



ARTICLE

Dietary fiber metabolites regulate innate lymphoid cell responses

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Innate lymphoid cells (ILCs) rapidly undergo expansion in population size and functional maturation in response to cytokines that signal infection, tissue damage, or changes in physiology. Optimal ILC responses are shaped, in part, by the microbiota but the mechanisms remain unclear. We report that short-chain fatty acids (SCFAs), produced by the commensal microbiota from dietary fibers, support optimal expansion of ILCs, including ILC1, ILC2, and ILC3 in the intestines through their G-protein-coupled receptors (GPCRs). While this function is primarily important for intestinal ILC populations, it can also boost ILC responses in other tissues depending on host condition. ILCs express multiple GPCRs that detect SCFAs. Interestingly, we found that the expression of SCFA receptors, such as Ffar2 and Ffar3, by ILCs is induced by SCFAs. GPCR triggering by SCFAs co-stimulates the activation of phosphoinositide 3-kinase (PI3K), Stat3, Stat5, and mammalian target of rapamycin (mTOR), which is important for ILC proliferation. While Ffar2 signaling promotes ILC2 proliferation, SCFAs can suppress ILC2 proliferation through a non-Ffar2-mediated mechanism. In conclusion, our findings indicate that SCFAs, as the major mediator of healthy microbiota and nutritional status, function to maintain optimal numbers of ILCs in peripheral tissues during infection and inflammatory responses.

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INTRODUCTION

The commensal microbiota plays profound roles in shaping the immune systems. Innate lymphoid cells (ILCs) are classified into group 1 (NK cells and non-NK ILC1), 2 (ILC2), and 3 (ILC3) based on their development and functional characteristics.¹ ILC1 express T-bet as the master transcription factor and produce IFN γ . ILC2 express GATA3 and produce IL-5, IL-9, and IL-13. ILC3 express ROR γ t and include natural cytotoxicity receptor (NCR)⁺ and NCR⁻CCR6⁺ ILC3 subsets.^{2,3} Lymphoid tissue-inducer (LTi) cells, inducing the formation of secondary lymphoid tissues during fetal development, also belong to the ILC3 group. ILC3 subsets express IL-22, IL-17A/F, and GM-CSF as the major effector cytokines. In addition, ILCs that express IL-10 for regulatory functions have been identified.^{4–6} Considerable levels of transdifferentiation between ILC1 and ILC3, as well as ILC2 and ILC3 have been observed.⁷ In line with their effector cytokine patterns, ILC1, ILC2, and ILC3 provide innate immunity against intracellular pathogens, helminths, and extracellular pathogens respectively. ILCs also regulate tissue remodeling/repair, adaptive immunity, and fat lipolysis in ILC subset-specific manners. ILCs, particularly ILC3, are required to maintain optimal symbiotic relationship with the microbiota.^{1,7–11} Deficiencies in ILCs cause dysbiosis and microbial tissue invasion.^{12,13} On the other hand, germ-free mice that lack the commensal microbiota have defective ILC functions.^{3,14,15}

The functions of microbiota in regulating ILCs are mediated, in part, through various cell types including epithelial cells, macrophages, and dendritic cells. These cells sense and activate ILCs by producing cytokines.¹⁶ Important cytokines for ILC differentiation include IL-7 for generation of all non-NK ILCs; IL-1 β and IL-23 for

ILC3; and thymic stromal lymphopietin (TSLP), IL-33, and IL-25 for ILC2. The development of ILCs, such as T-bet-expressing ILC1, is regulated by the microbiota.¹⁵ Microbiota can either increase or decrease ILC2 through distinct mediators such as TSLP and type I/II IFNs.^{17,18} Microbiota induces the expression of IL-1 β and IL-23 which, in turn, activate ILC3 cells.^{16,19–23}

A mounting body of evidence indicates that ILCs are regulated by the metabolites produced by the commensal microbiota. Thus far, activation of aryl hydrocarbon receptor (AHR) has been identified as a regulator of ILC3.^{21,24} AHR triggering promotes NCR⁺ ILC3 and LTi cells. For example, tryptophan metabolites, such as indole-3-acetate, can activate ILC3.²³ However, the functions of other microbial metabolites and their host receptors in regulating the ILC compartment remain largely unknown.^{25,26} In the intestine, the most abundant microbial metabolites are short-chain fatty acids (SCFAs). SCFAs are mainly produced from dietary fibers (DFs) by microbial fermentation.²⁷ Major SCFAs include acetate (C2), propionate (C3), and butyrate (C4). The combined luminal concentration of major SCFAs peaks in the proximal colon (~100 mmol/kg in humans), but SCFAs are also present in the distal colon (~80 mmol/kg) and small intestine (SI, ~10 mmol/kg) at significant levels.²⁸ SCFAs are also absorbed into the blood circulation. In mice, SCFAs are present at ~0.13 (C2), 0.015 (C3), and 0.15 (C4) μ mol/ml in the peripheral blood.²⁹ SCFAs promote IL-10 production by T cells and macrophages and support antibody production in the intestine in steady state but also boost the function of effector CD4⁺ and CD8⁺ T, and B cells in systemic sites during infection.^{29–35} Recently, several groups reported that SCFAs can regulate the activity of ILC2 and

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ILC3.^{36–38} However, the results are somewhat contradictory and limited to particular ILC subsets and tissue sites, warranting further studies to gain broad insights.

We investigated the function of DF metabolites and their receptors in regulating ILCs in mucosal and systemic tissue sites. We report that SCFA triggering of G-protein-coupled receptors (GPCRs) provide key signals to expand tissue populations of ILC1, ILC2, and ILC3. GPCR signaling synergizes with cytokine receptor signaling to expand ILC populations in the intestines. This establishes SCFAs as key mediators of nutritional and microbial status in shaping the peripheral ILC system.

RESULTS

DF administration expands intestinal T-bet⁺ and T-bet⁻ ILC3 populations

Soluble DFs, such as pectin and inulin, serve as prebiotics to increase SCFA levels in the intestine and the blood via microbial fermentation.^{39,40} These DFs boost immunity against enteric bacterial pathogens such as *Citrobacter rodentium* (*C. rodentium*) in mice.^{29,32} Because ILCs, particularly ILC3 subsets, play key roles in fighting *C. rodentium*, we examined the numbers of ILC3 in immunocompetent C57BL/6 mice fed low DF diet (LFD, 0% soluble DF) versus high DF (HFD, 15% soluble DF), which respectively produce low and high levels of SCFAs in the intestine and blood²⁹ but maintains comparable water content in feces (Fig. S1a). Considerable decreases in the expression of *RegIIIb*, *IL22*, *IL17a*, and *Ffar2* (also called *Gpr43*) were detected in colon tissues of LFD-fed mice compared with HFD mice (Fig. S1b). Significant differences in the number or frequency of T-bet⁻ and T-bet⁺ ILC3 cells were observed in both the SI and large intestine (LI) but not in the mesenteric lymph node (MLN) and spleen (Figs. 1a, S1c–e).

Because of the largely positive effect of DFs on ILC3 in immunocompetent WT mice, we performed a similar experiment with *Rag1*^{-/-} mice, which lack T and B cells. Soluble DFs increased both T-bet⁺ and T-bet⁻ ILC3 subsets in SI and LI (Fig. 1b, S1a–c). While the importance is unclear, moderate increases in ILC1 numbers in the spleen by LFD feeding was also observed. A clear positive effect of DFs on decreasing the enteric pathogen burden and inflammatory responses, based on colon length and neutrophil infiltration, was observed (Fig. 1c–e). Overall, DFs positively regulated intestinal ILC3 populations and improved intestinal immunity to *C. rodentium* infection.

ILCs express SCFA-sensing receptors

Because GPCRs, such *Ffar2*, *Ffar3*, *Olfir78*, and *Gpr109a*, mediate the functions of SCFAs in regulating cell functions,^{41–43} we examined the expression of these receptors in ILCs. RNA expression data retrieved from the publicly available ImmGen microarray data (contributed by the Colona Lab at the University of Washington) indicate that ILC3 subsets variably express the SCFA receptors (Fig. 2a). The expression of *Ffar2* was highest on ILC3 populations in the intestine. Most ILC3 subsets that express CCR6 or NKp46 expressed *Ffar2*. Single-cell RNA-seq data retrieved from the data contributed by Gury-BenAri et al.¹⁵ also show that ILC2 and ILC1, isolated from the SI, expressed *Ffar2* (Fig. 2b). Moreover, mRNAs for solute transporters that transport SCFAs into cells, such as *Slc16a1* and *Slc16a3*, were expressed at moderate to low levels in ILC subsets (Fig. 2a, b). Among ILCs, ILC2 expressed *Slc16a1* at the highest level (Fig. 2b). It is feasible that certain tissue factors in the intestine may induce the expression of SCFA receptors on intestinal ILCs. We examined the possibility that SCFAs themselves induce the expression of *Ffar2* and *Ffar3*. We found that C3 and C4 induce *Ffar2* and *Ffar3* at the mRNA level in vitro on flow cytometry-sorted spleen ILCs (Fig. 2c). This induction was largely blocked by pertussis toxin (PTX), which blocks the function of the heterotrimeric G proteins by inducing

ADP-ribosylation of the Gai subunit.⁴⁴ A class I/II HDAC inhibitor, trichostatin A (TSA), has moderate positive effects but this did not reach statistically significant levels. Similar patterns of the induction and suppression of SCFA transporters in ILCs were observed (Fig. 2d). These results indicate that ILCs express SCFA receptors and transporters, which is boosted by SCFAs. In vivo, *Ffar2* and *Ffar3* were more highly expressed in intestinal than splenic ILCs at mRNA level (Fig. 2e). Moreover, SCFAs increased Ki-67 expression and increased numbers of ILCs in culture (Figs. 2f, S3a). However, no significant effect of SCFAs on ILC survival was observed (Fig. S3b). While all major SCFAs increased the proliferation of splenic ILC3 from *Rag1*^{-/-} mice, only C4 had a small positive effect on the proliferation of splenic ILC1, and SCFAs, in general, had a negative effect on the expansion of lung ILC2 in vitro (Fig. 2g). These results indicate that SCFAs have proliferative effects on ILCs in vitro, but this appears to be dependent on ILC subsets and/or culture conditions.

