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Induction of Colonic Regulatory T Cells by Indigenous *Clostridium* Species

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Abstract

CD4$^+$ T regulatory cells (T_{regs}), which express the Foxp3 transcription factor, play a critical role in the maintenance of immune homeostasis. Here, we show that in mice, T_{regs} were most abundant in the colonic mucosa. The spore-forming component of indigenous intestinal microbiota, particularly clusters IV and XIVa of the genus *Clostridium*, promoted T_{reg} cell accumulation. Colonization of mice by a defined mix of *Clostridium* strains provided an environment rich in transforming growth factor–β and affected Foxp3$^+$ T_{reg} number and function in the colon. Oral

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Supporting Online Material
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inoculation of *Clostridium* during the early life of conventionally reared mice resulted in resistance to colitis and systemic immunoglobulin E responses in adult mice, suggesting a new therapeutic approach to autoimmunity and allergy.

The mammalian gastrointestinal tract harbors numerous species of commensal bacteria that constitute the "microbiota." The microbiota interacts with the host immune system, inducing the accumulation of several different lymphocyte populations at mucosal sites (1, 2). Recent reports have suggested that the induction of each lymphocyte subset may be regulated by a distinct component of the microbiota. For instance, segmented filamentous bacteria (SFB) strongly induce intestinal T helper 17 (T_H17) cells, which play a role in host resistance against intestinal pathogens and promote systemic autoimmunity (3–5).

$CD4^+$ regulatory T cells (T_{regs}) expressing the transcription factor forkhead box P3 (Foxp3) are present at higher frequencies in the gut lamina propria (LP), particularly in the colon, than in other organs (6) (fig. S1). It has been postulated that the number and function of mucosal T_{regs} are affected by the presence of intestinal bacteria. Indeed, daily treatment of mice with probiotic strains of bifidobacteria and lactobacilli modifies the inflammatory status of mice, presumably by inducing T_{regs} (7–9). Furthermore, colonization of mice with human commensal *Bacteroides fragilis* facilitates T_{reg} differentiation and interleukin-10 (IL-10) production (10). Given the importance of the community structure of "indigenous" microbial flora in the maintenance of intestinal homeostasis, and that its alteration (dysbiosis) correlates with inflammatory diseases (11), it is important to further investigate whether, and how, indigenous microflora affect the number and function of mucosal T_{regs}.

We first examined the appearance of T_{regs} during mouse ontogeny. The frequency of $Foxp3^+$ T_{regs} in colonic and small intestinal (SI) LP increased after weaning, whereas in inguinal lymph nodes (iLNs) it remained stable from the second week after birth (Fig. 1A). This temporal accumulation of intestinal T_{regs} suggested an influence of the intestinal microbiota. Therefore, we next examined germ-free (GF) mice. The percentage and absolute number of $Foxp3^+CD4^+$ T cells in SI, iLNs, Peyer's patches, and mesenteric LNs were unchanged, or increased, in GF mice and antibiotic-treated specific pathogen-free (SPF) mice compared with untreated SPF mice (Fig. 1B and fig. S2). These findings are consistent with previous observations that GF mice have increased or unchanged numbers of T_{regs} in SI (12, 13). In contrast, a significant decrease in the number of $Foxp3^+$ T_{regs} was observed in the colonic LP of GF mice or antibiotic-treated mice compared with SPF mice (Fig. 1B and fig. S2). This decrease may be attributed to the absence of specific signaling events induced by intestinal microbes rather than to a defect in the development of gut-associated lymphoid tissues (fig. S3). Indeed, when GF mice were colonized with fecal suspensions from SPF mice ("conventionalization"), a marked increase in the frequency of T_{regs} was observed in colonic LP (Fig. 1C). Therefore, we conclude that interactions between indigenous microflora and the host play a critical role in the accumulation of colonic LP, but not SI LP, $Foxp3^+$ T_{regs}.

