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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. While it is unclear how the microbiota regulates type 2 immunity, it is a strong inducer of pro-inflammatory T helper (Th) 17 cells and regulatory T cells (Tregs) in the intestine. Here, we report that microbiota-induced Tregs express the nuclear hormone receptor RORγt and differentiate along a pathway that also leads to Th17 cells. In the absence of RORγt$^+$ Tregs, Th2-driven defense against helminths is more efficient while Th2-associated pathology is exacerbated. Thus, the microbiota regulates type 2 responses through the induction of "type 3" RORγt$^+$ Tregs and Th17 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 (Th)2 cells and innate lymphoid cells (ILC)2, has not been documented. In contrast, symbionts are necessary for the differentiation of Th17 cells that produce interleukin (IL)-17 and IL-22 (*10*), cytokines involved in homeostasis and defense of mucosal surfaces, as well as of a subset of intestinal Tregs (*11*). Intriguingly, the absence of extrathymically generated Tregs leads to spontaneous type 2 pathologies at mucosal sites (*12*). As intestinal Tregs recognize bacterial antigens (*11*), the microbiota may regulate type 2 responses through the induction of extrathymically generated Tregs.

The nuclear hormone receptor RORγt is a key transcription factor for the differentiation of Th17 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 Treg marker FoxP3 (a transcription factor) and has regulatory functions (*15*). Furthermore, the generation of such RORγt$^+$ Tregs 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 Tregs expressed RORγt (Fig. 1A). The frequency of RORγt$^+$ Tregs increased with age, representing most intestinal Tregs 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 Tregs (*17*, *18*), in contrast to "conventional" RORγt$^-$ Tregs (Fig. 1B and fig. S1C). In the colon, most Helios$^-$ Tregs were absent in RORγt-deficient mice (Fig. 1B). RORγt$^+$ Tregs also expressed an activated CD44^{high} CD62L^{low} phenotype, as well as increased levels of ICOS, CTLA-4, and of the

nucleotidases CD39 and CD73 (fig. S1C), altogether indicating robust regulatory functions. Another major subset of intestinal Tregs expresses Gata3, responds to IL-33, and is involved in the regulation of effector T cells during inflammation (*19*, *20*). Gata3$^+$ Tregs were distinct from RORγt$^+$ Tregs, expressed Helios, as well as lower levels of IL-10 (fig. S2).

RORγt$^+$ Tregs were profoundly reduced in germfree or antibiotic-treated mice, whereas Helios$^+$ and Gata3$^+$ Tregs were unaffected (Fig. 1C and fig. S3). Recolonization of germfree mice with a specific pathogen free (SPF) microbiota restored normal numbers of RORγt$^+$ Tregs (fig. S4). Furthermore, a consortium of symbionts composed of 17 *Clostridia* species efficiently induces the generation of Tregs 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 Tregs through short chain fatty acids (SCFA) (*22*, *23*) and antigen (*11*). We found that the SCFA butyrate induced an increase mainly in RORγt$^+$ Tregs (fig S5). The generation of RORγt$^+$ Tregs was dependent on dendritic cells 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 (TCR) specific for that antigen (Fig. 1F). Altogether, these data show that RORγt$^+$ Tregs are induced by microbiota and oral antigen, and that microbiota-induced intestinal Tregs express RORγt.

Th17 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$^+$ Tregs differentiated following similar pathways. RORγt$^+$ Tregs were efficiently induced by SFB in the small intestine, even though more Th17 cells were induced in these conditions (Fig. 2A). Furthermore, similar to Th17 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 Th17 cells (*27*, *28*), developed significantly less RORγt$^+$ Tregs (Fig. 2B), whereas Gata3$^+$ Tregs 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 Tregs or RORγt$^+$ cells (Fig. 2B).

If Th17 cells and RORγt$^+$ Tregs are induced through similar pathways, how then is the development to Th17 cells or RORγt$^+$ Tregs regulated? In cell cultures, the vitamin A metabolite retinoic acid (RA) promotes the generation of Tregs (*29*) and of RORγt$^+$ Tregs (*15*) rather than of Th17 cells. We now find that feeding mice with vitamin A-deficient food, or treating mice with an inhibitor of the RA receptor (RARi), prevented the development of RORγt$^+$ Tregs, but not of Helios$^+$ Tregs or Th17 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$^+$ Tregs over Th17 cells, presumably to limit the number of pro-inflammatory cells present in the healthy intestine.