SCFAs boost cytokine signaling and mammalian target of rapamycin (mTOR) pathways in ILCs

Activation of ribosomal S6 protein (rS6), Stat5, and Stat3 by ILC-activating cytokines plays important roles in supporting ILC proliferation and effector function, and the activation of *Ffar2* and *Ffar3* induces Atf2 phosphorylation.^{45,46} SCFAs induced or enhanced phosphorylation of these molecules in cultured ILCs in collaboration with IL-7 (Fig. 3a). We next, examined the roles of Gai, phosphoinositide 3-kinase (PI3K), mTOR, and MEK1/2 in the SCFA-mediated ILC proliferation utilizing small molecule inhibitors (Fig. 3b). The inhibitors for Gai, PI3K, mTOR, and MEK1/2, while did not increase cell death, suppressed SCFA-induced ILC proliferation (Figs. 3b, S3c). SCFAs boosted cytoplasmic membrane-associated (3,4,5)-trisphosphate (PIP3)/PI3K activity induced by cytokines (IL-7, IL-1β, and IL-23), which was detected by an imaging assay utilizing a GFP-fused pleckstrin homology (PH) domain of AKT (Fig. 3c). We observed that Atf2 phosphorylation, which is downstream of ERK1/2 activation following *Ffar2* or *Ffar3* triggering,⁴⁶ was induced in WT but not *Ffar2*^{-/-} intestinal ILC3 (Fig. 3d). Similarly, the activation of Stat3 and Stat5 was diminished in intestinal ILC3 of *Ffar2*^{-/-} mice compared with WT mice. Thus, *Ffar2* signaling boosts cytokine signaling and mTOR activity, necessary to support ILC proliferation.⁴⁷

Ffar2 enhances the tissue population capacity of ILC1, ILC2, and ILC3

The positive effect of SCFAs on ILC proliferation in a GPCR-dependent manner and *Ffar2* expression by ILCs imply a potential role for *Ffar2* in supporting ILC populations in vivo. To determine this possibility, we cotransferred CD45.1⁺ WT and CD45.2⁺ WT or *Ffar2*^{-/-} ILCs at 1:1 ratio into ILC/T/B-deficient *Rag2*^{-/-}*IL2Rγ*^{-/-} mice (Fig. 4a). After 4–5 weeks, the relative frequencies of CD45.1⁺ and CD45.2⁺ ILCs in various tissues were examined. As expected, there was no difference between WT and WT ILCs in populating various tissues (Fig. S4a, b). *Ffar2*^{-/-} ILC3 were significantly less efficient in populating SI and LI compared with their WT counterparts (Figs. 4b, c, S4c). Interestingly, *Ffar2*^{-/-} ILC1 and ILC2 were also less efficient in populating the intestines compared with their WT counterparts. However, no significant difference between WT and *Ffar2*^{-/-} ILCs in populating the spleen and MLN was observed (Figs. 4d, S4c). Thus, *Ffar2* supports the tissue population of ILC1, ILC2, and ILC3 in the intestines.

Defective expansion of *Ffar2*^{-/-} ILC populations during infection
We, next, examined the size of ILC tissue populations in WT versus *Ffar2*^{-/-} mice in the steady state and during infection by *C. rodentium*. It has been previously determined that *Ffar2*^{-/-} mice are less effective in mounting immune responses to *C. rodentium*.⁴⁶ The ILC numbers in various tissues of WT and *Ffar2*^{-/-}

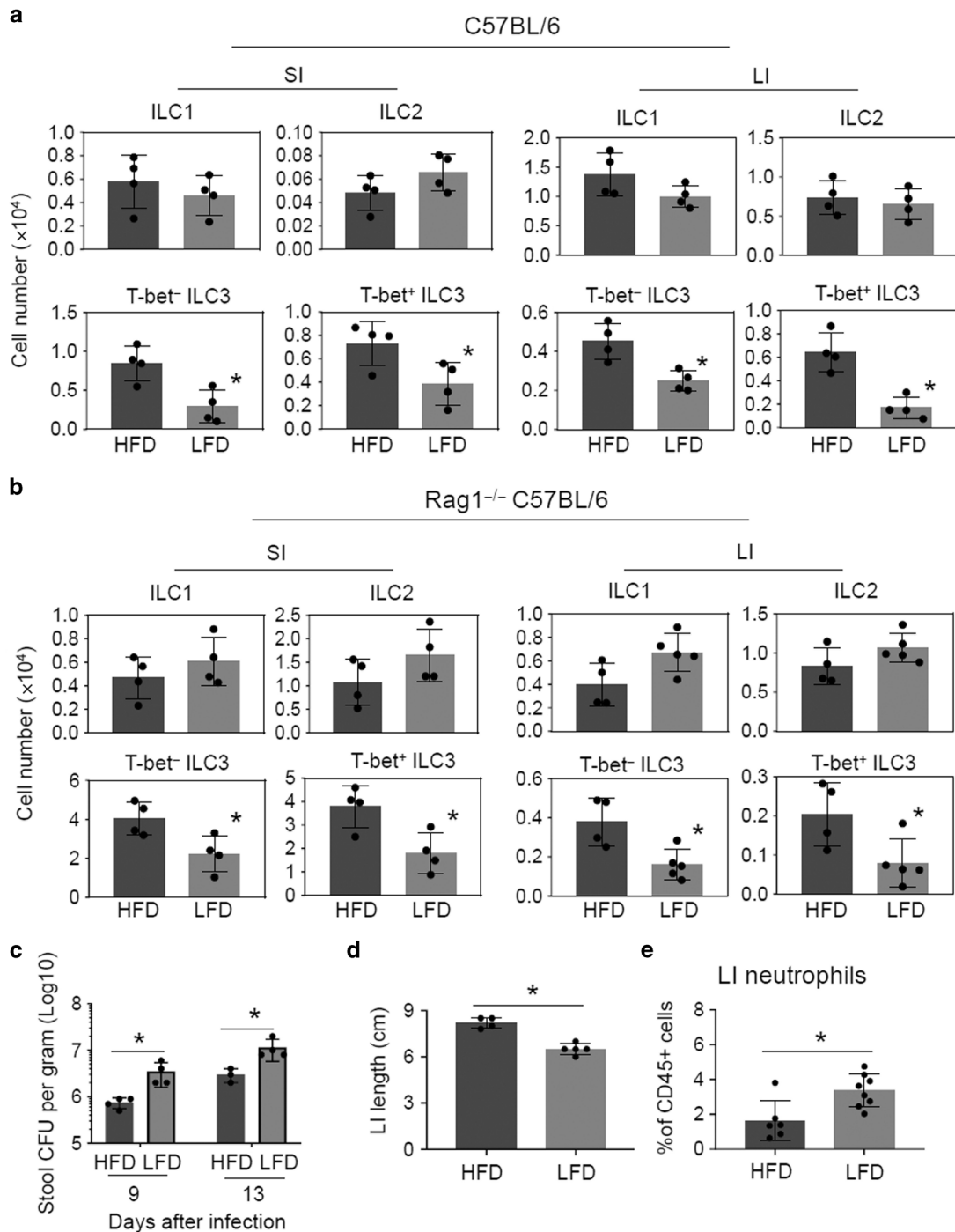


Fig. 1 Soluble dietary fibers increase the numbers of ILC3 in the small and large intestines. a Effects of DFs on ILCs in WT C57BL/6 mice. **b** Effects of DFs on ILCs in Rag1^{-/-} C57BL/6 mice. **c** Fecal *C. rodentium* burden. **d** Colon length. **e** Neutrophil numbers in colonic lamina propria cells. Mice were fed LFD or HFD for 3 weeks and infected with *C. rodentium*. Two weeks later, ILC numbers in various tissues were examined. Pooled data obtained from at least two different experiments ($n = 4-5$) are shown. Significant differences ($p < 0.05$) between groups. Error bars are SEM.

mice were not significantly different in the steady state (Fig. 5). However, *C. rodentium* infection expanded ILC1 and ILC3 populations in the SI of WT, but not *Ffar2*^{-/-}, mice (Figs. 5a, S5a). Also, *Ffar2*^{-/-} mice were defective in ILC expansion in the colon (Fig. 5b). *C. rodentium* primarily resides on the intestinal epithelial surface in immunocompetent mice but invades secondary lymphoid tissues in immunocompromised or *Ffar2*^{-/-} mice.^{37,46,48} Interestingly, we observed a defect in ILC expansion also in the spleen and MLN of *Ffar2*^{-/-} mice, which was not

detected without infection (Figs. 5c, d, S5b). To rule out the potential influence of strain or colony-specific microbial differences, we performed the same experiment on cohoused WT versus *Ffar2*^{-/-} mice. Despite minor differences from non-cohoused experiments, cohoused WT, and *Ffar2*^{-/-} mice maintained overall strain-specific differences in susceptibility to *C. rodentium* as well as ILC populations (Fig. S6a-c). Overall, these results indicate that major ILC subsets are regulated by *Ffar2* during *C. rodentium* infection.

