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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/349/6251/986/suppl/DC1
Materials and Methods
Figs. S1 to S10
Table S1
Movie S1
References (*30*–*52*)

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MUCOSAL IMMUNOLOGY

The microbiota regulates type 2 immunity through RORγt$^+$ T cells

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Changes to the symbiotic microbiota early in life, or the absence of it, can lead to exacerbated type 2 immunity and allergic inflammations. Although it is unclear how the microbiota regulates type 2 immunity, it is a strong inducer of proinflammatory T helper 17 (T_H17) cells and regulatory T cells (T_{regs}) in the intestine. Here, we report that microbiota-induced T_{regs} express the nuclear hormone receptor RORγt and differentiate along a pathway that also leads to T_H17 cells. In the absence of RORγt$^+$ T_{regs}, T_H2-driven defense against helminths is more efficient, whereas T_H2-associated pathology is exacerbated. Thus, the microbiota regulates type 2 responses through the induction of type 3 RORγt$^+$ T_{regs} and T_H17 cells and acts as a key factor in balancing immune responses at mucosal surfaces.

Allergic reactions are on the rise in industrialized nations, paralleling a decrease in the incidence of infectious diseases (*1*, *2*). The hygiene hypothesis proposes that exposure to pathogens reduces the risk of allergy, a notion that may be extended to exposure to the symbiotic microbiota. In support of this hypothesis, germfree mice, devoid of microorganisms, develop increased susceptibility to allergy (*3*–*6*). Furthermore, a developmental time window during childhood determines such susceptibility (*1*, *2*). Mice treated early with antibiotics, which deeply affect the microbiota, develop an increased susceptibility to allergy (*7*) that can last into adulthood (*8*), an effect also found in mice that remain germfree until weaning (*9*).

The mechanism by which the microbiota regulates type 2 responses remains unclear. A direct effect of microbiota on type 2 cells, such as T helper 2 (T_H) cells and innate lymphoid cells (ILC) 2, has not been documented. In contrast, symbionts are necessary for the differentiation of T_H17 cells that produce interleukin (IL)–17 and IL-22 (*10*), cytokines involved in homeostasis and defense of mucosal surfaces, and a subset of intestinal regulatory T cells (T_{regs}) (*11*). Intriguingly, the absence of extrathymically generated T_{regs} leads to spontaneous type 2 pathologies at mucosal sites (*12*). As intestinal T_{regs} recognize bacterial antigens (*11*), the microbiota may regulate type 2 responses through the induction of extrathymically generated T_{regs}.

The nuclear hormone receptor RORγt is a key transcription factor for the differentiation of T_H17 cells and ILC3s (*13*, *14*). In addition, a substantial fraction of RORγt$^+$ T cells residing in the lamina propria of the small intestine does not express IL-17, but rather IL-10, the T_{reg} marker FoxP3 (a transcription factor), and has regulatory functions (*15*). Furthermore, the generation of such RORγt$^+$ T_{regs} requires the microbiota (*16*). Using reporter mice for the expression of RORγt and Foxp3, we found that a majority of RORγt$^+$ T cells in the colon of adult mice expressed Foxp3, and, reciprocally, a majority of colon T_{regs} expressed RORγt (Fig. 1A). The frequency of RORγt$^+$ T_{regs} increased with age, representing most intestinal T_{regs} in 1-year-old mice (fig. S1A). These cells were not found in the thymus (fig. S1B) and did not express Helios or Neuropilin-1, markers of thymically derived T_{regs} (*17*, *18*), in contrast to conventional RORγt$^-$ T_{regs} (Fig. 1B and fig. S1C). In the colon, most Helios$^-$ T_{regs} were absent in RORγt-deficient mice (Fig. 1B). RORγt$^+$ T_{regs} also expressed an activated CD44^{high} CD62L^{low} phenotype, as well as increased levels of ICOS, CTLA-4, and the nucleotidases CD39 and CD73 (fig. S1C), altogether indicating robust regulatory functions. Another major subset of intestinal T_{regs} expresses Gata*3*, responds to IL-*33*, and is involved in the regulation of effector T cells during inflammation (*19*, *20*). Gata*3*$^+$ T_{regs} were distinct from RORγt$^+$ T_{regs} and expressed Helios, as well as lower levels of IL-10 (fig. S2).

