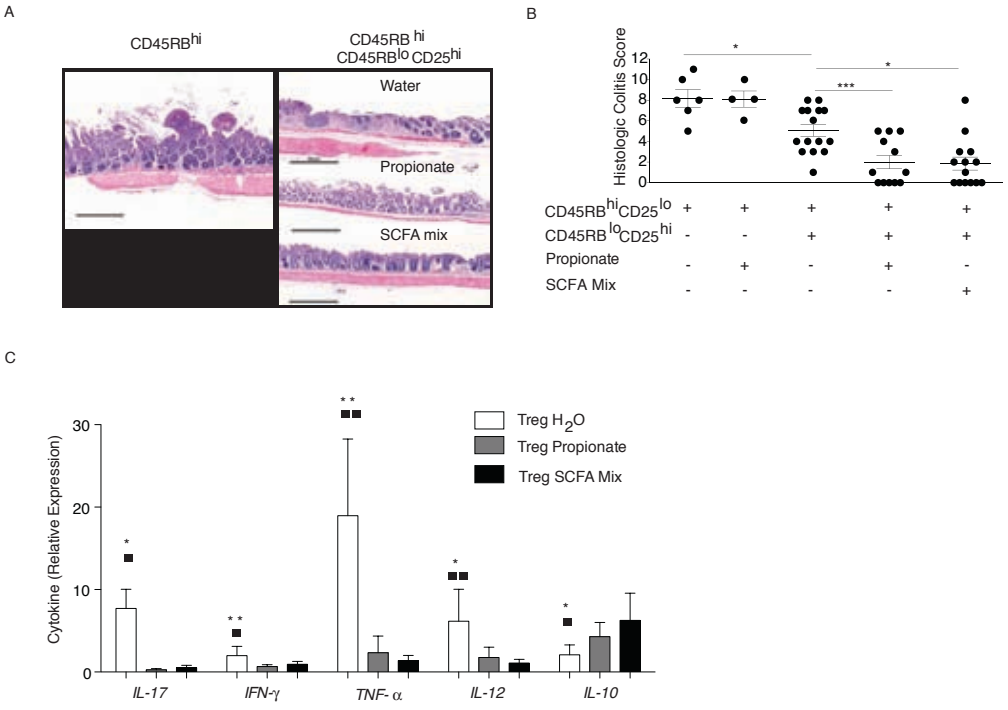

Fig. S20. SCFA treatment reduced colitis severity and proinflammatory cytokine expression and increased *IL-10* expression during T cell transfer colitis.

BALB/c *Rag2*$^{-/-}$ mice were injected with CD4^{+}CD45RB^{hi}CD25^{-} naive T cells alone or in combination with Tregs. Following injection, mice received propionate, SCFA mix, or pH and sodium-matched drinking water. (A) Representative H&E images are shown for the experimental groups. Propionate and SCFA treated mice show reduced degrees of colonic crypt injury, inflammation, and hyperplasia compared to control mice. A 100 μm scale bar is shown in the lower left of each image. (B) Histologic colitis score is shown along the y-axis, the treatment groups and experimental conditions are shown along the x-axis. (C) Colonic RNA was isolated from formalin fixed paraffin embedded (FFPE) blocks using the Ambion RecoverAll^{TM} total nucleic acid isolation kit for FFPE, and expression of the proinflammatory cytokines *IL-17*, *IFN-γ*, *TNF-α*, *IL-12* and the anti-inflammatory cytokine *IL-10* was determined by RTqPCR. Samples were the same as those in panel B and reflect three independent experiments. One-way ANOVA with Bonferroni post-hoc in panel B and Students' t-test in panel C to determine significance. For panel C, asterisks denote statistically significant differences between water and propionate samples and squares denote statistically significant differences between water and SCFA mix, ** indicate P <0.01, * indicates $P < 0.05$. Horizontal lines represent the mean and error bars the SD.

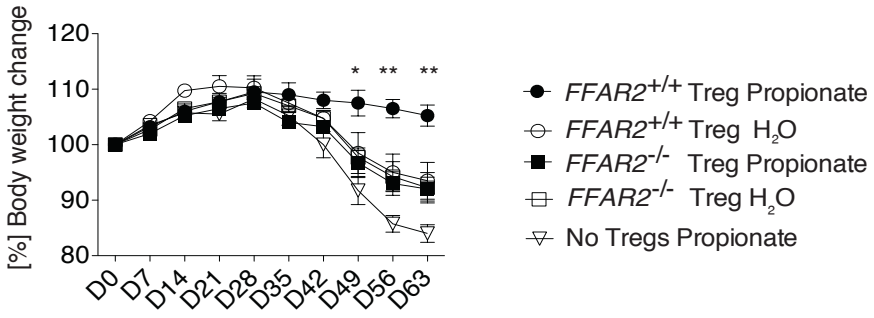

Fig. S21. *Ffar2* is required for SCFA-mediated protection against weight loss during T cell mediated colitis.

