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

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Abstract

Regulatory T cells (Tregs) that express the transcription factor Foxp3 are critical for regulating intestinal inflammation. Candidate microbe approaches have identified bacterial species and strain-specific molecules that can affect intestinal immune responses, including species that modulate Treg responses. Because neither all humans nor mice harbor the same bacterial strains, we posited that more prevalent factors exist that regulate the number and function of colonic Tregs. We determined that short chain fatty acids (SCFA), gut microbiota-derived bacterial fermentation products, regulate the size and function of the colonic Treg pool and protect against colitis in a *Ffar2*(GPR43)-dependent manner in mice. Our study reveals that a class of abundant microbial metabolites underlies adaptive immune microbiota co-adaptation and promotes colonic homeostasis and health.

The intestinal immune system has co-evolved with the gut microbiota for the maintenance of intestinal health (1). Disruption of this homeostasis leads to intestinal inflammation and disease (2, 3). Colonic regulatory T cells (cTregs) expressing the transcription factor Foxp3 are critical for limiting intestinal inflammation and depend on microbiota-derived signals for proper development and function (4-7). *Bacteroides fragilis* and clostridial species induce Treg responses (6, 7); however, how the gut microbiota affect cTreg responses across mammalian hosts remains unclear. While polysaccharide A from *B. fragilis* modulates Treg responses (6), such effects are also likely mediated through more common factor(s) produced by many bacterial genera.

Humans and mice rely on bacteria to breakdown undigestible dietary components, such as fibers (8). Short chain fatty acids (SCFA) are bacterial fermentation products and range in concentration between 50-100mM in the colonic lumen (9). We examined SCFA

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concentrations in specific pathogen-free (SPF), gnotobiotic Altered Schaedler Flora (ASF)-colonized mice, and germ-free (GF) mice and found that GF mice had reduced concentrations of the three most abundant luminal SCFA, acetic acid, propionic acid and butyric acid (Table S1), as previously reported (10). The decrease of these SCFA in GF mice suggested that SCFA may contribute to their immune defects, specifically reduced cTreg numbers. We provided SCFA in the drinking water (150mM) to GF mice for three weeks and found that SCFA individually or in combination (SCFA mix) increased cTreg frequency and number (Fig. 1A) but did not increase the number or frequency of splenic, mesenteric lymph node (MLN), or thymic Tregs (fig. S1). These effects coincided with increased luminal SCFA (Table S1). SCFA increased CD4⁺ T cell frequency and number (fig. S2) but did not alter colonic Th1 or Th17 cell numbers significantly (fig. S3).

Microbiota-induced cTreg development is associated with increases in *de novo* generation of inducible Tregs (iTregs) and not in Tregs of thymic origin (nTregs) (7). These populations can be distinguished by expression of *Helios*, which *in vivo* is restricted to nTregs (11). We found that *Helios*⁺ Treg frequency was similar between GF and SCFA-treated GF mice (fig. S4) but lower in SPF mice. SCFA-treatment increased *Helios*⁺ Treg numbers in GF mice, indicating that Tregs already present in the colonic lamina propria (cLP) were expanding.

To test if SCFA could affect cTregs in a GF setting, we isolated cTregs from GF mice treated with propionate *in vivo* for three weeks and examined expression of *Foxp3* and *interleukin (IL)-10*, a key cytokine in Treg-mediated suppression. We also isolated cTregs from GF mice and stimulated them *in vitro* with propionate. Both treatments significantly increased *Foxp3* and *IL-10* expression (Fig. 1B, C). *In vitro* treatment increased IL-10 production but not transforming growth factor- β (TGF- β), a Treg-mediated suppression factor, suggesting that SCFA specifically induce Foxp3⁺ IL-10-producing Tregs (Fig. 1B, C).

The antibiotic vancomycin, which targets Gram-positive bacteria and disrupts the gut microbiota (12), reduces cTregs to similar levels as observed in GF mice (7) (fig. S5). However, when SPF mice were treated with a combination of vancomycin and SCFA, the reduction in cTregs was completely restored (fig. S5). Collectively, these results suggest that SCFA, play a role in cTreg homeostasis.