We next assessed whether RORγt$^+$ Tregs regulate type 2 responses. Mice that lack only RORγt$^+$ Tregs were generated through a conditional knock-out of *Rorc* (which encodes RORγt) in Foxp3$^+$ cells. Such *Foxp3*^{Cre} x *Rorc(γt)*^{FL} mice developed increased frequencies of Gata3$^+$ T cells and Gata3$^+$ Tregs (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 to their wild-type littermates (Fig. 3B). In contrast, they were more resistant to infection by the helminth *Heligmosomoides polygyrus*, as they produced higher levels of IL-4, IL-5 and IL-13 during the infection (Fig. 3C). Th17 cells contributed to the control of type 2 responses, as a more pronounced increase in Th2 cells was observed in full RORγt-deficient mice (fig. S9B). Furthermore, RORγt-deficient mice expressed high levels of 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 Th17 or Th1 responses, even during acute intestinal inflammation induced by sodium dextran sulfate (DSS) (fig. S10). These data are in agreement with the spontaneous type 2 pathologies observed in mice lacking extrathymically generated Tregs (*12*), and indicate that microbiota regulate type 2 responses through RORγt$^+$ Tregs, and more generally RORγt$^+$ T cells.

What are the mechanisms by which RORγt$^+$ Tregs 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$^+$ Tregs when transferred into mice fed ovalbumin (Fig. 1F), whereas a majority of them developed into Gata3$^+$ Tregs when cells deficient in RORγt were transferred (Fig. 4A and fig. S11A). However, host Tregs were not affected, showing that a loss in RORγt affects Tregs only through a cell-intrinsic pathway. In contrast, the transfer of RORγt-deficient OT-II$^+$ cells affected both the generation of donor and host Th2 cells, but not Th17 cells, showing that RORγt$^+$ Tregs regulate Th2 cells also through a cell-extrinsic pathway. Furthermore, in RORγt$^+$ Tregs that lacked Stat3, the expression of Gata3 was deregulated and thus both transcription factors were co-expressed (Fig. 4B). This is in accordance with earlier data showing that IL-23 blocks the IL-33-mediated accumulation of Gata3$^+$ Tregs (*20*), and conversely, that the absence of Gata3 leads to the expansion of RORγt$^+$ Tregs (*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 Th17 cells (fig. S11B).

We next investigated the mechanisms of the cell-extrinsic regulation of Th2 cells by RORγt$^+$ Tregs. As RORγt$^+$ Tregs express high levels of IL-10 (Fig. 1D and fig. S2B) (*15*), we assessed whether RORγt$^+$ Tregs regulate Th2 cells through IL-10, the receptor of which activates Stat3 (*37*). However, IL-10-deficient mice showed massive expansion of Th17 cells but no expansion of Gata3$^+$ T cells (fig. S12). RORγt$^+$ Tregs 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 Tregs, Gata3$^+$ T cells were significantly expanded in the intestine, whereas Th1 and Th17 cells were not (Fig. 4D), and serum levels of IgE are increased (*38*). These data indicate that RORγt$^+$ Tregs regulate co-activator functions of DCs through CTLA4, and thereby regulate the generation of Th2 cells in the intestine. Finally, RORγt$^+$ Tregs express high levels of IRF4 (Fig. 4E), which endows Tregs with the ability to suppress Th2 responses (*39*).