RORγt$^+$ T_{regs} were profoundly reduced in germfree or antibiotic-treated mice, whereas Helios$^+$ and Gata*3*$^+$ T_{regs} were unaffected (Fig. 1C and fig. S3). Recolonization of germfree mice with a specific pathogen–free (SPF) microbiota restored normal numbers of RORγt$^+$ T_{regs} (fig. S4). Furthermore, a consortium of symbionts composed of 17 *Clostridia* species efficiently induces the generation of T_{regs} expressing IL-10 in the colon (*21*), the majority of which expressed RORγt (Fig. 1D). The microbiota has been shown to induce the generation of intestinal T_{regs} through short-chain fatty acids (SCFA) (*22*, *23*) and antigen (*11*). We found that the SCFA butyrate induced an increase

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mainly in RORγt$^+$ T_{regs} (fig. S5). The generation of RORγt$^+$ T_{regs} was dependent on dendritic cells (DCs) and major histocompatibility complex (MHC) class II (Fig. 1E) and induced by oral antigen (ovalbumin) in transferred naïve CD4$^+$ T cells expressing the OT-II transgenic T cell receptor specific for that antigen (Fig. 1F). Altogether, these data show that RORγt$^+$ T_{regs} are induced by microbiota and oral antigen, and that microbiota-induced intestinal T_{regs} express RORγt.

T_H17 cells are efficiently induced by the pathobiont segmented filamentous bacteria (SFB) that forms colonies on epithelial cells of the small intestine (*24*, *25*) and by cytokine signaling pathways involving the transcription factor Stat3 (*26*). Surprisingly, RORγt$^+$ T_{regs} differentiated following similar pathways. RORγt$^+$ T_{regs} were efficiently induced by SFB in the small intestine, even though more T_H17 cells were induced in these conditions (Fig. 2A). Furthermore, similar to T_H17 cells, innate receptors of the Toll-like receptor and NOD-like receptor families were not involved (fig. S6). In contrast, mice deficient for IL-6 or the p19 subunit of IL-23 (encoded by *Il23a*), both involved in the induction of T_H17 cells (*27*, *28*), developed significantly less RORγt$^+$ T_{regs} (Fig. 2B), whereas Gata3$^+$ T_{regs} were increased (fig. S7). In accordance with the fact that both cytokines signal through the transcription factor Stat3, similar results were obtained in mice that lack expression of Stat3 in T_{regs} or RORγt$^+$ cells (Fig. 2B).

If T_H17 cells and RORγt$^+$ T_{regs} are induced through similar pathways, how then is the development to T_H17 cells or RORγt$^+$ T_{regs} regulated? In cell cultures, the vitamin A metabolite retinoic acid (RA) promotes the generation of T_{regs} (*29*) and of RORγt$^+$ T_{regs} (*15*) rather than of T_H17 cells. We now find that feeding mice with vitamin A–deficient food, or treating mice with