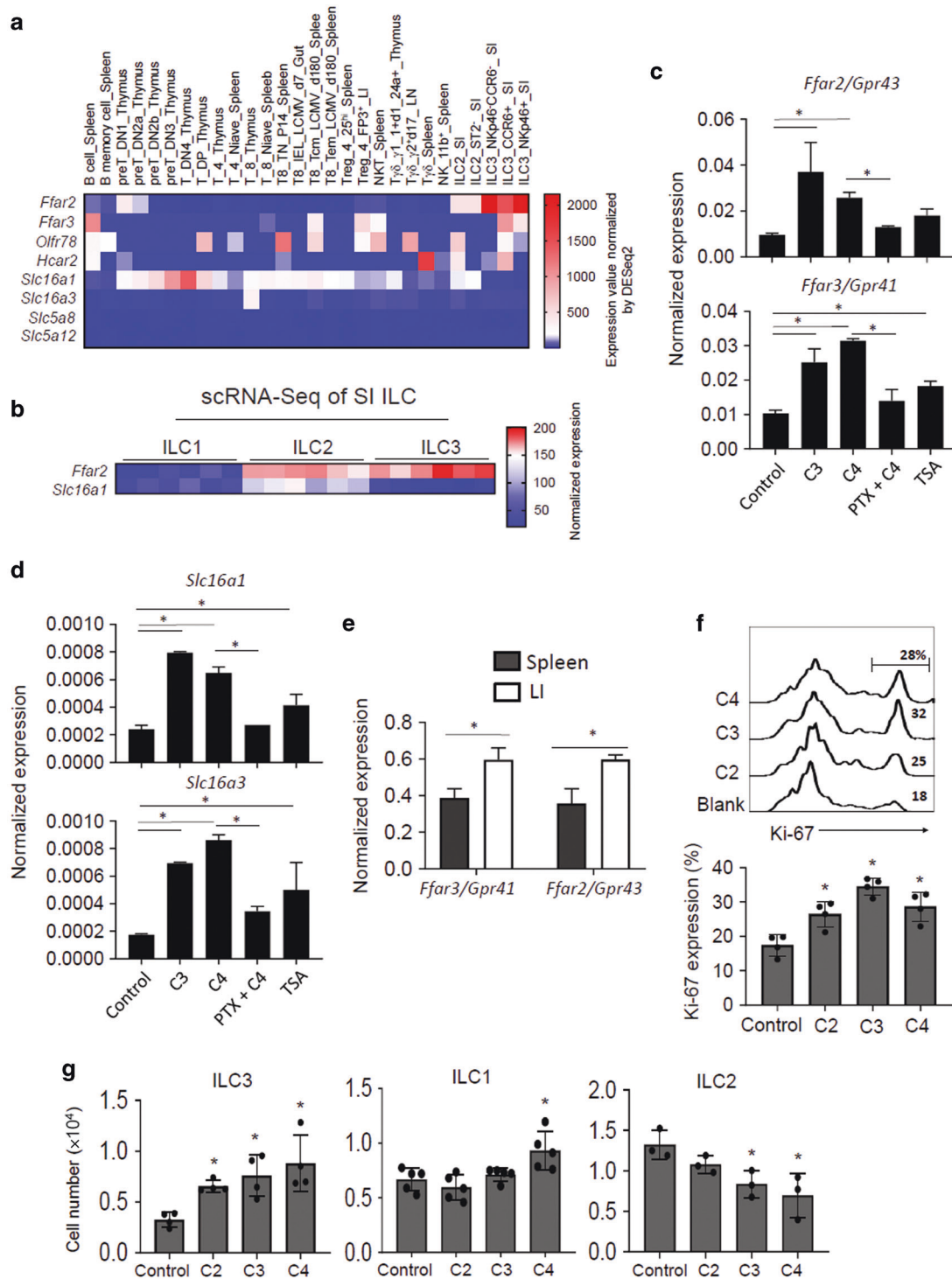


Fig. 2 SCFAs positively promote the expression of G-protein-coupled SCFA receptors in ILCs and regulate ILC proliferation. **a** Expression of SCFA receptors and SCFA transporters at mRNA level by ILCs versus other lymphocytes. Data for NK, ILC2, and NKp46⁺CCR6⁺ ILC3 subsets are shown. Microarray data available in the ImmGen database were plotted. **b** Expression of *Ffar2* and *Slc16a1* by Lin⁻CD127⁺RORgt⁺NKp46⁺ ILC1, Lin⁻CD127⁺RORgt⁺KLRG1⁺ ILC2, and Lin⁻CD127⁺RORgt⁺ ILC3 of SI origin. The scRNA-seq data were retrieved from the data contributed by Gury-BenAri et al.¹⁵ **c** Impact of SCFAs on the expression of *Ffar2* and *Ffar3* at mRNA level by ILCs. **d** Impact of SCFAs on the expression of *Slc16a1* and *Slc16a3* by ILCs. For **c** and **d**, *Rag1*^{-/-} spleen Lin⁻CD90⁺CD127⁺ cells were flow cytometry-sorted and cultured with IL-7, IL-23, and IL-1β for 3 days with or without PTX pretreatment. C2, C3, and TSA were added to culture on day 2. qRT-PCR was performed. **e** Comparison of spleen and intestinal ILCs for expression of *Ffar2* and *Ffar3*. Lin⁻CD90⁺CD127⁺ cells were flow cytometry-sorted, and mRNA expression for *Ffar2* and *Ffar3* genes was detected by qRT-PCR. **f** Effect of SCFAs on Ki-67 expression in ILCs cultured in the aforementioned ILC induction condition for 4 days. Sorted Lin⁻CD127⁺CD90⁺ cells from the spleen of *Rag1*^{-/-} mice were cultured. **g** Effects of SCFAs on proliferation of ILC3, ILC1, and ILC2 in vitro. Spleen total ILCs (for ILC1 and ILC3) or lung mononuclear cells (for ILC2) from *Rag1*^{-/-} mice were cultured in the presence of SCFAs (10 mM for C2, 0.5 mM for C3, and 0.01 mM for C4) in ILC1 (IL-7 and IL-15), ILC2 (IL-7 and IL-33), or ILC3 (IL-7, IL-23 and IL-1β) culture condition. Pooled data obtained from at least two different experiments (*n* = 3–4) are shown. An asterisk represents significant differences (*p* < 0.05) between groups.

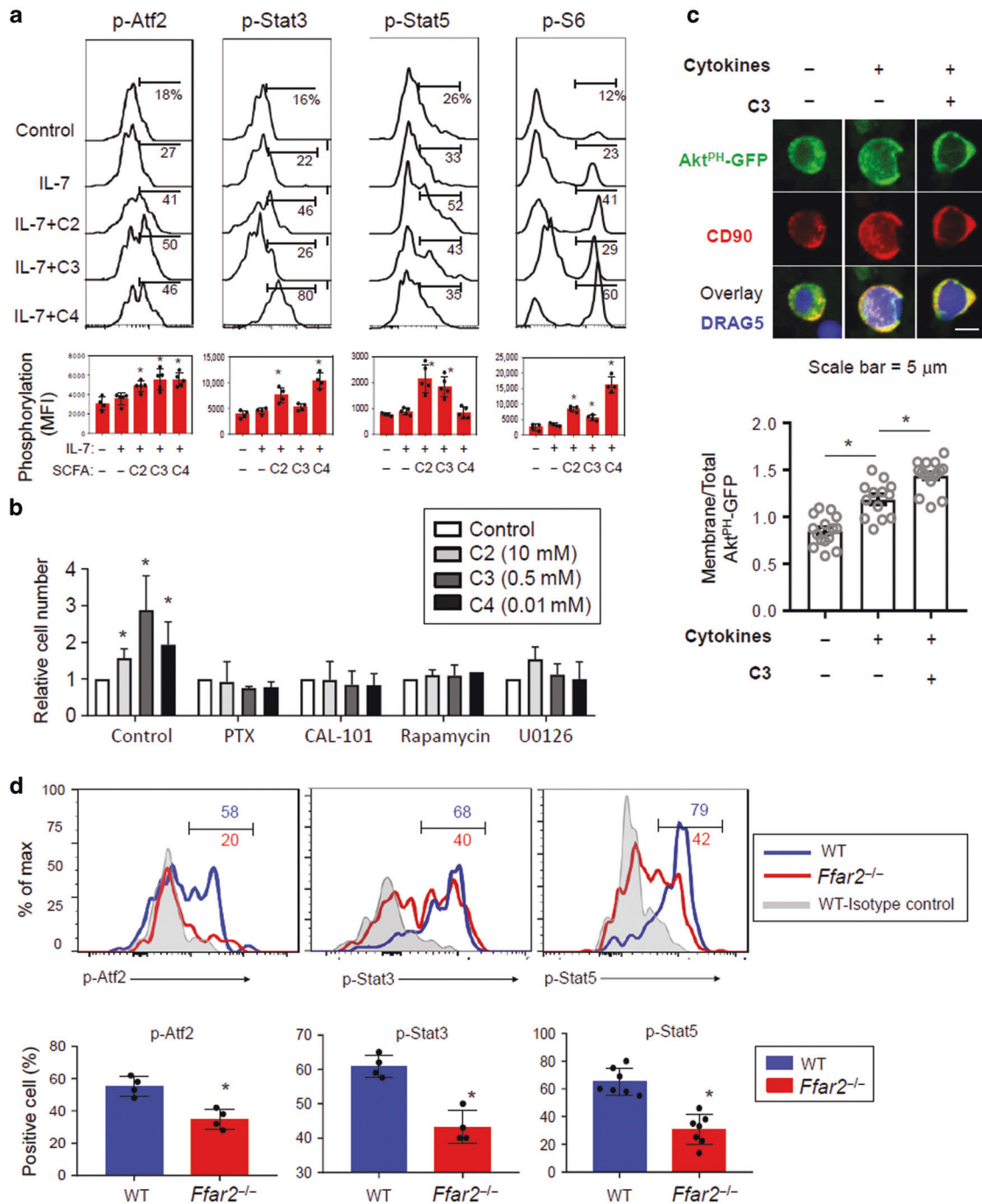


Fig. 3 Direct effects of SCFAs on ILCs. **a** Activation of signaling molecules (Arf2, Stat3, Stat5, and rS6) in ILCs. *Rag1*^{-/-} colonic LP cells were cultured for 4 h with IL-7 and SCFAs, and phosphorylation of indicated molecules in Lin⁻ CD127⁺ CD90⁺ ILCs was assessed. **b** Effects of small molecule inhibitors of Gαi, PI3K, mTOR, and MEK1/2 on SCFA-mediated ILC proliferation. *Rag1*^{-/-} spleen cells were cultured in an ILC3 condition (IL-7, IL-23, and IL-1β) for 4 days with the inhibitors, and numbers of Lin⁻ CD90⁺ CD127⁺ ILCs were determined by flow cytometry. **c** Detection of membrane PIP₃ in ILCs by expressing GFP-PH^{Akt}. CD90⁺ ILCs were enriched with magnetic sorting and were transfected with a plasmid expressing GFP-PH^{Akt} and activated for 16 h with cytokines (IL-7, IL-1β, and IL-23) and TSA (20 nM) followed by paraformaldehyde fixation and staining with anti-CD90 and DRAQ5 for confocal microscopy. **d** *Ffar2*-dependent phosphorylation of Atf2, Stat3, and Stat5 in ILCs. LI LP ILC3, isolated from WT and *Ffar2*^{-/-} mice, were stained for phosphorylation of the molecules. Pooled data obtained from at least three different experiments (*n* = 4–6) are shown. An asterisk represents significant differences (*p* < 0.05) between groups.