Type 2 responses are proposed to perform "house keeping" 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$^+$ Tregs, which express high levels of amphiregulin, an EGFR 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 where type 1, 2 and 3 responses, induced by intracellular threats, tissue injury, and extracellular threats, respectively, establish a healthy equilibrium. In that model, Treg 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 develop 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 pro-fibrotic and pro-allergic 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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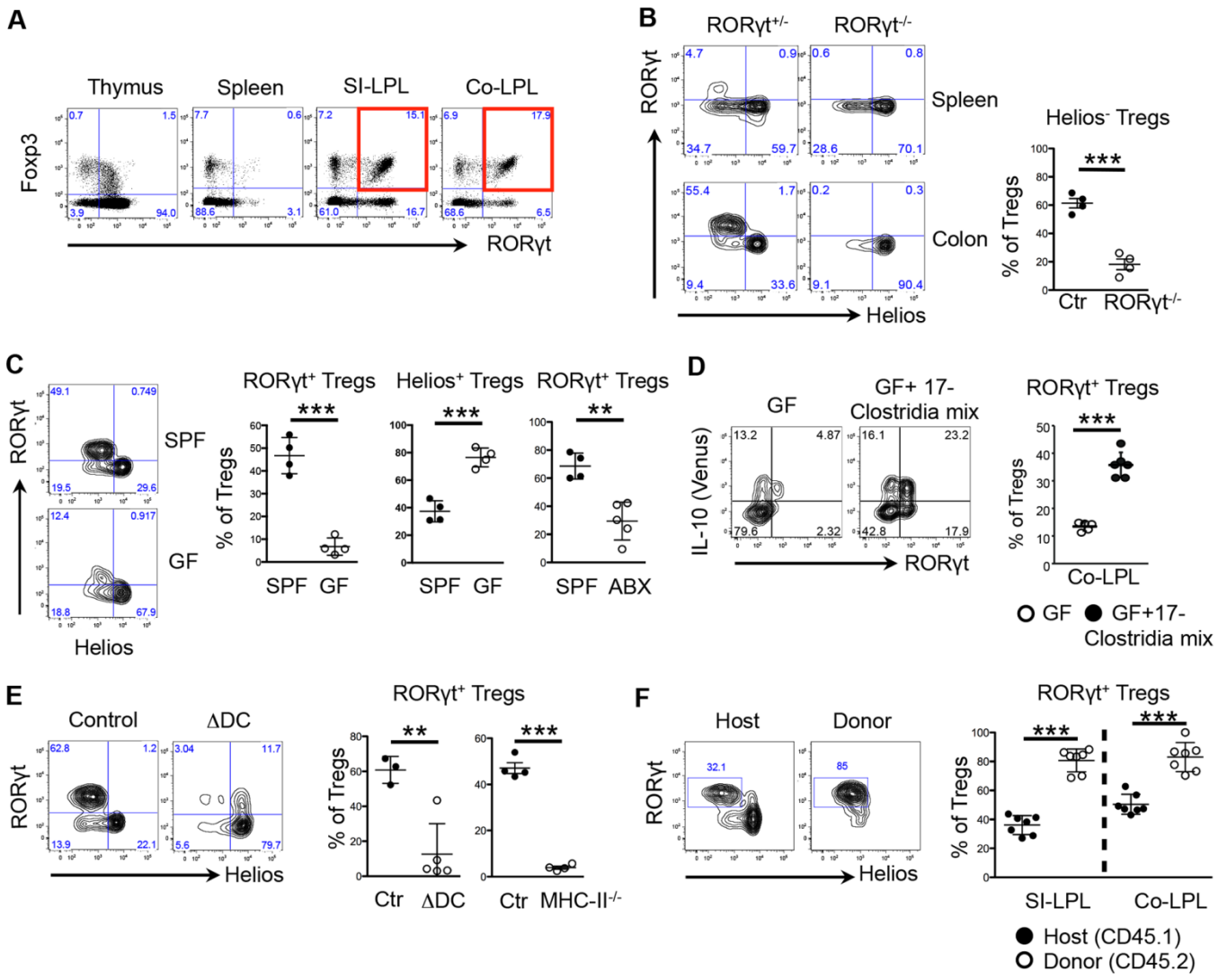