To determine whether a specific component of the intestinal flora induces colonic T_{reg} accumulation, we treated SPF mice with antibiotics that preferentially target Gram-positive (vancomycin) or Gram-negative (polymyxin B) bacteria (fig. S4). Compared with the controls, only mice treated with vancomycin had significantly lower frequencies of T_{regs} in the colon (Fig. 1D), suggesting a dominant role for Gram-positive commensal bacteria in T_{reg} accumulation. We next orally inoculated GF mice with 3% chloroform-resistant fecal microorganisms (spore-forming fraction) because this fraction has been shown to regulate intestinal T cell responses (4). Mice inoculated with chloroform-treated feces showed an increased number of T_{regs} (Fig. 1E) comparable to that in SPF mice or GF mice gavaged with untreated feces (Fig. 1, B and C). Collectively, our results suggest that a specific

component of the indigenous microbiota, belonging most likely to the Gram-positive, spore-forming fraction, plays a critical role in the induction of colonic T_{regs}.

Clostridia are one of the most prominent Gram-positive and spore-forming bacteria indigenous to the murine gastrointestinal tract (14). Moreover, *Clostridium* clusters IV and XIVa (also known as *Clostridium leptum* and *coccoides* groups, respectively) have been implicated in the maintenance of mucosal homeostasis and prevention of inflammatory bowel disease (IBD) (15, 16). We therefore explored the link between clostridia and the accumulation of colonic T_{regs}. Clostridia became prominent after weaning and persisted in the adult animals, in contrast to *Lactobacillus* or *Enterobacteriaceae*, which were more abundant during the neonatal period and declined thereafter (fig. S5). Furthermore, *Clostridium* clusters IV and XIVa were most abundant in the cecum and proximal colon, which correlated well with the distribution of T_{regs} (fig. S6).

To directly examine the effect of *Clostridium* on the induction of colonic T_{regs}, we generated "gnotobiotic" mice by colonizing GF mice with fecal material obtained from mice colonized with a cocktail of 46 strains of *Clostridium* (17). *Clostridium*-colonized gnotobiotic mice exhibited a robust accumulation of T_{regs} in colonic LP; however, the frequency of SI T_{regs} was not affected (Fig. 2A and fig. S7, A and B). The 46 strains of *Clostridium* used were originally isolated from chloroform-treated fecal material from conventionally reared mice (17). These strains primarily belong to clusters IV and XIVa (14) (see also fig. S8). They are normally present in the intestine of several colonies of commercially available SPF mice and in that of mice housed in our own animal facility (fig. S9A). They mainly colonized the cecum and proximal colon (fig. S9B) and formed a thick layer on the mucosal epithelium (Fig. 2B). The induction of colonic T_{regs} was specific to *Clostridium*-colonized mice. Colonization with a cocktail of three strains of *Lactobacillus*, or with SFB, had little effect on the number of colonic T_{regs} (Fig. 2A and fig. S7, A and B). Furthermore, colonization by *B. fragilis*, which is reported to induce IL-10 production in T_{regs} (10), did not significantly affect the frequency of colonic T_{regs} (fig. S7C). A cocktail of 16 strains of *Bacteroides* spp. isolated from the murine intestine did induce a significant increase in the number of T_{regs} in the colon, although the magnitude was dependent on the background of the mice and was less than that seen in *Clostridium*-colonized mice (Fig. 2A and fig. S7, A and B).

A substantial fraction of Foxp3$^+$ T_{regs} observed in SPF or *Clostridium*-colonized mice was negative for Helios, a transcription factor reported to be expressed in thymus-derived natural T_{regs} (18), suggesting that many of these T_{regs} could be "induced T_{regs}" (iT_{regs}) (Fig. 2C and fig. S10A). A high number of T_{regs} was maintained for at least 4 months after colonization (fig. S10B). Furthermore, *Clostridium*-mediated T_{reg} induction is vertically and horizontally transmissible (fig. S10C). *Clostridium* did not affect T_H1 cells but moderately induced T_H17 cells in the colon (fig. S10D). The 46 strains of *Clostridium* have been reported to affect the accumulation of CD8$^+$IELs (intraepithelial lymphocytes) in the colon (19). Therefore, *Clostridium* may modulate various aspects of the immune system.