SCFAs fail to expand *Ffar2*^{-/-} ILC populations
To determine the effect of SCFAs in vivo, we fed WT and *Ffar2*^{-/-} mice with LFD (to create a low SCFA condition) with or without SCFA feeding in drinking water (to create low and high SCFA conditions). This method has been tested to be effective in regulating SCFA levels.^{29,32} We infected these mice with *C.*

rodentium, and ILC populations were assessed in various tissues. SCFA feeding increased the resistance of WT but not *Ffar2*^{-/-} mice (Fig. S7a, b) as reported previously.^{29,32} On LFD without SCFA feeding, there were small differences in numbers of ILC3 subsets between WT and *Ffar2*^{-/-} mice mainly in the SI (Fig. 6a). With SCFA feeding, however, there were greater differences in numbers

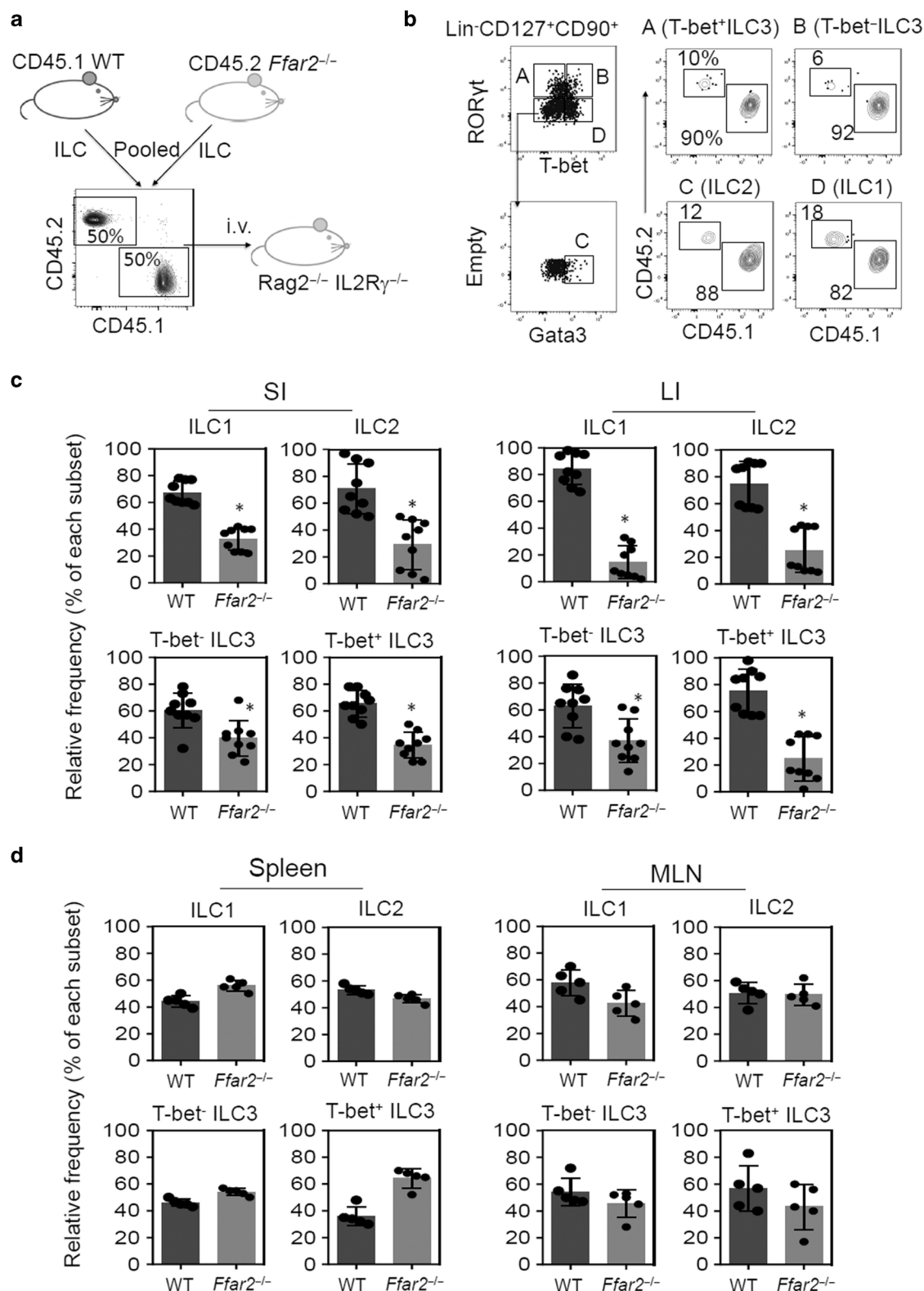


Fig. 4 *Ffar2* is required for effective population of ILCs in a lymphopenic condition. **a** CD45.1 WT and CD45.2 *Ffar2*^{-/-} Lin⁻CD127⁺ cells, isolated from the spleen, were cotransferred into *Rag2*^{-/-}*IL2Rγ*^{-/-} mice. Mice were euthanized ~4 weeks later, and ILC subsets were examined. **b** Representative data showing relative frequencies of WT versus *Ffar2*^{-/-} colon LP ILCs. **c** Relative frequency of WT versus *Ffar2*^{-/-} ILCs in the intestines. **d** Relative frequency of WT versus *Ffar2*^{-/-} ILCs in the secondary lymphoid tissues. Representative and pooled data obtained from two experiments are shown. An asterisk represents significant differences ($p < 0.05$, $n = 5-9$) from WT.

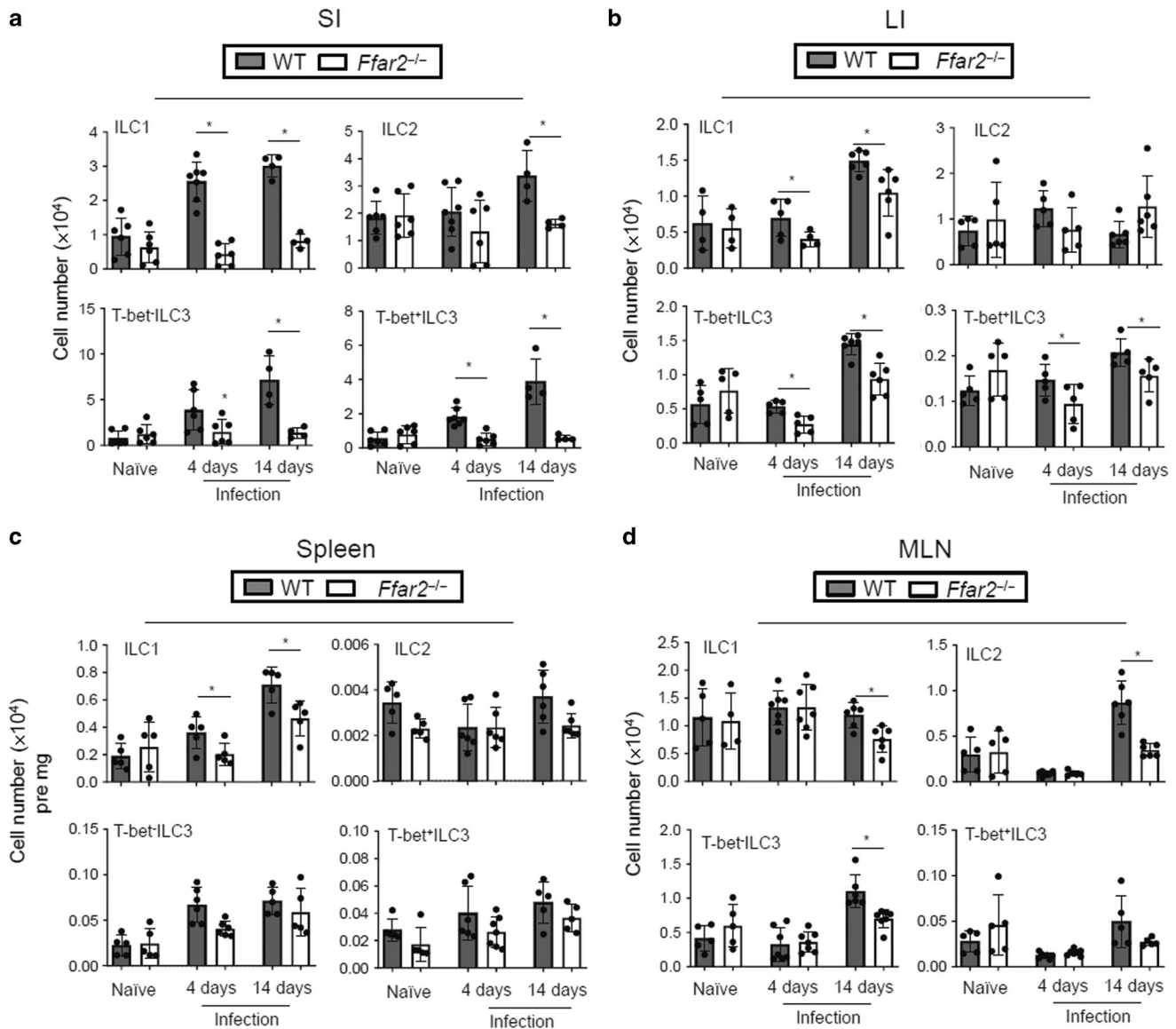


Fig. 5 *Ffar2* is required for normal expansion of ILC1 and ILC3 in both the intestine and systemic tissues following enteric bacterial infection. Numbers of ILCs in the intestines (a, b) and lymphoid tissues (c, d) of WT and *Ffar2*^{-/-} mice in noninfected mice or at 4 and 14 days after infection with *C. rodentium*. Pooled data obtained from at least three different experiments ($n = 5-10$) are shown. An asterisk represents significant differences ($p < 0.05$) from WT.

of ILC1 (SI, LI, MLN, and spleen), ILC2 (spleen and MLN), and ILC3 (SI, LI, spleen, and MLN) between WT and *Ffar2*^{-/-} mice (Figs. 6a–d, S7c).