Fig. 1. A majority of colon RORγ⁺ T cells are microbiota-induced RORγt⁺ Tregs. (**A**) Expression of Foxp3 and RORγt by CD4⁺ T cells in double reporter mice. SI, small intestine; Co, colon; LPL, lamina propria lymphocytes. (**B**) Expression of Helios and RORγt by Tregs (FoxP3⁺ CD4⁺ T cells) from spleen and colon lamina propria of littermate control (left) and RORγt-deficient mice (right). Right, frequency of colon Helios⁻ Tregs. *n* = 4. (**C**) Expression of RORγt and Helios by Tregs in the colon of SPF or germfree (GF) mice, and frequencies of RORγt⁺ Helios⁻ Tregs (left) and Helios⁺ Tregs (middle) in SPF and germfree mice, *n* = 4. Right, frequency of RORγt⁺ Tregs in the colon of SPF mice or mice treated from birth with a cocktail of antibiotics, *n* ≥ 4. (**D**) Expression of RORγt and IL-10 by Tregs, and frequency of RORγt⁺ Tregs in the colon of germfree mice (left) or mice recolonized for 3 weeks with a consortium of 17 strains of *Clostridia* (right), *n* ≥ 4. (**E**) Expression of RORγt and Helios by colonic Tregs (left) and frequency of RORγt⁺ Tregs (middle) in *Cd11c*^{Cre} x *Rosa26*^{Dta} (ΔDC) and littermate control mice, n ≥ 3. Right, frequencies of RORγt⁺ Tregs 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, subsequently fed for 7 days with 1.5% chicken ovalbumin in the drinking water. Expression of RORγt and Helios in small intestine Tregs (left) and frequency of RORγt⁺ Tregs in small intestine and colon (right) in host (CD45.1) and donor (CD45.2) cells, *n* = 5. Data are representative of at least two independent experiments. Error bars, s.d.; ns, not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, as calculated by Student's *t* test.