C57BL/6 *RAG2*$^{-/-}$ mice were injected with CD4^{+}CD45RB^{hi}CD25^{-} naive T cells alone or in combination with *Ffar2*$^{+/+}$ or *Ffar2*$^{-/-}$ Tregs. Following injection mice received propionate or pH and sodium-matched drinking water. Weekly % body weight change is shown across the experimental groups from experimental d0-d63. Each data point is the average of 5-7 individual mice and data are representative of 2 independent experiments. Students' t-test was performed to determine significance, ** indicates P <0.01, * P < 0.05. Horizontal lines represent the mean and error bars the SD.

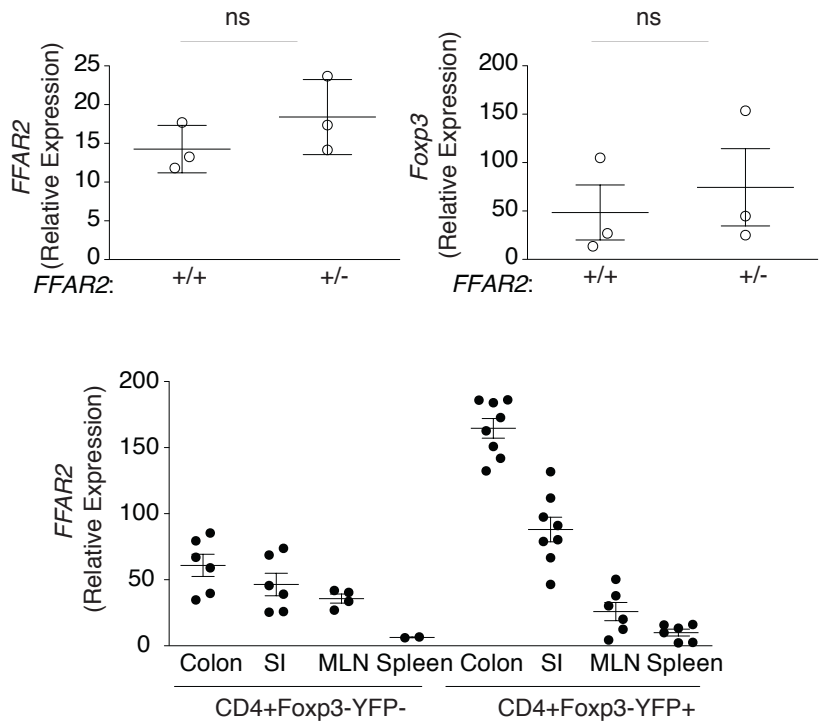

Fig. S22. *Ffar2* expression patterns.

Upper panels: Colonic LP lymphocytes were isolated from *Ffar2*$^{+/-}$ *and Ffar2*$^{+/+}$ littermates, purified by FACS staining for CD4, CD127, and CD25, and examined *ex vivo* for expression of *Ffar2* and *Foxp3* by RTqPCR. Each symbol represents data from 5-8 mice and data reflect three independent experiments. Lower panel: *Ffar2* expression levels were examined in Foxp3-YFP^{-} and Foxp3-YFP^{+} CD4+ T cells isolated from the colon, small intestine, mesenteric lymph node, and spleen by RTqPCR. Horizontal lines represent the mean and error bars the SD.