We questioned whether SCFA would augment cTregs in SPF mice. SCFA treatment of SPF mice increased Foxp3⁺ and Foxp3⁺IL-10⁺ cTreg frequency and number (Fig. 2A-C) but not Foxp3⁺TGF- β cTregs (fig. S6). We did not observe changes with SCFA treatment in small intestinal Treg numbers (fig. S7). Neither colonic Th17 and Th1 nor MLN and splenic Treg frequency and number) from SPF mice were affected by SCFA (fig. S8-S11).

These results may explain the benefits of dietary fibers and bacteria, such as clostridia and bifidobacteria (13) that can increase colonic luminal SCFA production and modulate inflammation in mice and humans. We measured SCFA production of species belonging to Clostridium cluster XI (*Clostridium bif fermentans*), XIV (ASF 356 and 492), XVII (*C. ramosum*), and the bacteroides species, *B. fragilis* as Clostridium cluster XIV members and *B. fragilis* affect cTregs (6,7). ASF 356 and 492 and *C. ramosum* generated more propionate (14-62 vs 0.05-1.1 μ mol/10⁵ CFU) and acetate (118-220 vs 0.1-2 μ mol/10⁵ CFU) as compared to the other strains (Table S1).

cTregs regulate intestinal homeostasis and control inflammation by limiting proliferation of effector CD4⁺ T cells (Teff). Addition of SCFA to cTreg and Teff co-cultures increased cTreg suppressive capacity (Fig. 2D and fig. S12). In SPF mice, SCFA are taken up by colonic epithelial cells but also diffuse through the epithelium into the lamina propria where they can mediate their effects directly (9, 14). To determine if SCFA directly affect cTregs,

we isolated cTregs from SCFA-treated SPF mice. *In vivo* treatment increased cTreg *Foxp3* and *IL-10* expression (Fig. 2E). We also isolated cTregs from SPF mice and incubated them with SCFA *in vitro*. *Foxp3* expression as well as *IL-10* expression and protein production increased (fig. S13), whereas TGF β levels remained unchanged (fig. S14). As enhanced suppressive activity (Fig. 2D) could be attributed not only to higher IL-10 levels per cTreg but also to increased cTreg proliferation, we examined cTreg proliferation. cTregs exhibited enhanced proliferation when cultured in the presence of propionate (fig. S15).

In addition, we analyzed the expression patterns of Treg trafficking molecules. Although levels of the chemokine receptor *CCR9* or $\alpha_4\beta_7$ integrin were not altered in propionate-treated GF and SPF mice, levels of the cTreg homing receptor, *GPR15* (15), did increase (fig. S16). Taken together, these data indicate that SCFA may have a beneficial effect in SPF mice through their ability to increase *Foxp3*⁺*IL-10* producing cTregs and cTreg proliferative capacity, as well as alter cTreg *GPR15* expression.

Considering that SCFA can influence cTregs directly, we asked if this was a receptor-mediated process. G coupled protein receptor (GPR) 43 (*Ffar2* is the gene that encodes GPR43) binds SCFA and through its expression on innate immune cells mediates resolution of inflammatory responses (3, 16). We found that intestinal Tregs and, particularly cTregs, had significantly higher levels of *Ffar2* than Tregs isolated from spleen or MLN (Fig. 3A, B), which was largely dependent upon microbiota-derived signals. As a reference, we compared cTreg *Ffar2* expression levels to colonic myeloid (CD11b⁺) cells, which are known to express *Ffar2* (3), and found that on average CD11b⁺ cells expressed 1.6 fold more *Ffar2* than cTregs (fig. S17).

To determine if *Ffar2* contributes to cTreg homeostasis, we treated *Ffar2*^{-/-} mice and *Ffar2*^{+/-} littermates with propionate, which has the highest affinity for *Ffar2* (17), and measured cTreg numbers. Propionate enhanced cTreg frequency and number in *Ffar2*^{+/-} but not *Ffar2*^{-/-} mice (Fig. 3C). SCFA-mediated, enhanced cTreg suppressive capacity was also dependent on *Ffar2* (Fig. 3D). In addition, propionate enhanced *Foxp3* and *IL-10* expression and IL-10 protein levels in *Ffar2*^{+/-}, but not in *Ffar2*^{-/-}, cTregs (fig. S18). Furthermore, we examined whether propionate could restore cTreg populations and numbers in the setting of vancomycin treatment and found that *Ffar2* was necessary (fig. S19).