We, further, assessed the function of *Ffar2* in potentiating the ILC effector function during *C. rodentium* infection with a cell transfer approach (Figs. 7a, S8). WT and *Ffar2*^{-/-} spleen ILCs were separately transferred into *Rag2*^{-/-}*IL2Ry*^{-/-} mice, and the host mice were infected by *C. rodentium*. Compared with the WT ILC-transferred mice, *Ffar2*^{-/-} ILC-transferred mice were more susceptible to *C. rodentium* infection based on body weight change, pathogen burden, and neutrophil infiltration (Fig. 7b). The numbers of donor-derived ILC3 in the intestines were significantly smaller in the mice transferred with *Ffar2*^{-/-} ILCs (Figs. 7c, S8). Also smaller were the numbers of intestinal ILC1 and ILC2.

In addition to *Ffar2*, we examined the role of *Ffar3*/*Gpr41* in regulating ILCs (Fig. 8). As reported previously,⁴⁶ *Ffar3*^{-/-} mice were more susceptible to *C. rodentium* infection, based on weight loss and pathogen burden (Fig. 8a–c), and had decreased numbers of most ILC subsets in SI, T-bet⁺ ILC3 in LI, and T-bet⁺

and T-bet⁻ ILC3 in the spleen (Figs. 8d, S9). However, the ILC deficiency in *Ffar3*^{-/-} mice was less severe compared with that of *Ffar2*^{-/-} mice.

Differential roles of *Ffar2* and SCFAs in regulating ILC2 response to IL-33

Regulation of ILC2 proliferation by SCFAs in vitro was different from other subsets. To better investigate the impact of *Ffar2* versus SCFAs on ILC2 responses in vivo, we employed an IL-33 injection model (Fig. 9a).⁴⁹ Repeated injection of IL-33 decreased ILC2P numbers in the bone marrow (BM) but increased ILC2 numbers in mucosal tissues (Fig. 9b, c). The decrease of BM ILC2P following IL-33 administration is in line with the emigration of ILC2 from the BM,⁵⁰ which is apparently not affected by *Ffar2* deficiency (Figs. 9b, S10a). In contrast, *Ffar2* deficiency decreased the peripheral expansion of the ILC2 population in the SI, LI, and lung (Figs. 9c, d, S10b). However, no significant effect of *Ffar2* deficiency on ILC2 in MLN and spleen was observed. We also performed a SCFA water-feeding experiment on WT and *Ffar2*^{-/-}

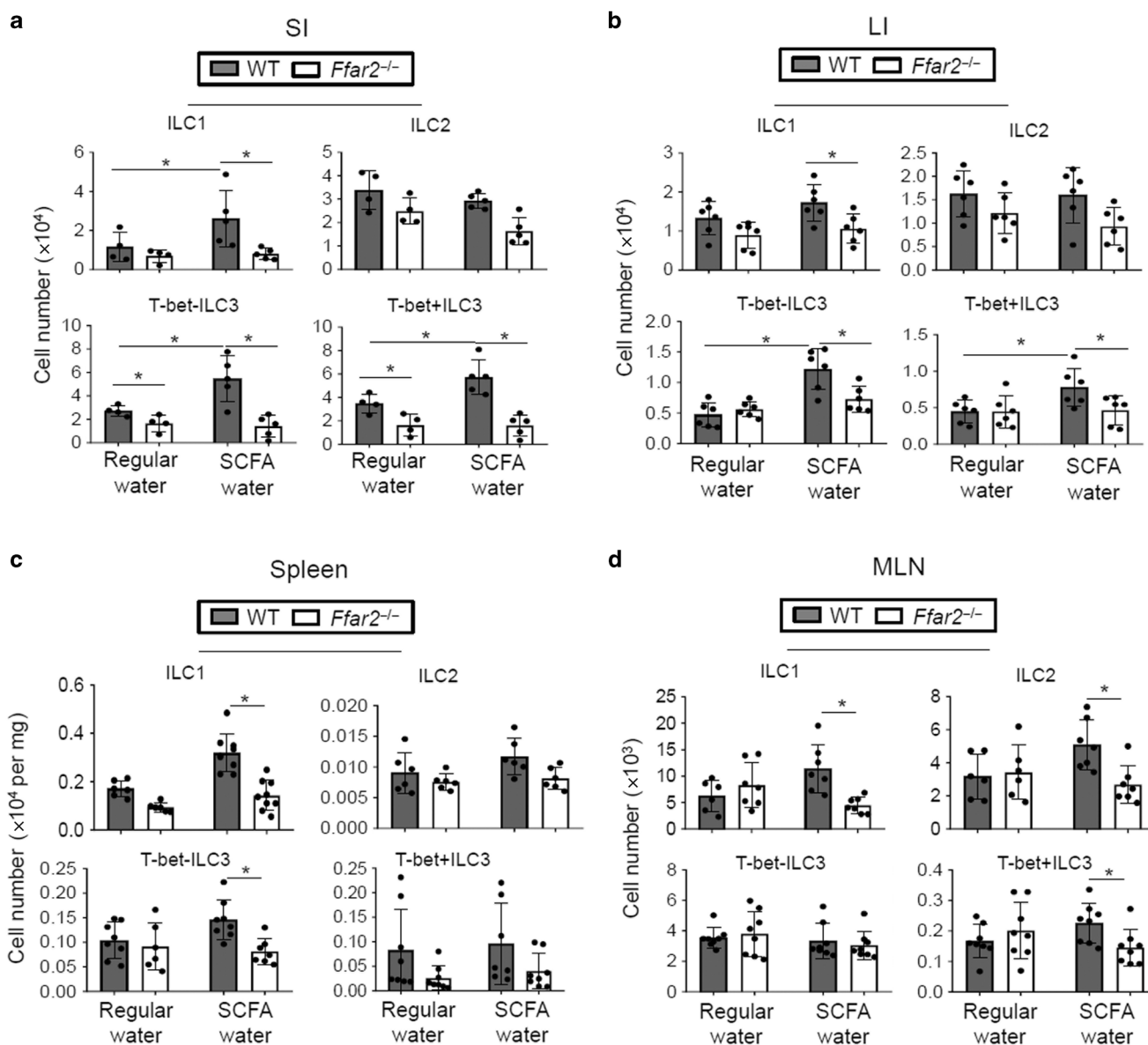


Fig. 6 SCFAs fail to expand *Ffar2*-deficient ILC1 and ILC3. WT and *Ffar2*^{-/-} mice were fed low-fiber diet (no soluble fiber, 10% cellulose) from the beginning and then on SCFAs (C2 at 70, C3 at 30, and C4 at 20 mM) in drinking water from the third week and then infected with *C. rodentium* 7 days later. At 14 days post infection, ILC numbers in indicated tissues were determined. Pooled (a–d) data obtained from two experiments are shown. An asterisk represents significant differences ($p < 0.05$, $n = 4–7$) from WT.

mice with the IL-33 administration (Figs. 9d, S10c). SCFA administration suppressed the IL-33-induced ILC2 response in not only WT, but also *Ffar2*^{-/-} mice. These results establish a positive role of *Ffar2*, but also surprisingly a negative role of SCFAs, mediated through a *Ffar2*-independent mechanism, in regulating the ILC2 response.

DISCUSSION

We investigated the roles of SCFAs and their receptors in regulating peripheral ILCs. We found that the DF microbial metabolites both induce and activate SCFA-sensing GPCRs, such as *Ffar2* and *Ffar3* on ILCs. Activation of these GPCRs generally signals the expansion of the peripheral ILC populations for effective effector functions. This process is important for optimal activity of peripheral ILCs, particularly ILC3, in the intestine and possibly other organs during active immune responses. We also

provide evidence that the functions of SCFAs and *Ffar2* can be different from each other in regulating ILC2.