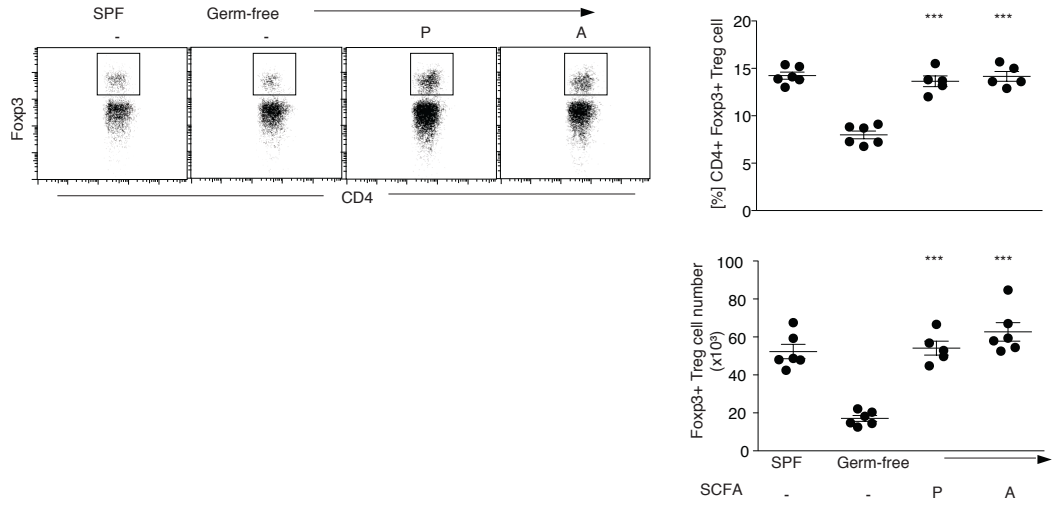

Fig. S23. SCFA increase colonic Treg populations and number in germ-free Swiss Webster mice.

Colonic LP lymphocytes were isolated and stained for CD4 and Foxp3. Upper left panel: Representative flowgrams and Upper right panel: Percentage of and Lower right panel: Number of $CD4^{+}Foxp3^{+}$ within the $CD45^{+}CD3^{+}$ population from SPF, GF, and GF mice treated with P or A in the drinking water for three weeks. Each symbol represents data from an individual mouse. Data reflect two independent experiments. Kruskal-Walls with Dunn's post-hoc was performed. Horizontal lines represent the mean and error bars the SD. *** indicates P <0.001.

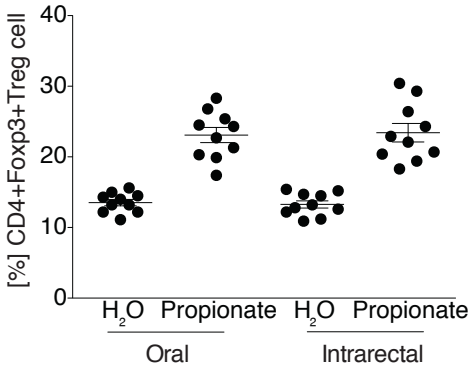

Fig. S24. Propionate in the drinking water and intrarectal instillation of propionate affect similar changes in colonic Treg populations in SPF mice.

Colonic LP Lymphocytes were isolated and stained for CD45, CD4, and Foxp3 from mice treated with propionate in the drinking water or treated with propionate intra-rectally for two weeks. Frequency of $CD4^{+}Foxp3^{+}$ within the $CD45^{+}$ population from SPF mice treated with pH- and sodium-matched water alone or propionate is shown. Each symbol represents data from individual mice and show data from two independent experiments. Horizontal lines represent the mean and error bars the SD.

Table S1. SCFA levels

Table 1 SCFA levels

Cecal or SI contents	SPF	ASF	GF	GF + P	GF + A	GF + B	GF + Mix	SPF +P	SPF + A	SPF + B	SPF + Vanco	SPF SI	SPF SI + P
µmol/g luminal contents													
Propionate	21.90 ± 0.122	18.83 ± 2.72	1.46 ± 0.162	14.93 ± 4.50	–	–	11.78 ± 6.38	43.33 ± 12.17	–	–	1.851 ± 1.41	2.69 ± 0.24	12.08 ± 3.69
Acetate	40.66 ± 5.86	28.69 ± 8.32	2.82 ± 0.534	–	16.18 ± 4.65	–	20.83 ± 0.47	–	53.95 ± 1.91	–	8.63 ± 1.73	15.19 ± 9.2	15.46 ± 3.69
Butyrate	18.52 ± 4.92	16.89 ± 1.46	2.13 ± 0.598	–	–	11.46 ± 1.46	20.6 ± 3.96	–	–	29.78 ± 6.202	1.04 ± 0.903	7.183 ± 2.39	6.45 ± 2.11

Select bacterial species	ASF 356 (XIV)	ASF 492 (XIV)	*Clostridium ramosum* (XVII)	*Clostridium bifermentans* (XI)	Bacteroides fragilis
µmol/10^5 CFU					
Propionate	62.39 ± 0.22	22.93 ± 0.109	14.74 ± 0.526	1.147 ± 0.008	0.0517 ± 0.001
Acetate	220.0 ± 0.435	123.2 ± 0.272	118.4 ± 0.526	1.973 ± 0.001	0.137 ± 0.001
Butyrate	ND	ND	ND	ND	ND

SCFA mix in mouse water bottle over time	Input (Day 0)	Day 1	Day 4	Day 7	Day 10	Day 14	Day 90
mM							
Propionate	200	200	199.6	201.24	202.9	203.2	41.9
Acetate	200	195	220.1	192.2	196.3	202.5	56.1
Butyrate	200	203.09	202.85	202.27	202.69	202.19	38