One mechanism by which SCFA mediate their effects is histone deacetylase (HDAC) inhibition (18). The HDAC inhibitor trichostatin-A (TSA) increases Treg gene expression and suppressive capacity (19); and HDAC6 and 9 down-regulate nTreg function (20). Given that SCFA promote cTreg homeostasis in our studies, we hypothesized that SCFA mediate their effect through HDAC inhibition. We treated *Ffar2*^{+/-} and *Ffar2*^{-/-} mice with propionate and measured HDAC expression. Propionate treatment of *Ffar2*^{+/-} mice reduced cTreg *HDAC6* and *HDAC9* (class Ib and IIa, respectively) expression, but did not reduce *HDAC1* and *HDAC2* (class I) or *HDAC7* (class IIa) expression (Fig. 3E). Western blot analysis demonstrated that propionate treatment of cTregs enhanced histone acetylation, all of which were dependent on *Ffar2* expression (Fig. 3F). These results suggest that SCFA via *Ffar2* may affect cTregs through HDAC inhibition.

To further evaluate the effects of SCFA on cTregs, we utilized the T cell transfer model of colitis (21). In this model, lymphopenic mice (e.g. *Rag2*^{-/-}) are injected either with naïve T cells, which results in severe colitis, or injected with naïve T cells in combination with Tregs, which reduces colitis severity. Propionate or SCFA mix-treated mice injected with naïve T cells and Tregs had less severe weight loss, reduced disease score, and lower levels of colonic pro-inflammatory cytokines than mice that received water alone (Fig. 4A and fig. S20). SCFA mix or propionate did not affect colitis severity in mice that received only naïve

T cells (fig. S20). The frequency and number of cLP Foxp3⁺ Tregs in mice receiving propionate and SCFA mix increased (Fig. 4B, C). SCFA, however, did not result in conversion of naïve T cells to cTregs (Fig. 4B, C: group 1 vs 2). To evaluate whether these effects were cTreg intrinsic and dependent upon *Ffar2*, we performed the T cell transfer colitis model using *Rag2*^{-/-} recipients, wild type naïve T cells, and *Ffar2*^{+/-} or *Ffar2*^{-/-} Tregs with or without propionate in the drinking water (Fig. 4D, fig. S21). The effect of propionate on intestinal inflammation was dependent upon *Ffar2* expression in Tregs as indicated by the colitis scores (Fig. 4D). Treg cell populations and cell numbers further substantiated that the propionate effects on cTregs were dependent on *Ffar2* (Fig. 4E, F).