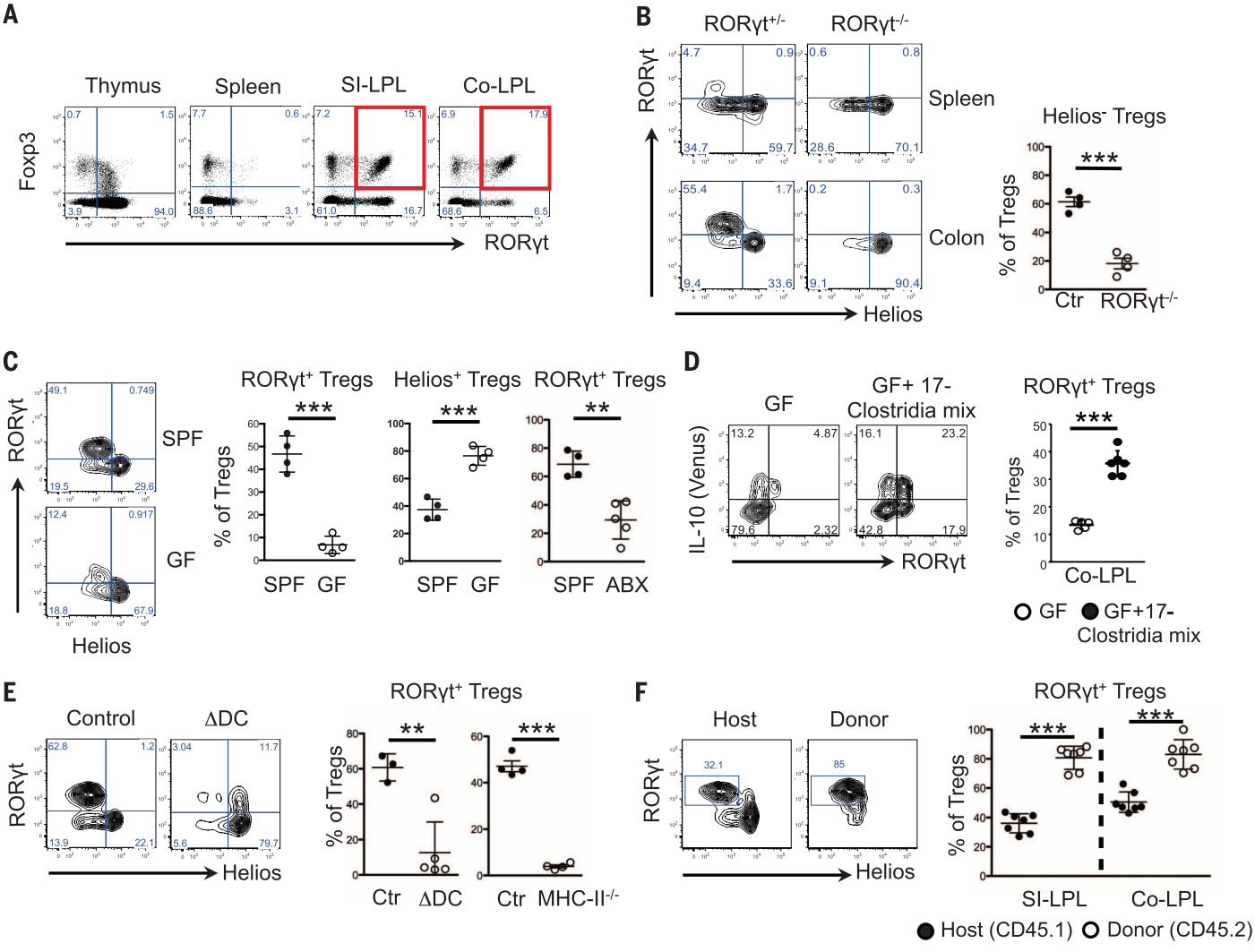

Fig. 1. A majority of colon RORγ$^+$ T cells are microbiota-induced RORγt$^+$ T_{regs}. (**A**) Expression of Foxp3 and RORγt by CD4$^+$ T cells in double reporter mice. SI, small intestine; Co, colon; LPL, lamina propria lymphocytes. (**B**) Expression of Helios and RORγt by T_{regs} (FoxP3$^+$ CD4$^+$ T cells) from spleen and colon lamina propria of littermate control (left) and RORγt-deficient mice (right). Right, frequency of colon Helios$^-$ T_{regs}. n = 4 mice per group. (**C**) Expression of RORγt and Helios by T_{regs} in the colon of SPF or germfree (GF) mice and frequencies of RORγt$^+$ Helios$^-$ T_{regs} (left) and Helios$^+$ T_{regs} (middle) in SPF and germfree mice; n = 4 mice per group. Right, frequency of RORγt$^+$ T_{regs} in the colon of SPF mice or mice treated from birth with a cocktail of antibiotics; $n \geq 4$. (**D**) Expression of RORγt and IL-10 by T_{regs} and frequency of RORγt$^+$ T_{regs} in the colon of germfree mice (left) or mice recolonized for 3 weeks with a consortium of 17 strains of *Clostridia* (right); $n \geq 4$ mice per group. (**E**) Expression of RORγt and Helios by colonic T_{regs} (left) and frequency of RORγt$^+$ T_{regs} (middle) in *Cd11c*^{Cre} x *Rosa26*^{Dta} (ΔDC) and littermate control mice, $n \geq 3$ mice per group. Right, frequencies of RORγt$^+$ T_{regs} in colon of control or MHC class II–deficient mice; n = 4. (**F**) Naïve CD4$^+$ T cells were isolated from CD45.2$^+$ OT-II Rag2$^{-/-}$ mice and adoptively transferred into CD45.1$^+$ congenic mice and subsequently fed for 7 days with 1.5% chicken ovalbumin in the drinking water. Expression of RORγt and Helios in small intestine T_{regs} (left) and frequency of RORγt$^+$ T_{regs} in small intestine and colon (right) in host (CD45.1) and donor (CD45.2) cells; n = 7 mice. Data are representative of at least two independent experiments. Error bars, mean ± 1 SD; ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001, as calculated by Student's t test.