Considering that transforming growth factor–β (TGF-β) is a critical regulator of T_{reg} development, we examined whether *Clostridium* colonization provides a TGF-β–rich environment within the colon. To this end, we cultured whole colons or colonic intestinal epithelial cells (IECs) from GF mice, or mice colonized by either *Clostridium* or *Lactobacillus*, and found a significant increase in the production of active-form TGF-β in the colon and IECs from *Clostridium*-colonized mice (Fig. 2, D and E). Addition of the culture supernatant of IECs from *Clostridium*-colonized mice to splenic CD4$^+$ T cells markedly enhanced the differentiation of Foxp3-expressing cells, and this differentiation was significantly inhibited by an antibody against TGF-β (Fig. 2F). Consistent with the production of TGF-β, transcripts for the genes encoding matrix metalloproteinase 2

(MMP2), MMP9, and MMP13, which have been reported to be involved in the activation of latent TGF-β (20), were expressed at higher levels by IECs from *Clostridium*-colonized mice than those from GF or *Lactobacillus*-colonized mice (fig. S11). IECs from *Clostridium*-colonized mice also expressed a high level of indoleamine 2,3-dioxygenase (IDO) (fig. S11), which has been implicated in the induction of T_{regs} (21). Collectively, our findings suggest that clostridia activate IECs to produce TGF-β and other T_{reg}-inducing molecules within the colon. We also examined the contribution of bacterial pattern-recognition receptors (PRRs) to the induction of T_{regs} by *Clostridium*. SPF mice deficient for *Myd88* (a signaling adaptor molecule for Toll-like receptors), *Rip2* (an adaptor molecule for NOD receptors), or *Card9* (a key transducer of Dectin-1 signaling) had a normal number of mucosal T_{regs}, compared to each of their littermate controls (fig. S12A). Furthermore, the colonization of GF *Myd88*-deficient mice with *Clostridium* induced robust accumulation of colonic LP T_{regs} (fig. S12B). These results indicate that clostridia induce T_{regs} independently of these PRR signaling pathways.

Intestinal $Foxp3^+$ T_{regs} exert their immunosuppressive activity, at least in part, through IL-10 production (22, 23). To further probe the nature of T_{regs} induced by *Clostridium*, we newly generated IL-10 reporter mice, in which a cassette containing an internal ribosomal entry site (IRES) and Venus, a brighter version of yellow fluorescent protein, was inserted immediately before the polyadenylation signal of the *Il10* gene (referred to as $Il10^{Venus}$ mice; fig. S13). In SPF $Il10^{Venus}$ mice, about 60% of $Foxp3^+$ T_{regs} in colonic LP were $Venus^+$, whereas only about 10% of T_{regs} in spleen or other organs were $Venus^+$, consistent with previous studies (24, 25) (Fig. 3, A and B, and fig. S14). Under GF conditions or after antibiotic treatment, the frequency and number of $Venus^+$ cells within the $CD4^+Foxp3^+$ T_{reg} population were significantly lower than those seen under SPF conditions (Fig. 3, A to C, and fig. S15). In contrast, no significant change in the frequency of any regulatory cell components within the $CD4^+$ T cell population in SI LP was observed in the absence, or reduction, of commensal bacteria (fig. S15). These results indicate that the indigenous microflora provide a signal for the accumulation of IL-10^+ T_{regs} in colonic LP, whereas different mechanisms are operating in the induction of $Foxp3^+$- and/or IL-10^+–regulatory cells in SI.