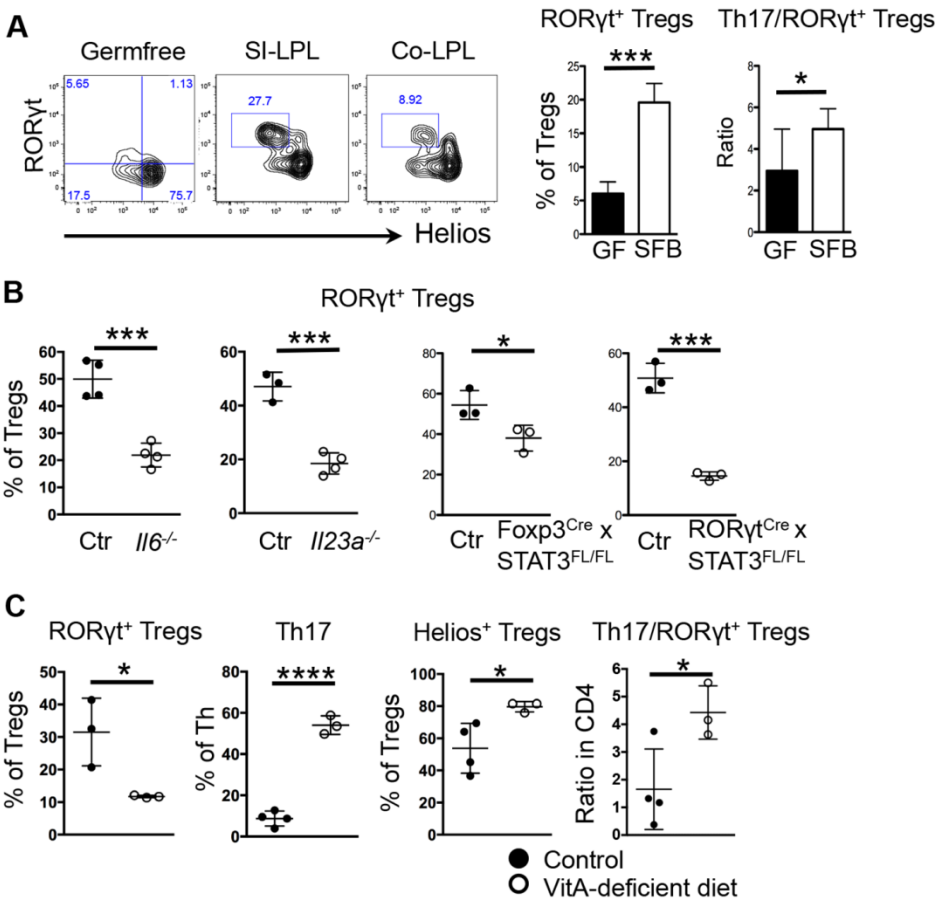

Fig. 2. A "Pro-inflammatory" pathway plus retinoic acid induce RORγt⁺ Tregs. (**A**) Expression of RORγt and Helios by Tregs in germfree mice and germfree mice recolonized for 3 weeks with SFB. Histograms show frequencies of RORγt⁺ Tregs and ratio of Th17 cells versus RORγt⁺ Tregs. $n \geq 4$. (**B**) Frequencies of (Helios⁻) RORγt⁺ Tregs in colon of *Il6*$^{-/-}$, *Il23a*$^{-/-}$, *Foxp3*^{Cre} x *Stat3*$^{Fl/Fl}$, *RORγt* ^{Cre} x *Stat3*$^{Fl/Fl}$ and littermate control mice, $n \geq 3$. (**C**) Frequencies of RORγt⁺ Tregs, Th17 cells, Helios⁺ Tregs, and ratio of Th17 cells versus RORγt⁺ Tregs in colon of mice fed a control diet or a vitamin A-deficient from birth for 10 weeks, $n \geq 3$. Data are representative of at least two independent experiments. Error bars, s.d.; $^{*}P < 0.05$, $^{***}P < 0.001$, as calculated by Student's *t* test.

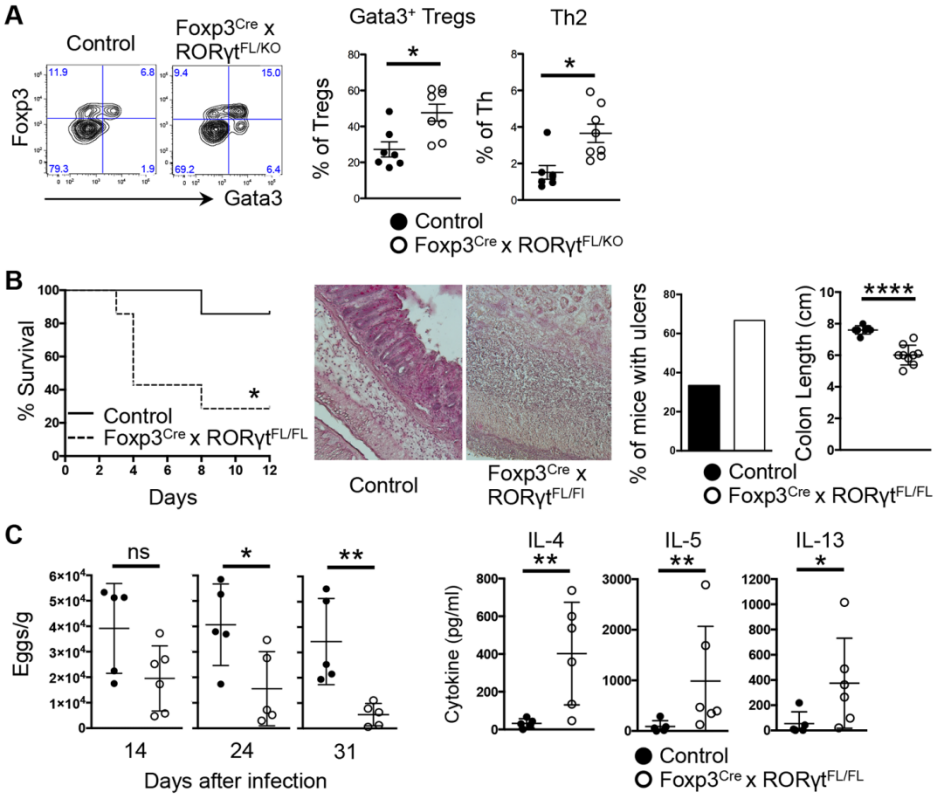

Fig. 3. RORγt⁺ Tregs regulate type 2 immune responses. (**A**) Expression of Foxp3 and Gata3 by small intestine CD4⁺ T cells (left), and frequency of Gata3⁺ Tregs (middle) and Gata3⁺ non-Tregs CD4⁺ T cells (right) in *Foxp3*^{Cre} x *Rorc(γt)*$^{Fl/KO}$ mice, $n = 7$. (**B**) Survival curve (left) of littermate control mice (straight line) or *Foxp3*^{Cre} x *Rorc(γt)*$^{Fl/Fl}$ mice (hatched line) treated with oxazolone, $n = 7$. 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), $n \geq 5$. Data are representative of at least two independent experiments. Error bars, s.d.; ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, as calculated by Student's *t* test or Mann-Whitney test.