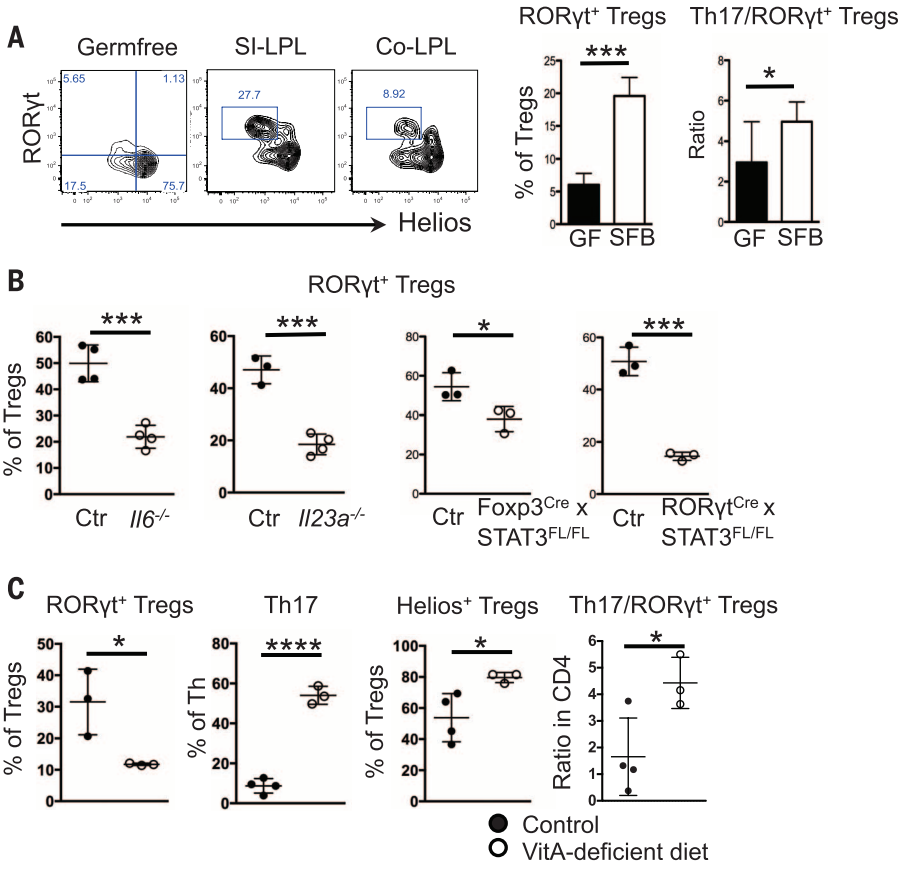

Fig. 2. A proinflammatory pathway plus retinoic acid induce RORγt$^+$ T$_{regs}$. (**A**) Expression of RORγt and Helios by T$_{regs}$ in germfree mice and germfree mice recolonized for 3 weeks with SFB. Histograms show frequencies of RORγt$^+$ T$_{regs}$ and ratio of T$_H$17 cells versus RORγt$^+$ T$_{regs}$. $n \geq 4$ mice per group. (**B**) Frequencies of (Helios$^-$) RORγt$^+$ T$_{regs}$ in colon of *Il6*$^{-/-}$, *Il23a*$^{-/-}$, *Foxp3*^{Cre} x *Stat3*$^{FL/FL}$, *RORγt* ^{Cre} x *Stat3*$^{FL/FL}$, and littermate control mice; $n \geq 3$ mice per group. (**C**) Frequencies of RORγt$^+$ T$_{regs}$, T$_H$17 cells, Helios$^+$ T$_{regs}$, and ratio of T$_H$17 cells versus RORγt$^+$ T$_{regs}$ in colon of mice fed a control diet or a vitamin A–deficient diet from birth for 10 weeks; $n \geq 3$ mice per group. Data are representative of at least two independent experiments. Error bars, mean ± 1 SD; *$P < 0.05$; ***$P < 0.001$, as calculated by Student's *t* test.