To determine the effect of *Clostridium* on IL-10 expression in colonic T_{regs}, we examined gnotobiotic $Il10^{Venus}$ mice colonized with 46 strains of *Clostridium*. The frequency and number of $Venus^+$ cells among $Foxp3^+$ cells in $Il10^{Venus}$ mice colonized with *Clostridium* were similar to those in SPF mice (Fig. 3, A to C), indicating that clostridia are sufficient to induce IL-10–expressing $Foxp3^+$ T_{regs}. The induction of IL-10^+ T_{regs} was, again, specifically observed in mice colonized with *Clostridium* but not other bacteria (Fig. 3, B and C). $Venus^+$ $Foxp3^+$ cells in the colonic LP of *Clostridium*-colonized mice expressed high levels of cytotoxic T lymphocyte antigen 4 (CTLA4) (Fig. 3D) and exhibited an in vitro suppressive activity similar to that of $Venus^+$ cells from SPF mice (Fig. 3E). These results indicate that clostridia sufficiently induce the accumulation of functionally competent IL-10^+ $CTLA4^{high}$ T_{regs}. Many of the $Venus^+$ $Foxp3^+$ cells in the colonic LP of SPF and *Clostridium*-colonized mice were found to be negative for Helios, and this cell fraction was particularly reduced in GF mice (fig. S16). These findings suggest that clostridia induce a shift in the composition of T_{regs} in the colonic LP, particularly through accumulation of the IL-10^+ $CTLA4^{high}$ $Helios^-$ subset of T_{regs} (presumably iT_{regs}). Furthermore, 3 weeks after *Clostridium* inoculation, we observed a substantial increase in the number of $Venus^+CD4^+$ cells in the liver, lung, and spleen, where otherwise a very small number of $Venus^+$ cells can be detected (fig. S17). This finding suggests that colonization of *Clostridium* also affects the extra-intestinal immune status.

Early exposure to the environment is known to be a key determinant of adult gut microbial ecology. To affect the *Clostridium* load, we orally inoculated 2-week-old neonatal SPF mice with feces from *Clostridium*-associated mice, kept them under SPF conditions, and examined their microbial composition and T_{reg} number in adulthood. Despite similar amounts of total bacteria in the feces of the *Clostridium*-treated and -untreated groups, there was a significant increase in the amounts of *Clostridium* clusters IV and XIVa in the treated mice (fig. S18A), accompanied by a significantly higher number of colonic $Foxp3^+$ T_{regs} (Fig. 4A). We then examined the effect of abundance of *Clostridium*, and the consequent increase in the number of T_{regs}, on local and systemic immune responses. Mice were subjected to dextran sodium sulfate (DSS)–mediated colitis, a model of colitis resembling human IBD. The symptoms of colitis, such as weight loss and rectal bleeding, were significantly suppressed in *Clostridium*-abundant mice compared with control mice (Fig. 4B). Colon shortening, edema, and hemorrhage were less severe in the colon of *Clostridium*-abundant mice than in that of control mice (Fig. 4C). Consistently, *Clostridium*-abundant mice exhibited milder histological disease characteristics, such as mucosal erosion, cellular infiltration, and crypt loss, than control mice (Fig. 4D). The *Clostridium*-abundant mice were also subjected to oxazolone-induced colitis, an experimental colitis mediated by T_H2-type cells (26). *Clostridium*-abundant mice had attenuated weight loss and fewer areas of mucosal erosion, edema, cellular infiltration, and hemorrhage (fig. S18, B and C). Finally, we investigated the effect of *Clostridium* on systemic immunoglobulin E (IgE) production. Ovalbumin (OVA)–specific IgE levels in sera were measured after intraperitoneal injection of alum-absorbed OVA. *Clostridium*-abundant mice showed significantly lower IgE levels than control mice (Fig. 4E). Moreover, splenocytes from *Clostridium*-abundant mice immunized with OVA plus alum showed lower IL-4 and higher IL-10 productions after restimulation with OVA in vitro than those from control mice (Fig. 4F). Therefore, the increased the proportion of *Clostridium* in the gut microbiota affected mucosal inflammation and systemic antibody responses. Although additional mechanisms and cell types could be involved, our findings suggest that *Clostridium*-mediated induction of T_{regs} in the colon may be responsible for these effects.