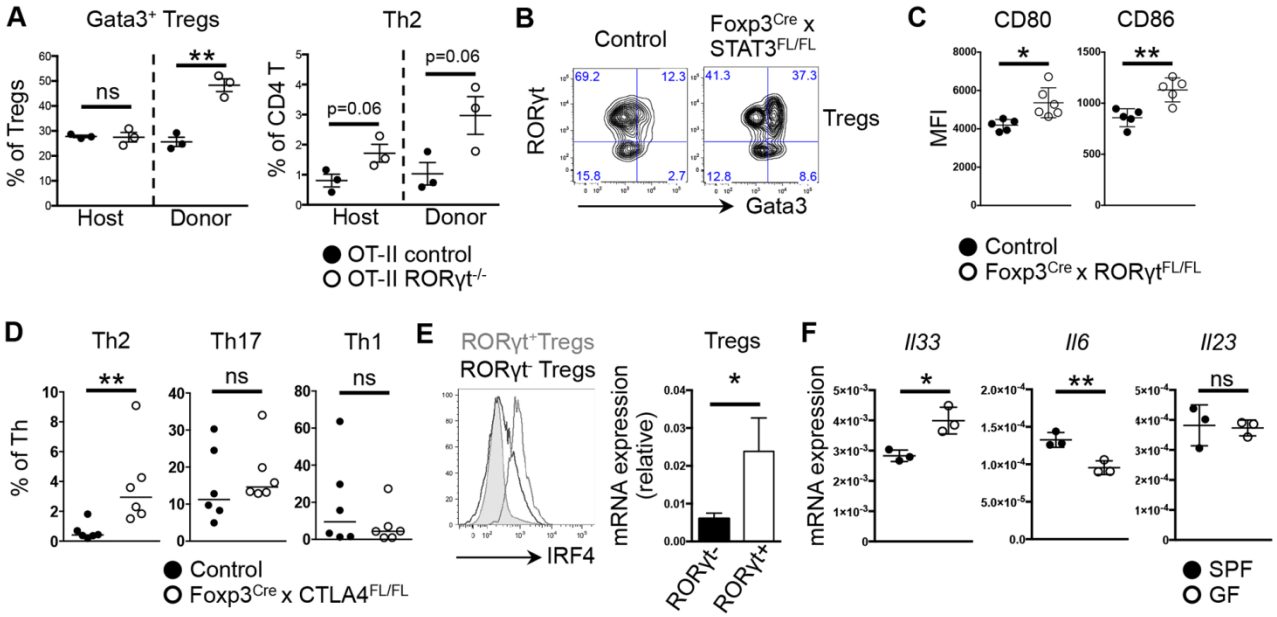

Fig. 4. Mechanisms of regulation by RORγt⁺ Tregs. (**A**) Naïve CD4⁺ T cells were isolated from CD45.2⁺ OT-II wild-type or CD45.2⁺ OT-II RORγt $^{-/-}$ mice and adoptively transferred into CD45.1⁺ congenic mice, subsequently fed for 7 days with 1.5% chicken ovalbumin. Frequency of Gata3⁺ Tregs and Th2 cells in the small intestine in host (CD45.1) and donor (CD45.2) cells, $n = 3$. (**B**) Expression of RORγt and Gata3 by Tregs 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, $n \geq 5$. (**D**) Frequency of Gata3⁺, RORγt⁺ or T-bet⁺ (a marker for Th1 cells) non-Treg CD4⁺ T cells in littermate control mice (filled symbols) or *Foxp3*^{Cre} x *CTLA-4*$^{Fl/Fl}$ mice (open symbols), $n = 6$. (**E**) Expression of IRF4 protein and transcripts by RORγt⁺ Tregs and RORγt⁻ Tregs (grey filled histogram represents effector T cells), $n = 3$. (**F**) Expression of transcripts for IL33, IL6 and IL23 in the ileum of SPF and germfree mice, as determined by qRT-PCR, $n = 3$. Data are representative of at least two independent experiments. Error bars, s.d.; ns, not significant, $^{*}P < 0.05$ (0.06 in A), $^{**}P < 0.01$, as calculated by Student's *t* test or Mann-Whitney test.