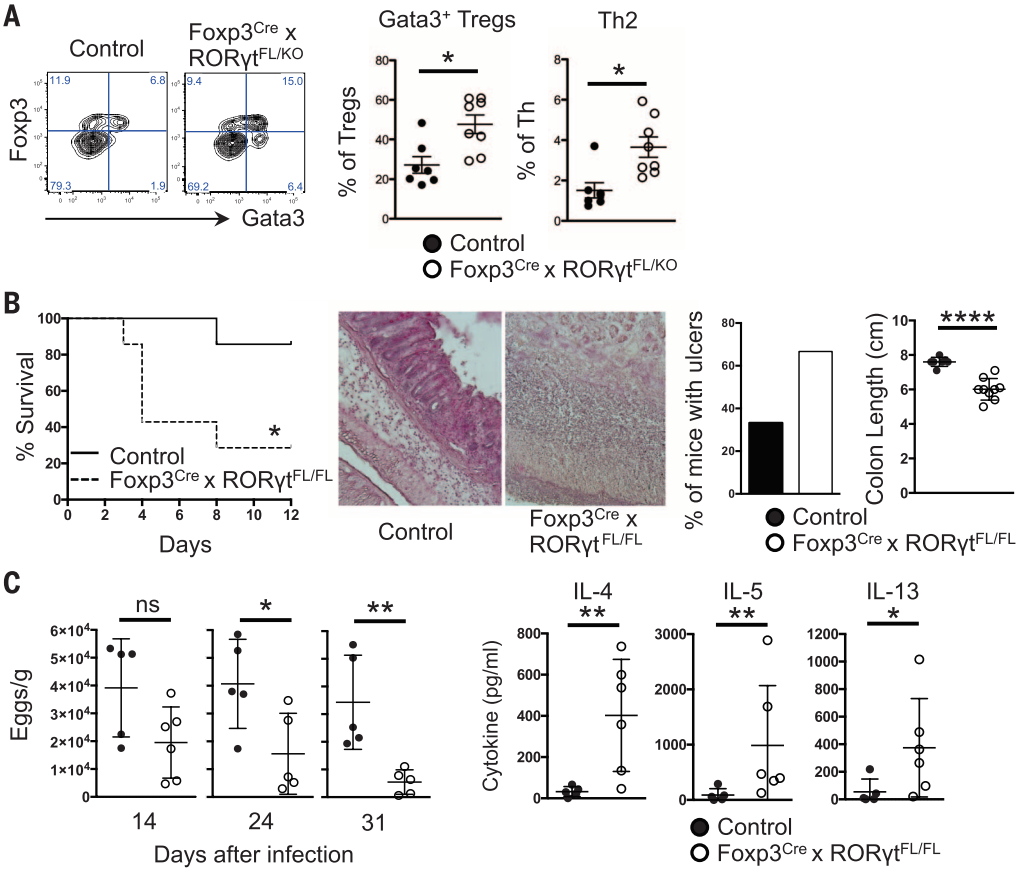

Fig. 3. RORγt$^+$ T$_{regs}$ regulate type 2 immune responses. (**A**) Expression of Foxp3 and Gata3 by small intestine CD4$^+$ T cells (left), and frequency of Gata3$^+$ T$_{regs}$ (middle) and Gata3$^+$ non-T$_{regs}$ CD4$^+$ T cells (right) in *Foxp3*^{Cre} x *Rorc(γt)*$^{FL/KO}$ mice; $n = 7$ mice per group. (**B**) Survival curve (left) of littermate control mice (straight line) or *Foxp3*^{Cre} x *Rorc(γt)*$^{FL/FL}$ mice (hatched line) treated with oxazolone; $n = 7$ mice per group. Periodic acid-Schiff staining of colons from the corresponding mice and ratio of mice with ulcers (middle) and length of colons 3 days after oxazolone challenge (right). (**C**) Egg burden 14, 24, and 31 days after infection with *H. polygyrus* and production of type 2 cytokines by T cells isolated from mesenteric lymph nodes 21 days after infection in control mice (filled symbols) or *Foxp3*^{Cre} x *Rorc(γt)*$^{FL/FL}$ mice (open symbols); $n \geq 5$ mice per group. Data are representative of at least two independent experiments. Error bars, ± 1 SD; ns, not significant; *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$, as calculated by Student's *t* test or Mann-Whitney test.

an inhibitor of the RA receptor (RARi), prevented the development of RORγt$^+$ T_{regs} but not of Helios$^+$ T_{regs} or T_H17 cells (Fig. 2C and fig. S8). RA also promotes the expansion of ILC3s over ILC2s (*30*) and the maturation of fetal ILC3s through RAR-mediated regulation of the *Rorc* locus encoding RORγt (*31*). Thus, vitamin A metabolism promotes the development of RORγt$^+$ cells and type 3 immunity, yet favors the development of RORγt$^+$ T_{regs} over T_H17 cells, presumably to limit the number of proinflammatory cells present in the healthy intestine.