SCFAs can regulate immune cell functions in several different ways. SCFAs activate GPCRs such as *Ffar2*, *Ffar3*, *Gpr109a*, and *Olfir78*.^{41,43,51} SCFAs inhibit HDACs, which regulate protein acetylation and gene expression.^{32,52} SCFAs are metabolized in cells to produce acetyl-CoA and ATP.^{29,53} The regulatory effects of SCFAs on ILCs are likely to be mediated by combinations of these pathways. We observed that ILC3 numbers in mice were increased when animals were fed soluble DF (pectin and inulin). These DF-containing diets produce different levels of SCFAs in the colon.^{29,32} SCFAs are also produced in the SI at relatively lower but significant concentrations.²⁸ This explains the regulation of SI ILCs by SCFA feeding or *Ffar2* signaling. SCFAs are absorbed and transported to the blood circulation at ~400 and 80 μ M in portal and peripheral veins, respectively, in humans.^{28,29} It remains unknown if the systemic levels of SCFAs can effectively regulate

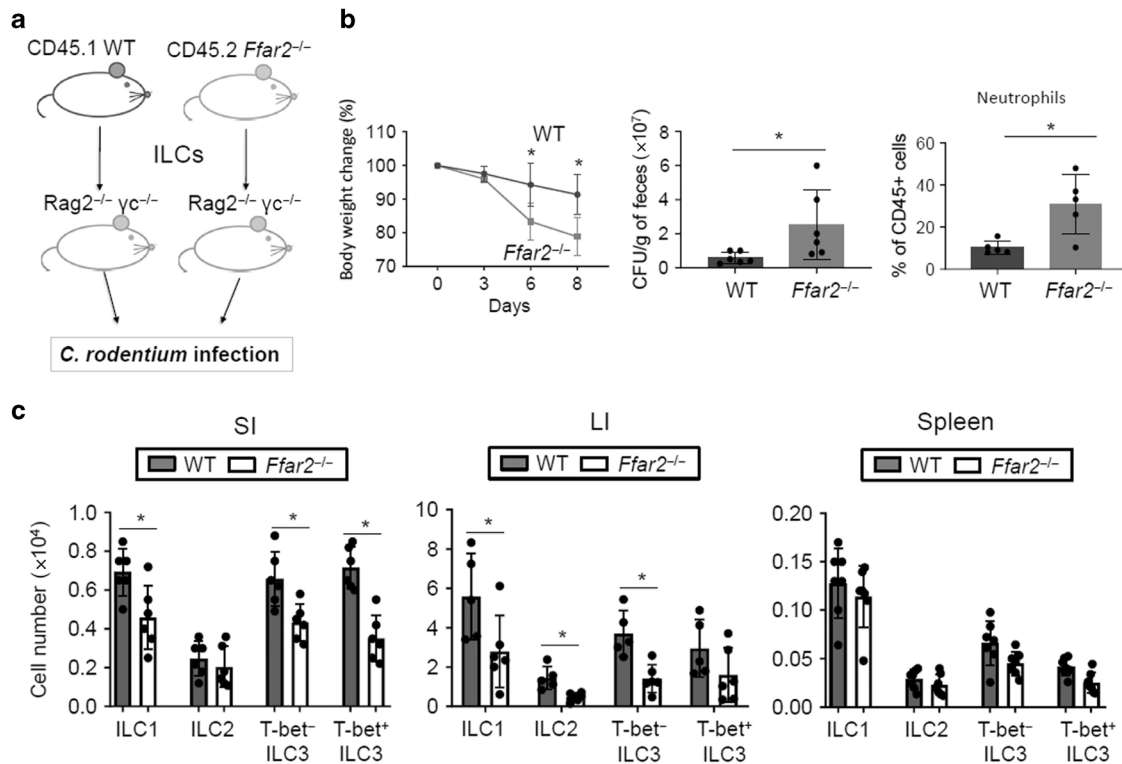


Fig. 7 ILC-expressed *Ffar2* is necessary for normal intestinal ILC immunity. **a** WT and *Ffar2*^{-/-} ILCs (~200,000 Lin⁻ CD127⁺ cells per mouse), isolated from spleen, were separately transferred into *Rag2*^{-/-}*IL2Ry*^{-/-} mice. Four weeks later, mice were infected with *C. rodentium* and the mice were sacrificed 8 days later. **b** Weight change, pathogen burden, and neutrophil numbers among colonic LP cells. **c** Numbers of ILCs in the intestines and lymphoid tissues of host mice transferred with WT and *Ffar2*^{-/-} ILCs. Pooled data obtained from two experiments are shown. An asterisk represents significant differences ($p < 0.05$, $n = 6$) from WT.

ILCs. All major SCFAs, such as C2, C3, and C4, can promote ILC3 proliferation. However, SCFAs suppress ILC2 proliferation. For ILC1, only C4 was able to increase ILC1 production. Thus, SCFAs variably regulate the proliferation of ILC subsets. It is also considered that SCFA levels may be altered during infection.

ILCs variably express SCFA receptors at the mRNA level. Of these, the most highly expressed is *Ffar2*, which is more highly expressed in intestinal ILCs compared with spleen ILCs. This indicates that certain gut tissue factors induce the SCFA receptors on ILCs. We found that SCFAs, themselves, can induce the expression of *Ffar2* and *Ffar3*. This raises a question about the mechanism of the induction. Because the induction of SCFA receptors by SCFAs was largely suppressed by PTX, it appears that *Ffar2* triggering by SCFAs, with potential assistance from other stimulation, induces the expression of SCFA receptors. Intestinal ILC3 and ILC2 expressed *Ffar2* at levels higher than that of ILC1, which is in line with the relatively small effect of SCFAs on ILC1 proliferation.

Ffar2 triggering activates the function of heterotrimeric G proteins, composed of G α i and G β y subunits. G β y protein triggers the downstream signaling pathways, including PLC β and PI3K.^{54,55} Importantly, the function of SCFAs in expanding ILC numbers was inhibited by PTX. This indicates that GPCR signaling is a major signal that drives the induction of SCFA receptors and activation of ILCs. In line with this, SCFAs can increase the activation of key signaling molecules such as ERK/Atf2, Stat3, Stat5, and mTOR. Activation of Stat3 and Stat5 occurs downstream of general and ILC subset-specific activating cytokines.⁵⁶ Co-stimulation of the cytokine signaling pathways appears to be a major mechanism for the positive function of *Ffar2*. Moreover, enhanced activity of mTOR along with the aforementioned signaling pathways by GPCR signaling can support the metabolic activity required for ILC proliferation and population expansion.⁴⁷

Our results, together with the recent findings of others,^{37,38,57} indicate that SCFAs regulate most ILC subsets, which is apparently mediated by multiple mechanisms. We provided evidence that *Ffar2* affects all major ILC subsets. The numbers of intestinal ILC1, ILC2, and ILC3 in *Ffar2*^{-/-} mice in the steady state were not significantly different from those of WT mice. However, upon *C. rodentium* infection, there was a clear defect in ILC expansion in *Ffar2*^{-/-} mice. This is somewhat different from Chun et al.³⁸ in that the defect in the colonic ILC3 compartment in *Ffar2*^{-/-} mice was detectable even at steady state without infection. They also found that SCFAs and GPR43 agonism increased the numbers of ILC3 in the colon in the steady state. Potential microbial differences across animal facilities may be a reason for the heterogeneous sensitivity of ILC3 to *Ffar2* signaling at steady state. The positive effect of *Ffar2* on ILC3, observed in our study, is in line with their report on the positive role of *Ffar2* in proliferation of CCR6⁺ ILC3. Another group reported that IL-22 secretion by ILC3 is promoted by acetate potentially via upregulation of the expression of IL-1 β receptor in a *C. difficile* infection model.⁵⁸ Our results additionally indicate that GPR43 can also support ILC2 expansion in the colon in response to cytokine stimulation.³⁸ Our competitive population data for WT versus *Ffar2*^{-/-} ILCs in ILC/T/B-deficient *Rag2*^{-/-}*IL2Ry*^{-/-} mice indicate that steady state numbers of intestinal ILCs are also regulated by *Ffar2*. ILC expansion in a lymphopenic condition is driven by IL-7⁵⁹ and, therefore, *Ffar2* appears to promote the IL-7-mediated ILC expansion.

Ffar3 also has a positive effect on ILC populations in the intestines and other tissues, but the effect of *Ffar3* appears to be considerably weaker than that of *Ffar2*. This is explained, in part, by the relatively low expression of *Ffar3* by ILCs.

The in vivo effects of DFs and SCFAs during *C. rodentium* infection largely mirror that of *Ffar2* in regulating the ILC3 subsets in the intestine. However, the effects of SCFAs and GPCR