Gut microbiota-host immune misadaptation has been implicated in the rising incidence of IBD, other inflammatory diseases, and obesity (22). The western dietary pattern, specifically reduced ingestion of plant-based fibers, may be a critical factor that links the gut microbiome to disease (23). While the gut microbiota composition is divergent across individuals, functional gene profiles are quite similar (24, 25), and alterations to common gut microbial metabolic pathways may affect production of symbiotic factors, such as SCFA, which regulate intestinal adaptive immune responses and promote health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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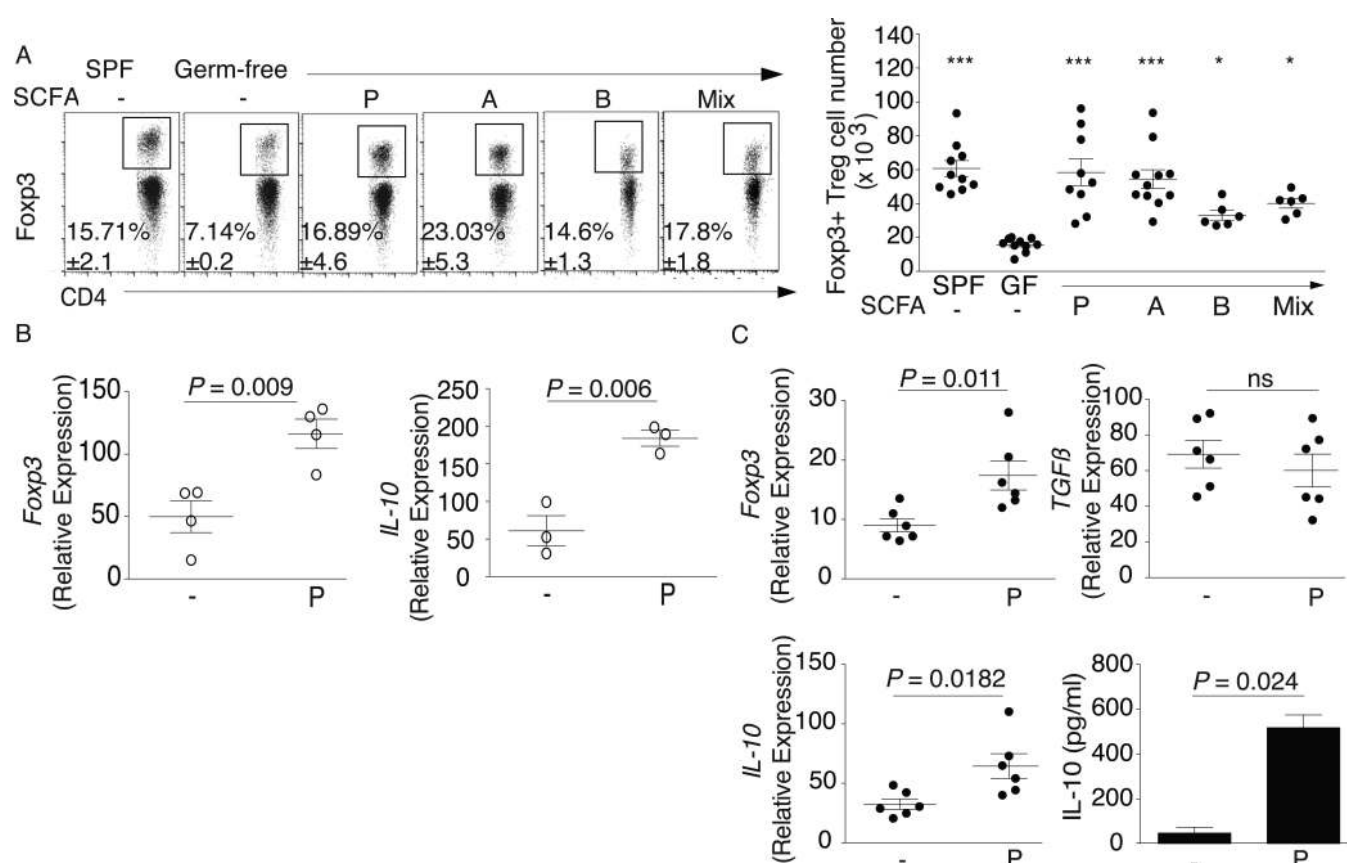


Fig. 1. SCFA restore colonic Treg populations and function in germ-free mice

(A) Colonic lamina propria (LP) lymphocytes were isolated and stained for CD4 and Foxp3. Left panel: Representative dot plots and percentage of CD4⁺Foxp3⁺ within the CD45⁺CD3⁺ population from SPF, GF, and GF mice treated with propionate (P), acetate (A), butyrate (B), or the SCFA mix in the drinking water. Right panel: Numbers of Foxp3⁺ Tregs for the left panel. (B) cTregs were isolated from *in vivo* propionate-treated GF mice, sorted by CD4, CD127, and CD25, and examined *ex vivo* for expression of *Foxp3* and *IL-10* by RTqPCR. (C) cTregs were isolated from GF mice and purified as in 1B, cultured for 24 hrs in the presence or absence of 0.1 mM propionate and examined for expression of *Foxp3*, *TGFβ* and *IL-10* by RTqPCR and IL-10 protein production by ELISA. For panel A, symbols represent data from individual mice. Horizontal lines show the mean and error bars the SD. For panels B and C, each symbol or bar represents pooled cTregs from 3-5 mice. All data shown are representative of at least 3 independent experiments. A Kruskal-Wallis test with a Dunn's post hoc test was performed in panel A, *** $P < 0.001$ and * $P < 0.05$. A Mann-Whitney U test was performed in panels B and C.