We next assessed whether RORγt$^+$ T_{regs} regulate type 2 responses. Mice that lack only RORγt$^+$ T_{regs} were generated through a conditional knockout of *Rorc* in Foxp3$^+$ cells. Such *Foxp3*^{Cre} x *Rorc*(γt)^{FL} mice developed increased frequencies of Gata3$^+$ T cells and Gata3$^+$ T_{regs} (Fig. 3A), and, as a consequence, T cells produced higher amounts of the type 2 cytokines IL-4 and IL-5 (fig. S9A). These mice developed a more severe and lethal form of oxazolone-induced colitis, a model of ulcerative colitis dependent on the type 2 cytokines IL-4 (*32*) and IL-13 (*33*), as compared with their wild-type littermates (Fig. 3B). In contrast, they were more resistant to infection by the helminth *Heligmosomoides polygyrus*, because they produced higher levels of IL-4, IL-5, and IL-13 during the infection (Fig. 3C). T_H17 cells contributed to the control of type 2 responses, because a more pronounced increase in T_H2 cells was observed in full RORγt-deficient mice (fig. S9B). Furthermore, RORγt-deficient mice expressed high levels of immunoglobulin E (IgE) (fig. S9C), a hallmark of type 2 immunity, at levels sometimes similar to those found in germfree mice (*3*). In contrast, *Foxp3*^{Cre} x *Rorc*(γt)^{FL} mice did not develop increased T_H17 or T_H1 responses, even during acute intestinal inflammation induced by sodium dextran sulfate (fig. S10). These data are in agreement with the spontaneous type 2 pathologies observed in mice lacking extrathymically generated T_{regs} (*12*) and indicate that microbiota regulate type 2 responses through RORγt$^+$ T_{regs} and more generally RORγt$^+$ T cells.

What are the mechanisms by which RORγt$^+$ T_{regs} regulate type 2 immunity? We find that both cell-intrinsic and cell-extrinsic mechanisms of regulation are involved. Naïve OT-II$^+$ CD4$^+$ T cells developed into RORγt$^+$ T_{regs} when transferred into mice fed ovalbumin (Fig. 1F), whereas a majority of them developed into Gata3$^+$ T_{regs} when cells deficient in RORγt were transferred (Fig. 4A and fig. S11A). However, host T_{regs} were not affected, showing that a loss in RORγt affects T_{regs} only through a cell-intrinsic pathway. In contrast, the transfer of RORγt-deficient OT-II$^+$ cells affected the generation of both donor and host T_H2 cells, but not T_H17 cells, showing that RORγt$^+$ T_{regs} regulate T_H2 cells also through a cell-extrinsic pathway. Furthermore, in RORγt$^+$ T_{regs} that lacked Stat3, the expression of Gata3 was deregulated, and thus both transcription factors were coexpressed (Fig. 4B). This is in accordance with earlier data showing that IL-23 blocks the IL-33–mediated accumulation of Gata3$^+$ T_{regs} (*20*), and, conversely, that the absence of Gata3 leads to the expansion of RORγt$^+$ T_{regs} (*19*, *34*). This cross-inhibition may act directly, as Foxp3 binds to Gata3, Stat3 (*35*), and RORγt (*15*, *36*), and Gata3 binds to the *Rorc* promoter (*19*). In contrast, the expression levels of Gata3 remained unchanged in Stat3-deficient T_H17 cells (fig. S11B).

We next investigated the mechanisms of the cell-extrinsic regulation of T_H2 cells by RORγt$^+$ T_{regs}. Because RORγt$^+$ T_{regs} express high levels of IL-10 (Fig. 1D and fig. S2B) (*15*), we assessed whether RORγt$^+$ T_{regs} regulate T_H2 cells through IL-10, the receptor of which activates Stat3 (*37*). However, IL-10–deficient mice showed massive expansion of T_H17 cells but no expansion of Gata3$^+$ T cells (fig. S12). RORγt$^+$ T_{regs} also express high levels of CTLA4 (fig. S1C), shown to regulate the expression of CD80 and CD86 on DCs (*38*). As a consequence, the expression of both CD80 and CD86 by intestinal DCs was increased in *Foxp3*^{Cre} x *Rorc*(γt)^{FL} mice (Fig. 4C). Furthermore, in mice that lack CTLA4 expression in T_{regs}, Gata3$^+$ T cells were significantly expanded in the intestine, whereas T_H1 and T_H17 cells were not (Fig. 4D), and serum levels of IgE were increased (*38*). These data indicate that RORγt$^+$ T_{regs} regulate coactivator functions of DCs through CTLA4 and thereby regulate the generation of T_H2 cells in the intestine. Finally, RORγt$^+$ T_{regs} express high levels of interferon regulatory factor