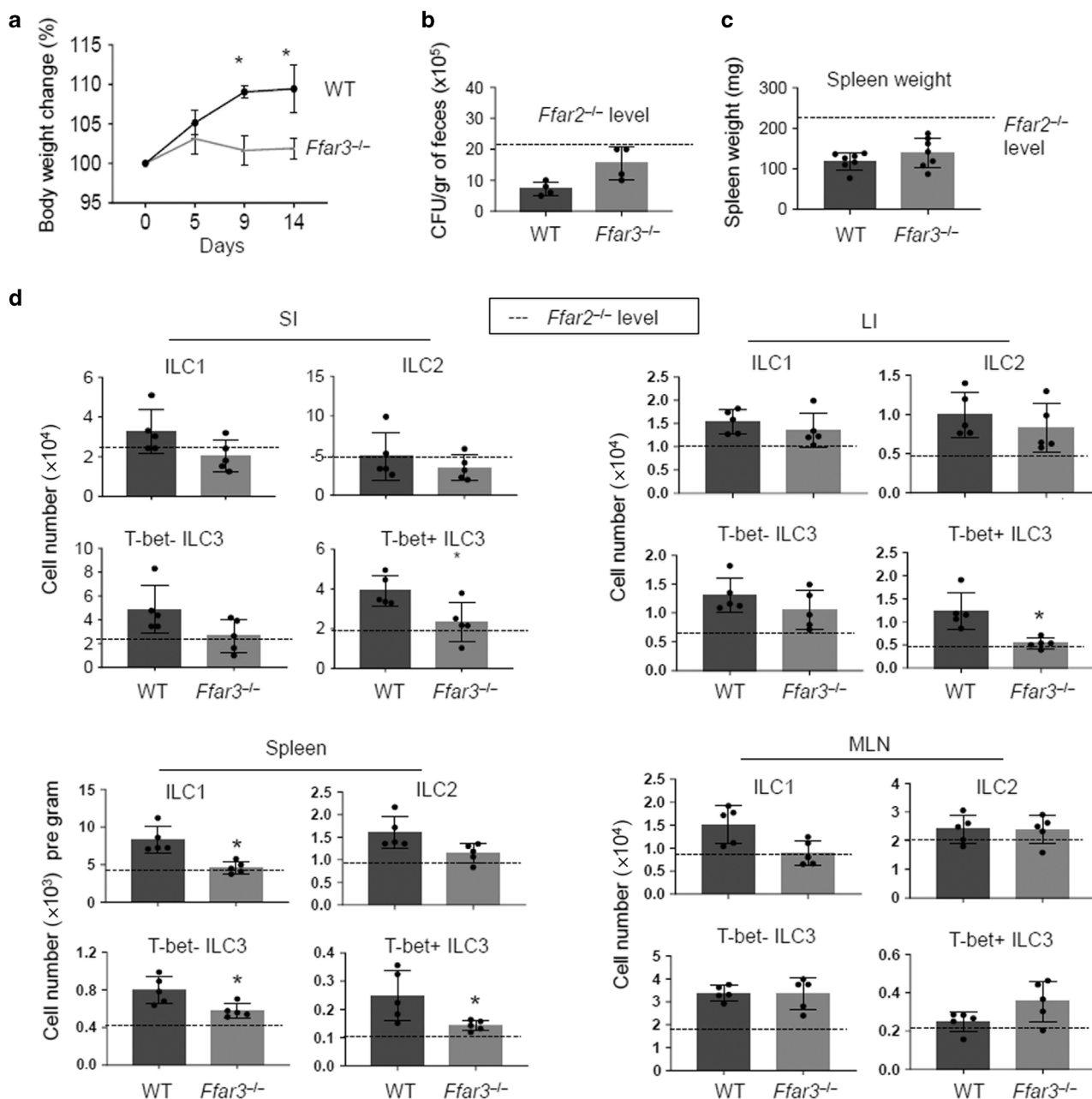


Fig. 8 ILC responses to enteric bacterial infection in *Ffar3*^{-/-} mice. WT and *Ffar3*^{-/-} mice were infected with *C. rodentium* and numbers of ILC subsets were determined. **a–c** Body weight change, pathogen burden, and spleen weight following the bacterial infection were examined. **d** Numbers of indicated ILCs in the intestine, spleen, and MLN were examined by flow cytometry. Pooled data obtained from at least three different experiments ($n = 4–7$) are shown. An asterisk represents significant differences ($p < 0.05$) from WT.

triggering on ILCs are not the same. *Ffar2* positively regulates the size of most ILC populations in both the intestine and lymphoid tissues, whereas DFs and SCFAs primarily affect intestinal ILC3. SCFAs also increase intestinal ILC1 numbers. While ILC2 expansion in response to IL-33 was boosted by *Ffar2* signaling, it was suppressed by SCFAs. Indeed, these results are in line with the recent report that SCFAs suppress an ILC2 response in airway inflammation.^{37,60} The HDAC inhibition activity of SCFAs, which is independent of the GPCR signaling, has been proposed to mediate the suppression of ILC2.⁶⁰ Among ILC subsets, the ILC2 subset had highest expression of *Slc16a1*, which transports SCFAs into cells. This suggests that ILC2 may absorb SCFAs more effectively, leading to heightened intracellular functions of SCFAs, such as HDAC inhibition. In terms of the negative role of SCFAs, it

has been documented that SCFAs can suppress ILC3 in Peyer's patches.³⁶ We reason that the differential regulation of ILCs in different tissues by SCFAs is likely due to (1) differential roles of GPCRs versus other mechanisms such as HDAC inhibition in regulating ILC proliferation; (2) differential GPCR expression among ILC subsets; and (3) distinct responses of ILCs depending on their maturation status or tissue environment. These issues should be studied in depth in the future.

Another potentially important regulation of ILCs by SCFAs and their receptors may be mediated indirectly through other cell types. Indeed, antigen presenting cells and tissue cells can be regulated by SCFAs and this can, in turn, regulate other immune cells.^{34,46,61,62} This is supported, in part, by the ILC regulation by SCFAs in *Ffar2* or *Ffar3*-deficient mice. Moreover, microbial

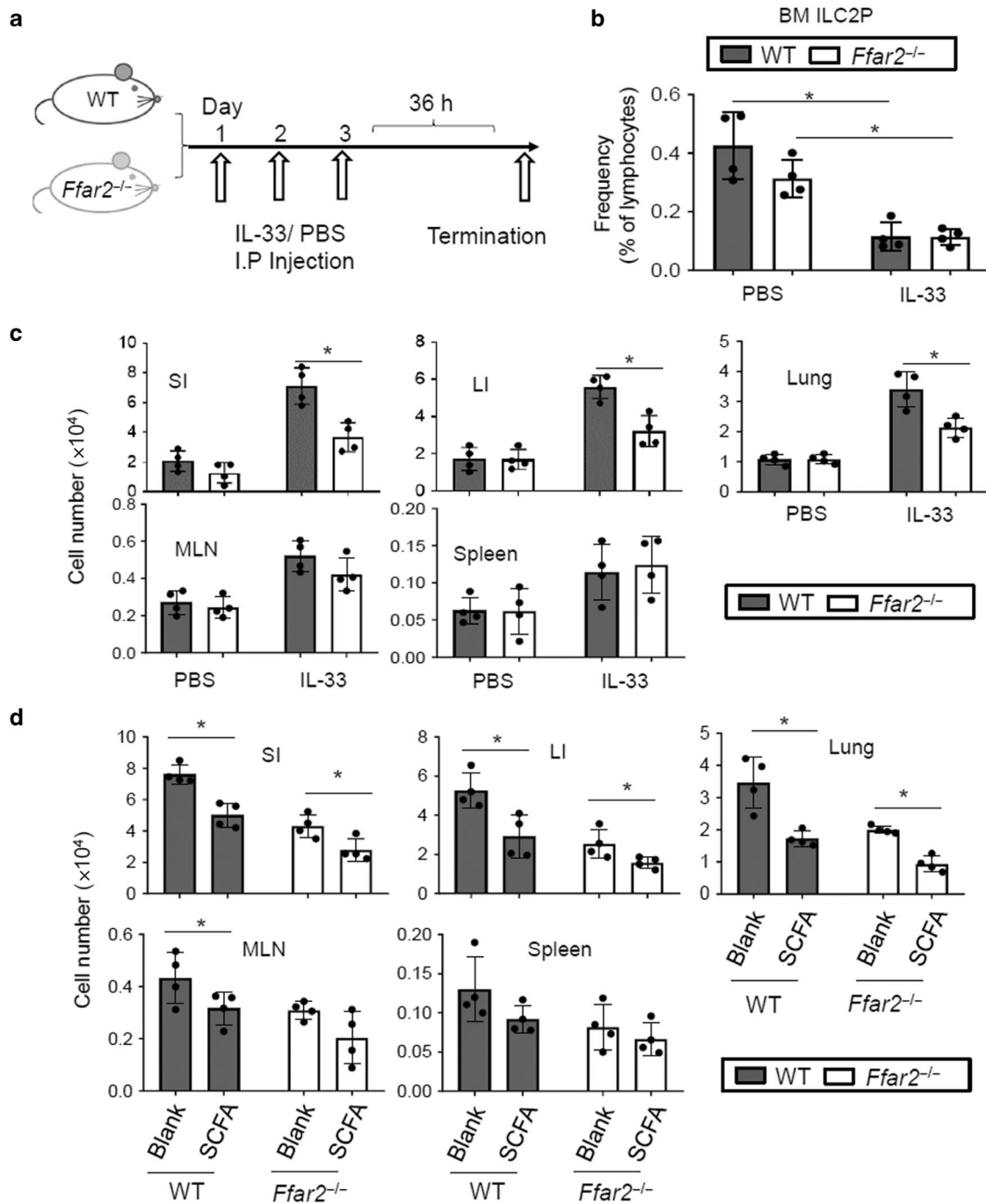


Fig. 9 ILC2 responses to IL-33 in *Ffar2*^{-/-} mice with or without regulation of SCFA levels. **a** WT and *Ffar2*^{-/-} mice were injected i.p. with IL-33 (500 ng per injection) or PBS control for three consecutive days, and ILC numbers in indicated tissues were examined 36 h later. **b** Frequency of ILC2P in the bone marrow (BM). **c** Combined data showing ILC numbers in indicated tissues. **d** Impact of SCFAs on the ILC2 response to IL-33 in WT versus *Ffar2*^{-/-} mice. For **d**, mice were fed LFD for 2 weeks and then fed SCFA water for 1 week prior to IL-33 injection (three times). The data were obtained from two independent experiments using total four mice each group. An asterisk represents significant differences ($p < 0.05$) from WT.

changes occur during infection, including *C. rodentium* infection,⁶³ and this has the potential to change ILC responses via SCFA-dependent and independent pathways. However, it appears that *C. rodentium* infection does not alter SCFA levels.⁶⁴

The defect of ILCs in the SI of *Ffar2*^{-/-} mice is interesting. As mentioned, there are still significant levels of luminal SCFAs in the SI²⁸ to regulate ILCs, particularly in the ileum. Other microbial metabolites/products may antagonize or modulate the effect of SCFAs. These include retinoic acid, bile acid metabolites,

tryptophan metabolites, amino acids, and pattern recognition receptor ligands, all of which have the potential to modulate the SCFA-GPCR signaling effects on ILCs. Differential presence of these factors in different segments of the intestine can contribute to the heterogeneous effects of SCFAs and their receptors on ILCs in the small versus LI.

In conclusion, our findings establish an important role of SCFAs in regulating the ILC responses (Fig. S11). This regulation is mediated by the functions of SCFAs in inducing the expression

and triggering of SCFA-sensing GPCRs on ILCs, but other direct and indirect mechanisms may also play significant roles. This function is particularly important for ILC3, but other subsets such as ILC1 and ILC2 are also regulated depending on tissue sites and host conditions in a subset-dependent manner.