Our findings show that T_{regs} are abundant in intestinal LP, and their accumulation in the SI and colon is differentially regulated. The induction of colonic T_{regs} is dependent on commensal microorganisms with specialized properties. Among the indigenous commensal bacteria, *Clostridium* spp. belonging to clusters IV and XIVa are outstanding inducers of T_{regs} in the colon. Although alternative mechanisms may also be involved, our findings are consistent with a model in which the presence of *Clostridium* induces the release of active TGF-β and other T_{reg}-inducing factors from IECs, which presumably cooperate with dendritic cells to induce a general accumulation of T_{regs} in the colon and at the same time affect the proportions of individual T_{reg} subsets through the preferential induction of $IL\text{-}10^+$ $CTLA4^{high}$ iT_{regs}. Several recent studies have focused on the microbial composition in the intestine during health and disease. Notably, *Clostridium* clusters IV and XIVa constitute a smaller proportion of the fecal community in patients with IBD than in healthy controls (15). Furthermore, some patients with IBD have a specific reduction in *Faecalibacterium prausnitzii*, a bacterium belonging to *Clostridium* cluster IV (16). These reports are consistent with our findings and raise the possibility that indigenous *Clostridium*-dependent induction of T_{regs} may be required for maintaining immune homeostasis in mice and humans. The factors derived from *Clostridium* that are required for the induction of mucosal T_{regs} are currently unknown. Because gnotobiotic mice colonized with three strains of *Clostridium* showed an intermediate pattern of T_{reg} induction between GF mice and mice inoculated with all 46 strains (fig. S19), we speculate that a diverse set of metabolites that are most efficiently produced by the 46 strains of *Clostridium* as a whole may be required for the optimal induction of T_{regs}. Identifying these metabolites and the molecular mechanisms underlying the *Clostridium*-host crosstalk will provide invaluable information

toward understanding how the gut microbiota regulates immune homeostasis and may suggest potential therapeutic options for treating human IBD and allergies.

Supplementary Material

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Acknowledgments

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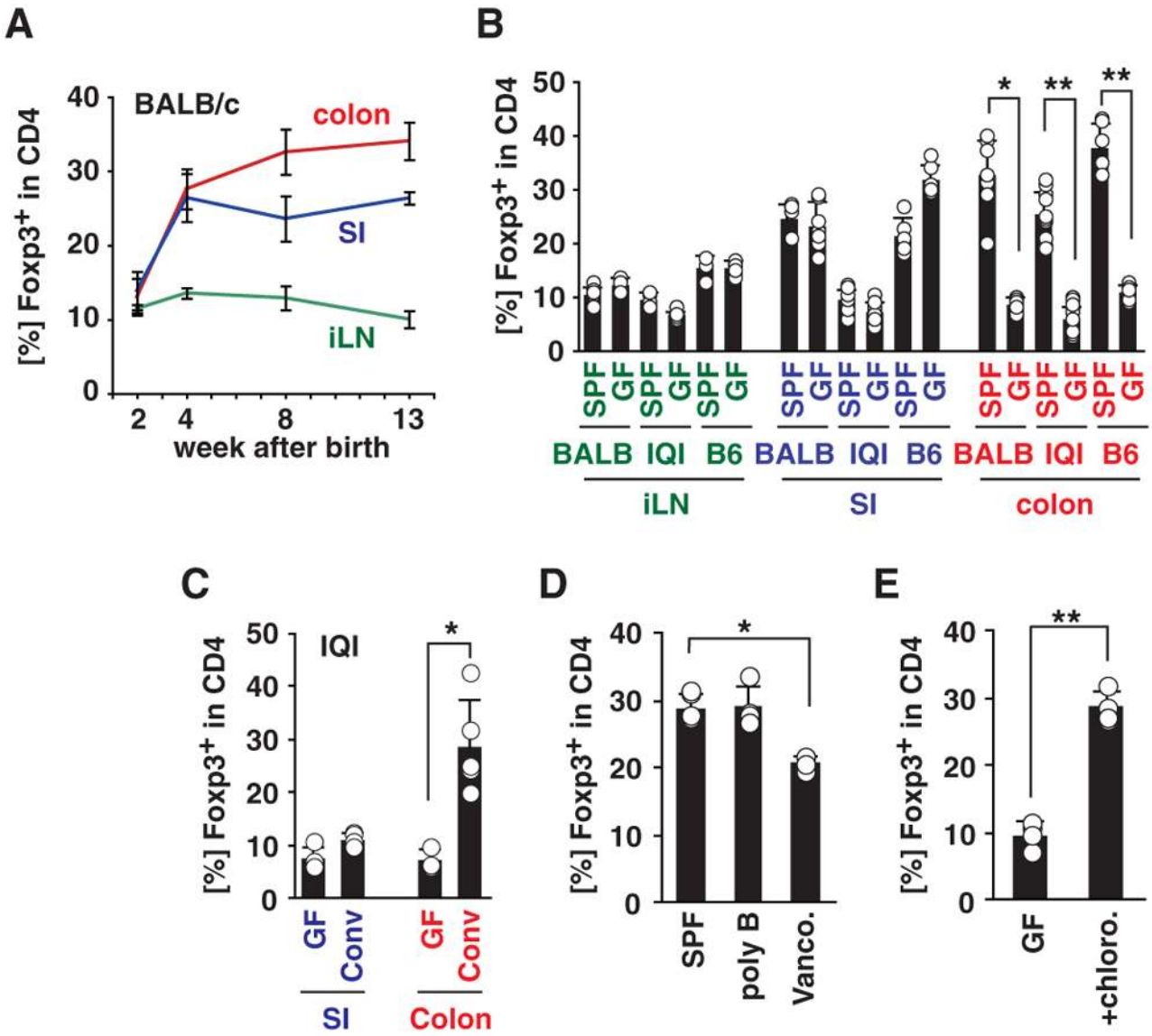