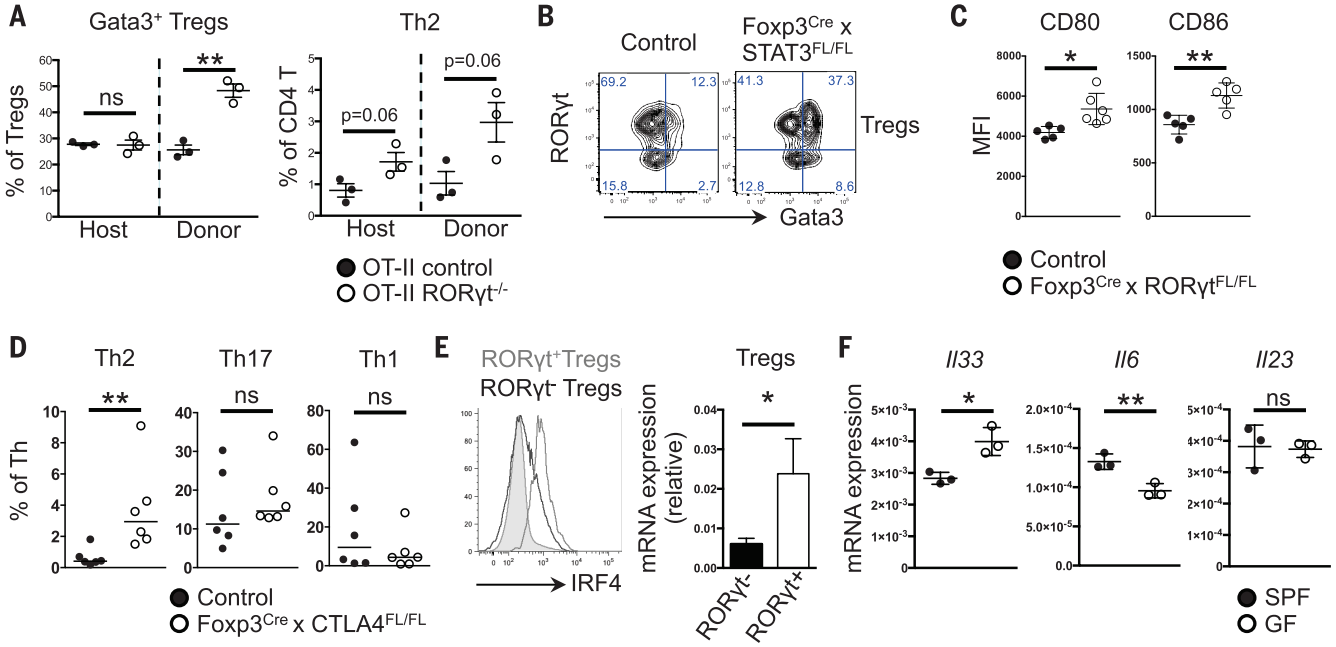

Fig. 4. Mechanisms of regulation by RORγt$^+$ T_{regs}. (**A**) Naïve CD4$^+$ T cells were isolated from CD45.2$^+$ OT-II wild-type or CD45.2$^+$ OT-II RORγt $^{-/-}$ mice, adoptively transferred into CD45.1$^+$ congenic mice, and subsequently fed for 7 days with 1.5% chicken ovalbumin. Frequency of Gata3$^+$ T_{regs} and T_H2 cells in the small intestine in host (CD45.1) and donor (CD45.2) cells; n = 3 mice. (**B**) Expression of RORγt and Gata3 by T_{regs} in colon of littermate control mice and of *Foxp3*^{Cre} x *Stat3*$^{FL/FL}$ mice. (**C**) Mean fluorescence intensity (MFI) of CD80 and CD86 on colon DCs; $n \geq 5$ mice per group. (**D**) Frequency of Gata3$^+$, RORγt$^+$, or T-bet$^+$ (a marker for T_H1 cells) non-T_{reg} CD4$^+$ T cells in littermate control mice (filled symbols) or *Foxp3*^{Cre} x *CTLA-4*$^{FL/FL}$ mice (open symbols); n = 6 mice per group. (**E**) Expression of IRF4 protein and transcripts by RORγt$^+$ T_{regs} and RORγt$^-$ T_{regs} (gray filled histogram represents effector T cells); n = 3 mice (left) or in triplicates (right). (**F**) Expression of transcripts for IL-33, IL-6, and IL-23 in the ileum of SPF and germfree mice, as determined by quantitative reverse transcriptase polymerase chain reaction; n = 3 mice per group. Data are representative of at least two independent experiments. Error bars, ±1 SD; ns, not significant; *P < 0.05 [0.06 in (A)]; **P < 0.01, as calculated by Student's t test or Mann-Whitney test.