MATERIALS AND METHODS

Animals, diets, and infection

Animal protocols were approved by the Animal Care and Use Committee at the University of Michigan. C57BL/6, CD45.1, *Rag1*^{-/-}, and *Rag2*^{-/-}*IL2Rg*^{-/-} mice were originally from the Jackson Laboratory and housed at the University of Michigan. *Ffar2*^{-/-} and *Ffar3*^{-/-} mice on the C57BL/6 background were described previously.⁴⁶ All mice were housed in a specific pathogen-free condition. When indicated, mice were fed on AIN 93-based diet containing low or high content soluble DFs (LFD for 0% pectin and inulin; HFD for 7.5% of pectin and 7.5% inulin).²⁹ When indicated, mice were fed SCFAs in drinking water (sodium acetate/C2 at 70 mM, sodium propionate/C3 at 30 mM, and sodium butyrate/C4 at 20 mM, pH 7.4, Sigma-Aldrich). For the cohoused mouse experiment, WT and *Ffar2*^{-/-} mice were cohoused from 2 weeks before infection and until termination. When indicated, mice were infected with *C. rodentium* (DBS100, ~10¹⁰ CFU/mouse) via oral gavage and monitored for weight change, stool consistency, and *C. rodentium* load as previously described.⁴⁶

IL-33-induced in vivo response of ILC2

For IL-33-induced inflammation,⁴⁹ carrier-free recombinant mouse IL-33 (500 ng/mouse; BioLegend), or PBS was injected i.p into mice on days 1, 2, and 3. Mice were sacrificed and tissues were collected 36 h later.

Cell isolation and culture

Preparation of BM cells, spleen, and intestinal cells for flow cytometry or cell isolation were previous described.⁶⁵ Total ILCs were sorted from the spleen of *Rag1*^{-/-}, WT, and *Ffar2*^{-/-} mice by a BD Melody sorter (BD Biosciences) or sequential magnetic sorting (lineage depletion and positive selection of CD127⁺ cells). For in vitro culture, *Rag1*^{-/-} spleen or lung mononuclear cells or sorted Lin⁻CD127⁺ cells were cultured in complete RPMI-1640 medium supplemented with 10% FBS. For isolation of lung mononuclear cells, lung tissues from *Rag1*^{-/-} mice were digested with 0.5 mg/ml collagenase IV and isolated by density cut in 40/80% Percoll gradient. For ILC culture, IL-7 and IL-15 for ILC1; IL-7 and IL-33 for ILC2; and IL-7, IL-1β, and IL-23 for ILC3 were used at 20 ng/ml. When indicated, SCFAs at 10 (C2), 0.5 (C3), or 0.01 (C4) mM were added to culture. TSA was added at 20 nM when indicated. For PTX treatment, cells were preincubated with PTX (0.5 μg/ml) for 30 min and were further cultured with SCFAs and cytokines. Inhibitors used for the cultures were U0126 (1 μM), rapamycin (25 nM), and CAL 101 (200 nM) from Cayman Chemical.

Flow cytometry

For ILC detection by flow cytometry, cells were stained with various antibodies to antigens. Lineage cocktail antibodies include CD3ε/145-2C11, CD5/53-7.3, CD8α/53-6.7, CD19/6D5, B220/RA3-6B2, CD11b/M1/70, CD11c/N418, Ter119/Ter-119, F4/80/BM8, Gr-1/RB6-8C5, TCRβ/H57-597, TCRγδ/GL3, CD49b/DX5, FcεR1α/MAR-1, NK1.1/PK136, and CD25/3C7. Antibodies to CD45.2/104, CD45.1/A20, CD90.2/53-2.1, CD127/A7R34, Sca-1/D7, KLRG-1/2F1, α4β7/DATK32, Flt3/A2F10, c-kit/2B8, and/or Nkp46/29A1.4 were used to differentiate ILC subsets and their donor origin. The cells were fixed and further stained with antibodies for intracellular antigens (T-bet/eBio-4B10, GATA3/TWJ, and RORγt/AFKJ5-9). Most of the antibodies were from BioLegend or eBioscience unless indicated otherwise. ILC1 was identified by CD45⁺Lin⁻CD127⁺CD90⁺RORγt⁻Tbet⁺ phenotype. ILC2 was

identified by CD45⁺Lin⁻CD127⁺CD90⁺RORγt⁻Tbet⁻GATA3⁺. ILC3 was identified as CD45⁺Lin⁻CD127⁺CD90⁺RORγt⁺Tbet⁺ cells. A 12-color NovoCyte Flow Cytometer (ACEA Biosciences) was used, and the data were analyzed by FlowJo (BD Biosciences).

For phosphorylation of Akt2 and Stat3/5, primary or cultured cells were fixed with 4% formaldehyde and permeabilized with ice-cold graded methanol (100 → 90%) at -20 °C overnight. Cells were stained with Rabbit mAb to phospho-ATF-2 (ATF2T71-G3), phospho-Stat3 (Tyr705) (4/P-STAT3), and phospho-Stat5 (Tyr694) (C11C5) (Cell Signaling Technology), and then further stained with PE-labeled anti-rabbit IgG antibody (BioLegend). For phosphorylation of rS6 (pS235/236), cells were fixed and permeabilized by BD Phosflow™ Lyse/Fix Buffer/Permeabilization Kits (BD Biosciences) according to the manufacturer's instruction. Cells were washed and stained with Alexa Fluor[®] 647 anti-mouse-S6 (D57.2.2E). Nuclear Ki-67 was stained with an antibody (16A8, BioLegend) after surface antigen staining for ILCs and then fixation in cold ethanol (70%). For intracellular staining of cytokines, such as IL-5, IL-9, IL-17, and IFN-γ, cells were activated with phorbol myristate acetate and ionomycin or IL-23 in the presence of monensin for 4 h and the cells were fixed in 1% paraformaldehyde and permeabilized with saponin buffer followed by staining with antibodies to the cytokines (BioLegend). For cell survival or gating for live cells, cells were stained with Ghost dye Violet 450 (Tonbo Biosciences) together with antibodies to surface antigens.

ILC transfer into *Rag2*^{-/-} *IL2Rg*^{-/-} mice

Spleen Lin⁻ cells were isolated from the spleen of CD45.1⁺WT, CD45.2⁺WT, or CD45.2⁺ *Ffar2*^{-/-} mice by magnetic depletion using antibodies to CD19, CD3, CD11b, Gr-1, and CD11c (Miltenyi Biotec) and further sorted by a BD Melody sorter to isolate Lin⁻CD127⁺CD90⁺ ILCs. These cells were injected separately or co-injected at 1:1 ratio i.v. into *Rag2*^{-/-}*IL2Rg*^{-/-} mice (~0.1 million cells per mouse). The cotransferred mice were sacrificed 3–4 weeks later and ILC1/2/3 subsets in various organs were examined. The *Rag2*^{-/-}*IL2Rg*^{-/-} mice, separately transferred with WT or *Ffar2*^{-/-} ILCs and rested for 3–4 weeks, were infected with *C. rodentium* and sacrificed 8 days later.

RNA expression

Total RNA from indicated cells were isolated using the RNeasy micro Kit (Qiagen) and reverse-transcribed with Sensiscript RT Kit (Qiagen). Gene expression was analyzed by qRT-PCR with SYBR™ Green PCR Master Mix (Thermo Fisher). Oligonucleotide primers used were β-actin-F (AGAAGAGCTACGAGCTGCCTGAC) and β-actin-R (TACTCCTGCTTGCTGATCCACAT); Slc16a3-F (GAGGTGCTCATGGCTATC) and Slc16a3-R (GCTCCACCTCCCTCGAG); Slc16a1-F (GGATTTGCCTTTGGTTGGCTC) and Slc16a1-R (GACATCGGTGCTGG CCTCG). Primers for other genes were described previously.⁴⁶ The data were normalized by β-actin or glyceraldehyde 3-phosphate dehydrogenase levels. Publicly available microarray (ImmGen) and RNA-seq data (GEO GSE85154)¹⁵ for ILCs were analyzed for indicated genes.

Confocal microscopy of PI3K activation

Membrane PIP₃, an enzymatic product of PI3K, was detected by GFP-PH^{Akt} expression. CD90⁺ cells, enriched with magnetic sorting (anti-CD90-PE and anti-PE beads from Miltenyi Biotec) from *Rag1*^{-/-} spleen cells, were transfected with a plasmid expressing GFP-PH^{Akt} with a Nucleofector (AMAXA). The transfected cells were cultured for 16 h with cytokines (IL-7, IL-1β, and IL-23) and TSA (20 nM) first, and then further stimulated with C3 (0.5 mM) for 1 h followed by paraformaldehyde fixation and staining with anti-CD90-PE and DRAQ5 (DNA/nucleus). The cells were spun onto Cell-Tak coated slides, mounted and imaged on the Nikon confocal microscope with the 60× oil objective. ImageJ software was used to quantify total cellular versus membrane GFP signals.

Statistical analysis

Student's *t* test (paired two-tailed unless indicated otherwise) was used to determine the significance of differences between two groups for most data. For analysis of three or more groups, one-way ANOVA was performed. *p* values ≤ 0.05 were considered significant. Error bars are standard error of the mean (SEM) in all figures.

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AUTHOR CONTRIBUTIONS

C.H.K. conceived the project, obtained funding, designed most experiments, and drafted the manuscript based on the figures and information prepared by A.S., Q.Y.L., and L.F. Author Q.Y.L. made the initial observation of altered SI ILC1/2/3 in *Ffar2*^{-/-} mice, set up IL-33-injected mice, and examined intestinal ILCs. A.S. set up *C. rodentium* infection, competitive population, and DF/SCFA feeding experiments, the data from which were analyzed by A.S. and Q.Y.L. Most figures were prepared by A.S. (LI/lymphoid tissue ILCs and RT-PCR data) and Q.Y.L. (intestinal ILCs, ILC1/ILC2 responses, and protein phosphorylation data) except Fig. 3c, which was prepared by L.F. All authors participated in verifying the manuscript for submission.

ADDITIONAL INFORMATION

The online version of this article (<https://doi.org/10.1038/s41385-020-0312-8>) contains supplementary material, which is available to authorized users.

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