Fig. 1.

Indigenous intestinal bacteria-dependent accumulation of colonic T_{regs}. (**A**) The percentage of Foxp3$^+$ cells within the CD4$^+$ cell population isolated from iLNs or LP of colon or SI of SPF BALB/c mice at the indicated age was analyzed by flow cytometry. (**B**) Lymphocytes from SI, colon, and iLN of 8-week-old BALB/c, IQI and C57BL/6 (B6) GF, and SPF mice were analyzed for CD4 and Foxp3 expression. (**C**) GF IQI mice were conventionalized (Conv) by oral administration of the fecal suspension from B6 SPF mice purchased from Jackson Laboratory. Colonic LP lymphocytes were isolated 3 weeks later and analyzed for Foxp3 expression. (**D**) Four-week-old SPF B6 mice were treated with polymyxin B (poly B) or vancomycin (Vanco) for 4 weeks and analyzed for the percentage of Foxp3$^+$ cells within the CD4$^+$ cell population. (**E**) GF mice were gavaged with chloroform-treated feces from SPF mice (+chloro) and analyzed for the percentage of Foxp3$^+$ cells within the CD4$^+$ cell population. Each circle in (B) to (E) represents an individual mouse, and error bars indicate the SD. Data were obtained from more than two independent experiments with similar results ($n \geq 4$ mice per group). *$P < 0.01$; **$P < 0.001$, unpaired t test.

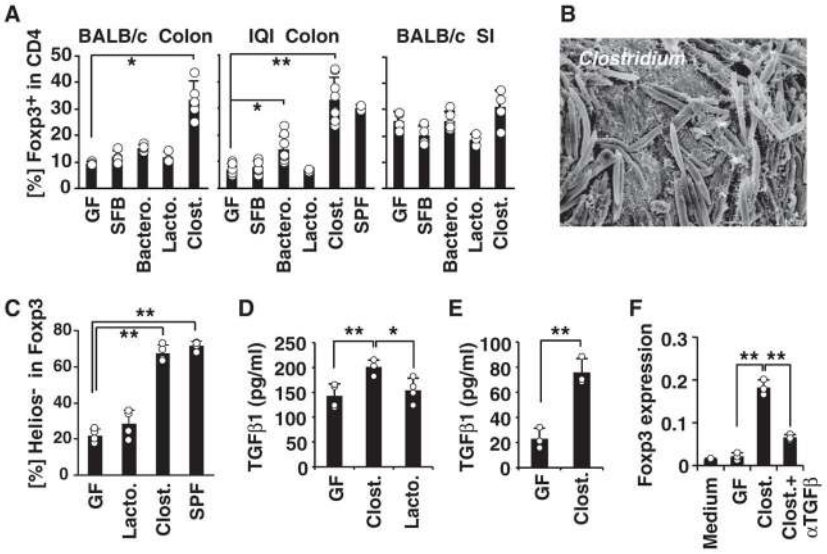

Fig. 2.