4 (IRF4) (Fig. 4E), which endows T_{regs} with the ability to suppress T_H2 responses (*39*).

Type 2 responses are proposed to perform "housekeeping" repair functions co-opted for defense against large parasites (*40*). In germfree mice, type 2 immunity is exacerbated (*3*–*6*), possibly as a consequence of deregulated repair responses. In accordance with this view, expression of the type 2 cytokine IL-33 by epithelial cells is increased in germfree mice (Fig. 4F and fig. S11C). IL-33 promotes the accumulation and function of microbiota-independent (fig. S3) Gata3$^+$ T_{regs}, which express high levels of amphiregulin, an epidermal growth factor receptor ligand involved in tissue repair (*20*). In contrast, the microbiota induces type 3 responses through cytokines such as IL-6 and IL-23 (Fig. 4F) and thereby suppresses the default type 2 responses (Fig. 3 and fig. S13).

A model of the immune system may therefore be proposed in which type 1, 2, and 3 responses, induced by intracellular threats, tissue injury, and extracellular threats, respectively, establish a healthy equilibrium. In that model, T_{reg} subsets are part of each type of responses and play an essential role in balancing the number of effectors that are generated during steady state, infection, or injury. As we have evolved and developed in the presence of microbes, an absence of microbes leads to a loss in type 1 (*41*) and type 3 responses and, therefore, to deregulated type 2 responses associated with profibrotic and proallergic pathologies (*42*). A similar mechanism may account for the increase, in industrialized nations, of autoimmune pathologies associated with type 3 immunity (*1*).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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MUCOSAL IMMUNOLOGY

Individual intestinal symbionts induce a distinct population of RORγ$^+$ regulatory T cells

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T regulatory cells that express the transcription factor Foxp3 (Foxp3$^+$ T_{regs}) promote tissue homeostasis in several settings. We now report that symbiotic members of the human gut microbiota induce a distinct T_{reg} population in the mouse colon, which constrains immunoinflammatory responses. This induction—which we find to map to a broad, but specific, array of individual bacterial species—requires the transcription factor Rorγ, paradoxically, in that Rorγ is thought to antagonize FoxP3 and to promote T helper 17 (T_H17) cell differentiation. Rorγ's transcriptional footprint differs in colonic T_{regs} and T_H17 cells and controls important effector molecules. Rorγ, and the T_{regs} that express it, contribute substantially to regulating colonic T_H1/T_H17 inflammation. Thus, the marked context-specificity of Rorγ results in very different outcomes even in closely related cell types.

FoxP3 regulatory T (Foxp3$^+$ T_{reg}) cells are essential regulators of immunologic homeostasis and responses (*1*). Beyond their well-described role in regulating the activity of other immunocytes, T_{regs} located in parenchymal tissues control other, nonimmunological, processes. These "tissue T_{regs}" include those that reside in visceral adipose tissue and regulate metabolic parameters (*2*, *3*) and those that help channel inflammatory and regenerative events in injured muscle (*4*). The activities, transcriptomes, and T cell receptor (TCR) repertoires of these tissue T_{regs} are distinct from their counterparts in secondary lymphoid organs.

Another essential and specific population of tissue T_{regs} resides in the lamina propria (LP) of the digestive tract, in particular in the colon, where these cells modulate responses to commensal microbes [reviewed in (*5*)]. Colonic T_{regs} are an unusual population that has provoked some contradictory observations. TCRs expressed by colonic T_{regs} show marked reactivity against microbial antigens that seem to be important drivers of their differentiation and/or expansion (*6*, *7*). Many of them appear to arise by conversion from FoxP3$^-$ conventional CD4$^+$ T cells (T_{conv}) (*6*, *7*), although arguments for a

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