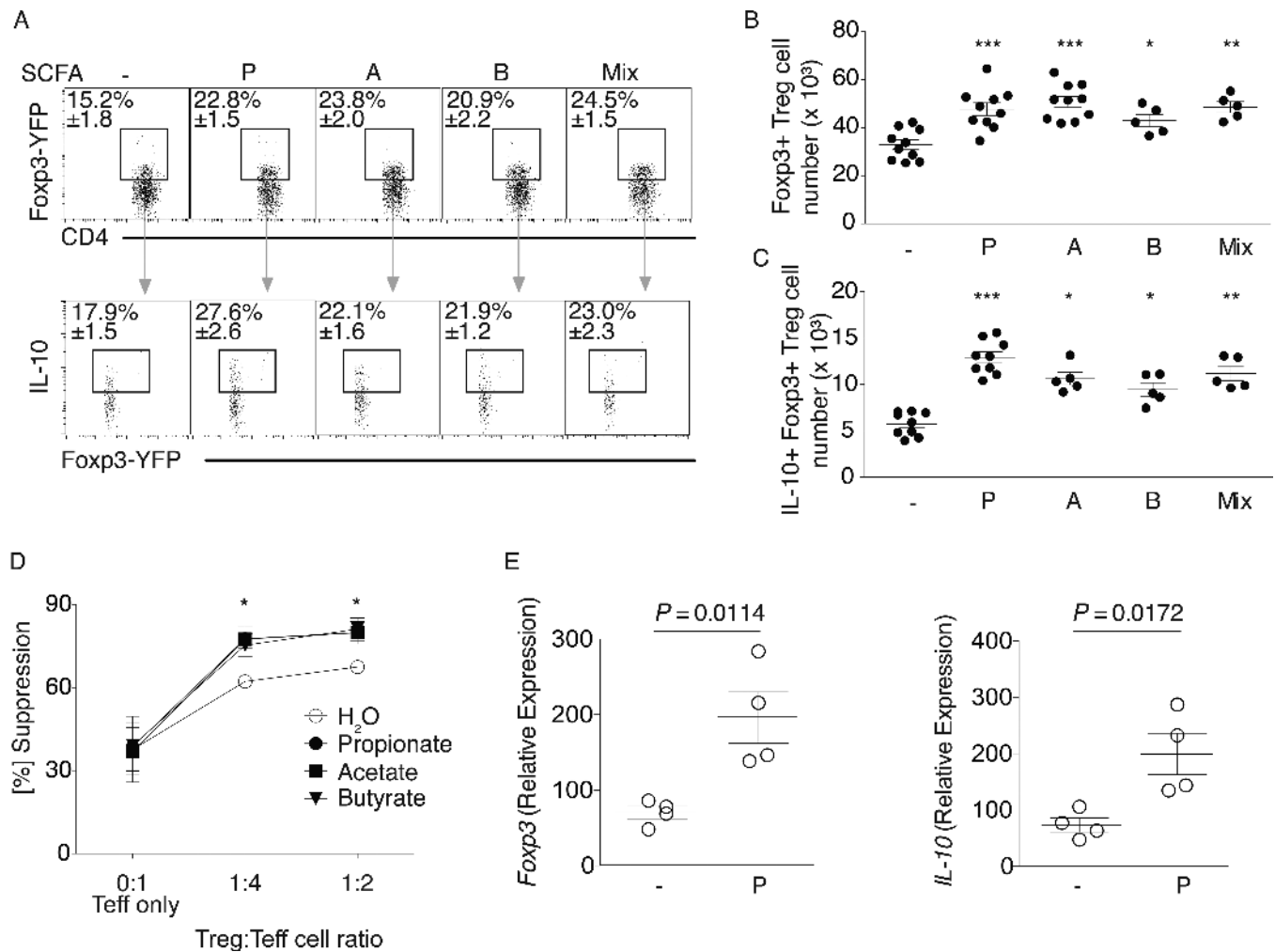


Fig. 2. SCFA augment colonic Treg population size and function in SPF mice

(A) SPF Foxp3^{YFP-Cre} mice were treated with water alone (-) or P, A, B, or the mix. Colonic LP lymphocytes were isolated and stained for CD4 and IL-10. Upper panel: Representative dot plots and percentage of CD4⁺ Foxp3-YFP⁺ within the CD45⁺CD3⁺ population. Lower panel: Representative dot plots and percentage of the CD4⁺Foxp3⁺IL-10⁺ population. (B) Cell numbers for the data in (A) upper panel. (C) Cell numbers for the data in (A) lower panel. Symbols in B and C represent data from individual mice and represent 4 independent experiments. (D) cTregs were co-cultured with splenic effector T cells (Teff) and P, A, B, or media (sodium and pH matched) for 96 hrs. Percent suppression (y-axis), Treg:Teff ratios (x-axis). Symbols represent mean values and error bars SD for four independent experiments. * $P < 0.01$. (E) cTregs were isolated from the LP of *in vivo* propionate treated SPF Foxp3^{YFP-Cre} mice, sorted for CD4 and YFP, and examined for *ex vivo* expression of *Foxp3* and *IL-10*. Each symbol represents pooled cTregs from 3-5 mice, horizontal lines show the mean and error bars SD. Four independent experiments were performed. A Kruskal-Wallis test with a Dunn's post hoc test was performed in panels B, C, D, *** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$. A Mann-Whitney U test was performed for experiments in panel E and P -values are shown where significant.