Clostridia induce T_{reg} accumulation in colonic LP. (**A**) GF BALB/c or IQI mice were colonized with segmented filamentous bacteria (SFB), 16 strains of *Bacteroides* (Bactero.), 3 strains of *Lactobacillus* (Lacto.), or 46 strains of *Clostridium* (Clost.) for 3 weeks. The percentage of $Foxp3^+$ cells within the $CD4^+$ cell population in the colon and SI of individual mice was analyzed by flow cytometry ($n \geq 5$ mice per group). (**B**) Electron micrograph showing the proximal colon of Clost.-colonized B6 mice. (**C**) Lymphocytes from the colonic LP of indicated mice were analyzed for the expression of CD4, Foxp3, and Helios by flow cytometry. The percentage of $Helios^-$ cells within $Foxp3^+CD4^+$ lymphocytes is shown (n = 4 mice per group). (**D** and **E**) Whole colons (D) or IECs (E) from GF, Lacto.-, or Clost.-colonized mice were cultured for 24 hours. The concentration of TGF-β1 in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA). (**F**) Splenic $CD4^+$ T cells were cultured with antibodies against CD3 and CD28 and 50% conditioned medium from cultured IECs isolated from GF or Clost.-colonized mice in the presence or absence of anti–TGF-β. After 5 days, T cells were collected and assayed for Foxp3 expression by real-time reverse transcription–polymerase chain reaction. Each circle represents a mouse or a sample, and error bars indicate the SD ($n \geq 3$ per group). $^*P < 0.02$; $^{**}P < 0.001$, unpaired t test. Data are representative of at least two independent experiments with similar results.

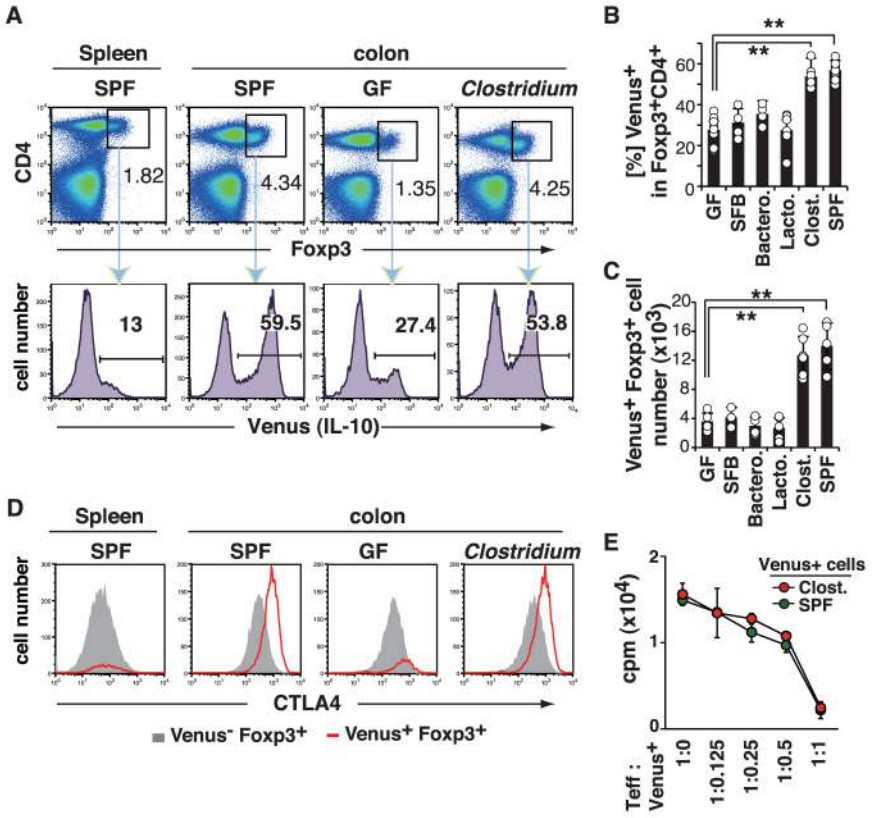

Fig. 3.