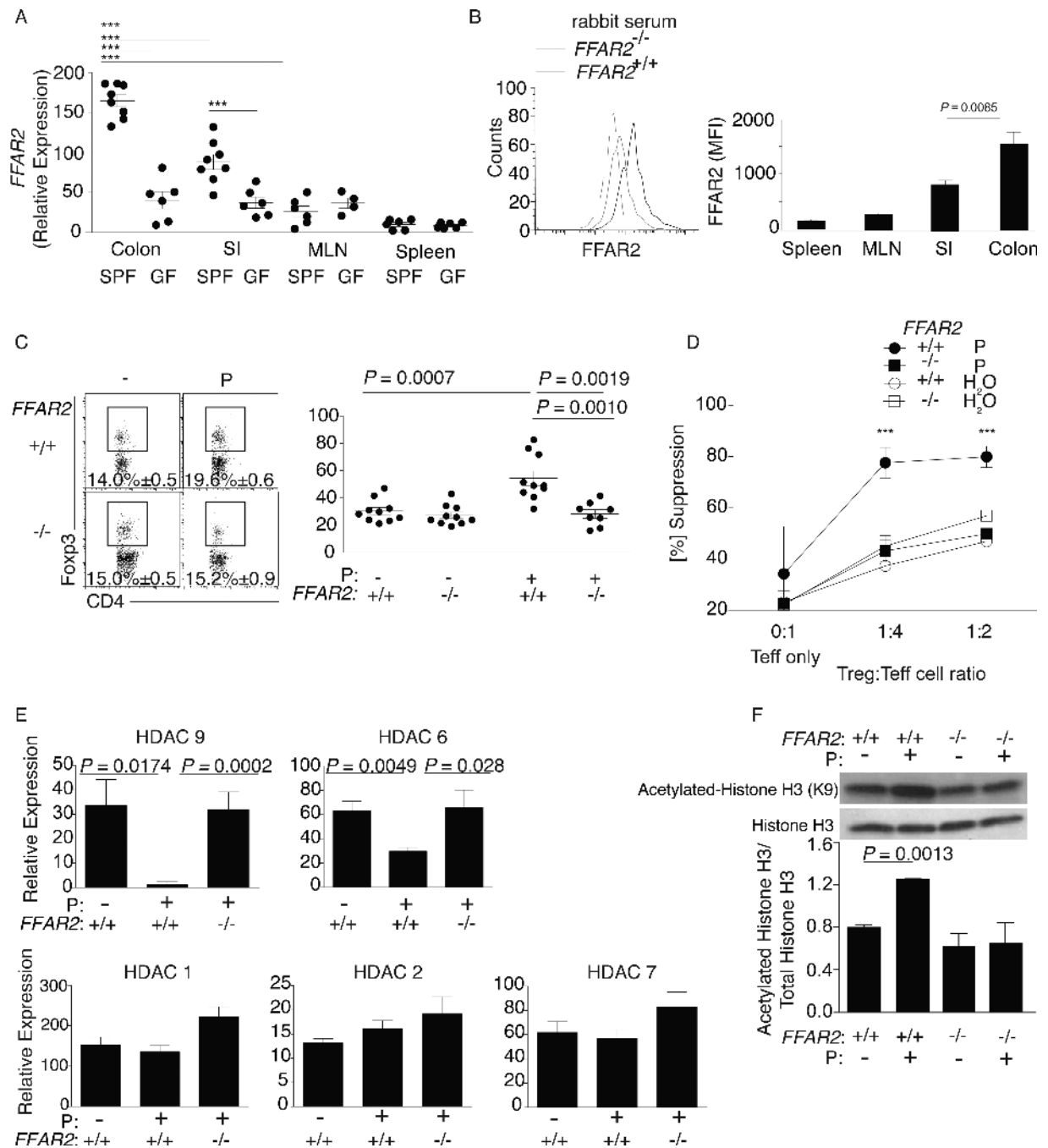


Fig 3. *Ffar2* mediates SCFA effects on cTregs

(A) Tregs were isolated from the colon, small intestine, spleen and MLN of SPF and GF BALB/c mice, purified as in 1B and *Ffar2* expression examined by qPCR. Each symbol represents data from 3-5 individual mice, horizontal lines show the mean and error bars the SD. Data reflect 3-7 independent experiments. (B) Lymphocytes were isolated from the colon, small intestine, spleen, and MLN of SPF *Ffar2*^{-/-} and littermate *Ffar2*^{+/+} mice. Cells were stained for CD4, Foxp3, and *Ffar2*. Left panel depicts a representative flow cytometry histogram comparing colonic *Ffar2* expression in *Ffar2*^{-/-} vs littermate *Ffar2*^{+/+} mice. Right panel shows the MFI for *Ffar2* for Tregs from the indicated sites. Bars show the mean, error bars SD, and data reflect 4 independent experiments. (C) Colonic LP lymphocytes were

isolated from *Ffar2*^{-/-} and littermate *Ffar2*^{+/+} mice exposed to propionate (P) or water alone and stained for CD4 and Foxp3. Left panel: Representative dot plots with percentage of CD4⁺Foxp3⁺ within the CD45⁺CD3⁺ population. Right panel: Foxp3⁺ Treg number for the left panel. Each symbol represents data from individual mice, horizontal lines show the mean and error bars the SD. **(D)** *Ffar2*^{-/-} and littermate *Ffar2*^{+/+} cTregs were co-cultured with splenic Teff cells in media with or without propionate for 96 hrs. Percent suppression (y-axis) and Treg:Teff ratios (x-axis). Symbols represent the mean of 3 independent experiments and error bars show the SD. **(E)** cTregs were isolated from the LP of *Ffar2*^{-/-} and littermate *Ffar2*^{+/+} mice, purified as in 1B, cultured in the presence of 0.1 mM propionate or media (pH and sodium matched) for 24hrs and examined for expression of *HDAC 1,2,6,7* and *9* by RTqPCR. Bars show the mean and error bars the SD of 3 independent experiments **(F)** Whole cell extracts were generated from cTregs isolated from the LP of *Ffar2*^{-/-} and littermate *Ffar2*^{+/+} mice, purified as in 1B, and cultured in the presence of 0.1 mM propionate or media (pH and sodium matched) for 24hrs. Samples were analyzed by western blotting for histone acetylation by examining levels of acetylated histone (H3K9), total histone levels were used as a loading control. The western blot shown is representative of two independent experiments with cTregs cell lysates pooled from 10-12 mice per group. A bar graph of densitometry ratios of acetylated Histone H3:total Histone H3 is shown. Bars represent the mean and error bars the SD. A Kruskal-Wallis test with a Dunn's post hoc test was performed for panels A and D, *** $P < 0.001$. The Mann-Whitney U test was performed for panels C and E. The student's t-test was performed for panel B and F.