Clostridia induce IL-10–expressing T_{regs} in the colon. (**A** to **D**) Splenic and colonic LP lymphocytes from SPF, GF, or *Clostridium*-colonized *Il10*^{Venus} mice were analyzed for expression of CD4, Foxp3, Venus, and CTLA4 by flow cytometry. Representative dot-plots and histograms are shown in (A) and (D), and the percentage and absolute number of Venus$^+$ cells within the CD4$^+$Foxp3$^+$ cell population in individual mice are shown in (B) and (C). Each symbol represents a mouse, and error bars indicate the SD. Data are representative of at least two independent experiments with similar results ($n \geq 5$ mice per group). $^{**}P < 0.001$ versus GF, unpaired t test. (**E**) Purified CD4$^+$ Venus$^+$ cells from the colonic LP of SPF or *Clostridium*-colonized *Il10*^{Venus} mice were cultured with splenic CD4$^+$CD25$^-$ T cells (T_{eff}) in the presence of irradiated splenic CD11c$^+$ cells and anti-CD3 for 72 hours at the indicated ratios. Proliferation was measured by [^{3}H]thymidine uptake. Data represent the mean ± SD of triplicate cultures.

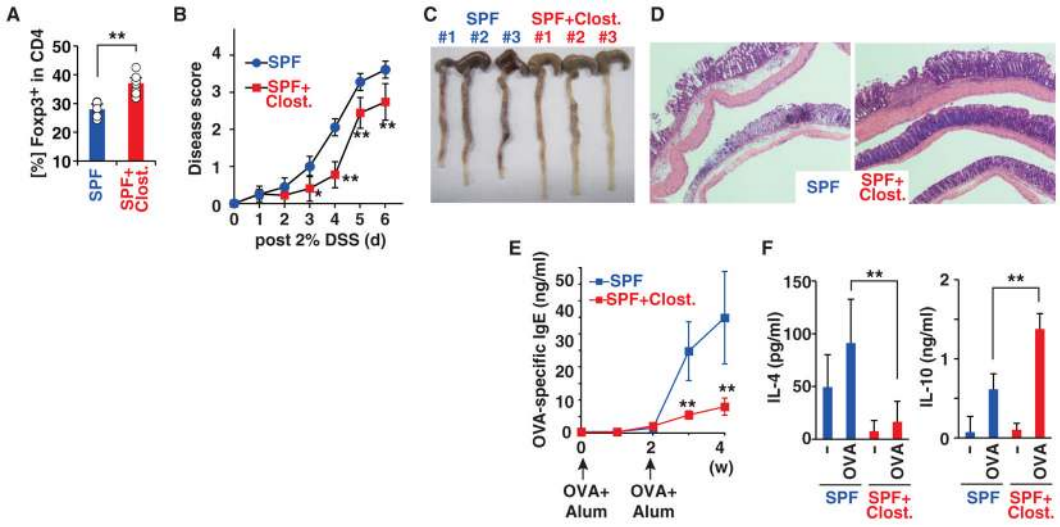

Fig. 4.

Effect of *Clostridium* abundance on regulation of colitis and IgE response. (**A**) Two-week-old SPF mice were orally inoculated with *Clostridium* (SPF+Clost.) or untreated (SPF). After 6 weeks, the percentage of $Foxp3^+$ cells within $CD4^+$ cells in colonic LP was analyzed. The experiment was repeated more than three times with similar results. Each circle represents a mouse, and error bars represent the SD ($n = 7$ mice per group). (**B** to **D**) SPF and SPF+Clost. mice were treated with 2% DSS and monitored and scored for body weight loss, stool consistency, and bleeding for 6 days ($n = 7$ mice per group). (B) On day 6, the colons were collected (C) and dissected for histological analysis by hematoxylin and eosin staining (D). (**E** and **F**) SPF and SPF+Clost. mice were immunized with OVA + alum twice at a 2-week interval. Sera were collected and examined for OVA-specific IgE levels by ELISA (E). Splenocytes were collected from mice in each group and examined for IL-4 and IL-10 production upon restimulation with OVA in vitro (F). Error bars represent the SD ($n = 5$ mice per group). $*P < 0.02$; $**P < 0.001$, unpaired t test.