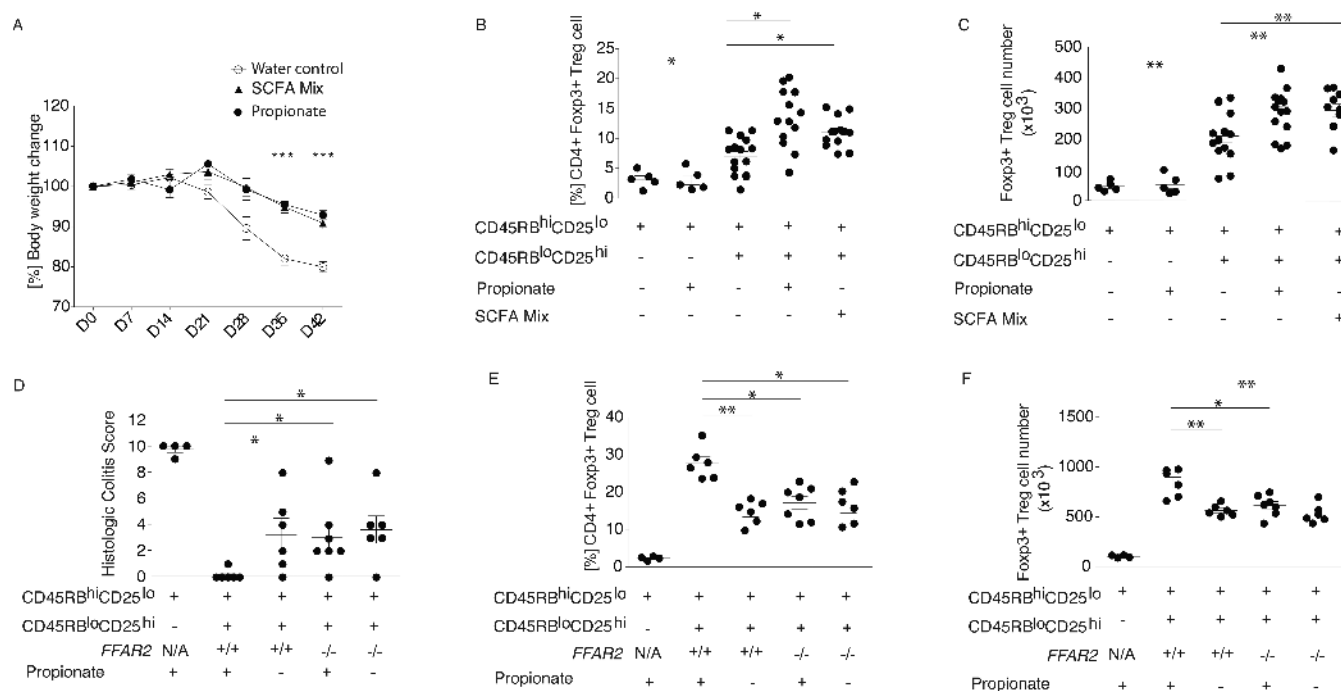


Fig. 4. SCFA exposure ameliorates T cell transfer colitis in a Treg-intrinsic, *Ffar2*-dependent manner

BALB/c *Rag2*^{-/-} mice were injected with CD4⁺CD45RB^{hi}CD25^{lo} naive T cells alone or in combination with Tregs. Following injection, mice received propionate, SCFA mix, or pH and sodium-matched drinking water. **(A)** Weekly percentage body weight change is shown across the experimental groups from experimental d0-d42. Symbols show the mean and error bars the SD. Data reflect three independent experiments. Colonic LP lymphocytes were isolated and stained for CD4 and Foxp3 and **(B)** percentage and **(C)** number of CD4⁺Foxp3⁺ within the CD45⁺CD3⁺ population are shown. Symbols represent data from individual mice, horizontal lines show the mean and error bars the SD. **(D-F)** C57BL/6 *Rag2*^{-/-} mice were injected with CD4⁺CD45RB^{hi}CD25^{lo} naive T cells alone or in combination with *Ffar2*^{+/+} or *Ffar2*^{-/-} Tregs. Following injection mice received propionate or pH and sodium-matched drinking water. **(D)** Histologic colitis score is shown along the y-axis, the treatment group and experimental conditions are shown along the x-axis. Colonic LP lymphocytes were isolated and **(E)** percentage and **(F)** number of CD4⁺Foxp3⁺ within the CD45⁺CD3⁺ population are shown. Symbols represent data from individual mice. Horizontal lines show the mean and error bars the SD. Panels D-F represent data from 2 independent experiments. The Kruskal-Wallis test with a Dunn's post hoc test was performed for panels A-F. ** *P* < 0.01, * *P* < 0.05.