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Ravi Verma, Changhon Lee, Eun-Ji Jeun, Jaeu Yi, Kwang Soon Kim, et al.. Cell surface polysaccharides of *Bifidobacterium bifidum* induce the generation of Foxp3<sup>+</sup> regulatory T cells. *Science Immunology*, 2018, 3 (28), pp.eaat6975. 10.1126/sciimmunol.aat6975 . hal-01901482

**HAL Id: hal-01901482**

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Submitted on 22 Oct 2018

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## MICROBIOME

# Cell surface polysaccharides of *Bifidobacterium bifidum* induce the generation of Foxp3<sup>+</sup> regulatory T cells

Ravi Verma<sup>1</sup>, Changhon Lee<sup>2</sup>, Eun-Ji Jeun<sup>2</sup>, Jaeu Yi<sup>2</sup>, Kwang Soon Kim<sup>1</sup>, Ambarnil Ghosh<sup>1</sup>, Seohyun Byun<sup>2</sup>, Choong-Gu Lee<sup>1</sup>, Hye-Ji Kang<sup>1</sup>, Gi-Cheon Kim<sup>1</sup>, Chang-Duk Jun<sup>3</sup>, Gwenaël Jan<sup>4</sup>, Chang-Hee Suh<sup>5</sup>, Ju-Yang Jung<sup>5</sup>, Jonathan Sprent<sup>2,6</sup>, Dipayan Rudra<sup>1,2</sup>, Cristina De Castro<sup>7,8</sup>, Antonio Molinaro<sup>8,9</sup>, Charles D. Surh<sup>1,2</sup>, Sin-Hyeog Im<sup>1,2,\*</sup>

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Dysregulation of intestinal microflora is linked to inflammatory disorders associated with compromised immunosuppressive functions of Foxp3<sup>+</sup> T regulatory (T<sub>reg</sub>) cells. Although mucosa-associated commensal microbiota has been implicated in T<sub>reg</sub> generation, molecular identities of the “effector” components controlling this process remain largely unknown. Here, we have defined *Bifidobacterium bifidum* as a potent inducer of Foxp3<sup>+</sup> T<sub>reg</sub> cells with diverse T cell receptor specificity to dietary antigens, commensal bacteria, and *B. bifidum* itself. Cell surface β-glucan/galactan (CSGG) polysaccharides of *B. bifidum* were identified as key components responsible for T<sub>reg</sub> induction. CSGG efficiently recapitulated the activity of whole bacteria and acted via regulatory dendritic cells through a partially Toll-like receptor 2–mediated mechanism. T<sub>reg</sub> cells induced by *B. bifidum* or purified CSGG display stable and robust suppressive capacity toward experimental colitis. By identifying CSGG as a functional component of T<sub>reg</sub>-inducing bacteria, our studies highlight the immunomodulatory potential of CSGG and CSGG-producing microbes.

## INTRODUCTION

The mammalian gastrointestinal tract harbors numerous species of commensal organisms that constitute the intestinal microbiota. Dysregulation in the composition and diversity of microbiota (dysbiosis) is closely associated with diverse immune disorders, such as allergy, autoimmunity, and gastrointestinal inflammatory disorders. Compared with specific pathogen-free (SPF) conditions, mice raised in complete germ-free (GF) settings display disorganized lymphoid tissues and abnormal immune responses; these deficits are corrected by the provision of commensal microbial species (1–3). The intestinal microbiota have also been implicated in diversified functions of host immunity by potentiating generation of particular immune cell types with reciprocal functions, notably proinflammatory T helper 17 (T<sub>H</sub>17) or anti-inflammatory regulatory T (T<sub>reg</sub>) cell populations (1, 4–7). T<sub>reg</sub> cells are a subset of CD4<sup>+</sup> T cells with suppressive function and are characterized by expression of the transcription factor Foxp3 (8, 9). T<sub>reg</sub> cells are generally formed in the thymus (nT<sub>reg</sub>) but can also arise in the periphery from normal CD4<sup>+</sup> cells (pT<sub>reg</sub>).

In recent years, individual species of the microbial community have been shown to shape the immune composition of the host. For example, colonization with segmented filamentous bacteria (SFB) results in strong induction of T<sub>H</sub>17 cells in the small intestine, which is associated with host resistance against intestinal pathogens while exacerbating autoimmune syndromes (10–12). Several studies have implicated specific strains or a mixture of the commensal microbiota in homeostasis and function of mucosa-associated T<sub>reg</sub> cells (13).

In particular, a mixture of 17 strains residing within clusters IV, XIVa, and XVIII of *Clostridia* was shown to enhance colonic T<sub>reg</sub> cell differentiation and function in mice (14). Also, colonization of mice with human commensal *Bacteroides fragilis* (*Bf*) potentiates interleukin-10 (IL-10)–producing T<sub>reg</sub> cells (2, 15–17). We have previously reported that a mixture of five probiotic strains, IRT5, has the capacity to induce Foxp3<sup>+</sup> T<sub>reg</sub> cells and to suppress diverse immune responses in several experimental disease models, such as atopic dermatitis, rheumatoid arthritis, myasthenia gravis, and multiple sclerosis (18–20).

Although these studies demonstrate immunomodulatory properties of specific bacterial species, the underlying molecular events driving these processes remain elusive. Several bacteria-derived metabolites and cell wall components have previously been implicated. For example, butyrate was reported as a key effector molecule produced by *Clostridia*, which can potentiate colonic T<sub>reg</sub> cell induction (21). The polysaccharide A (PSA) of *Bf* was identified as a key effector molecule for IL-10–producing T<sub>reg</sub> induction (2, 22).

Supplementing patients with missing microorganisms might be effective in restoring dysregulated microbiota. Fecal microbiota transplants (FMTs) from healthy donors to patients have been shown to have beneficial effects in treating gastrointestinal disorders (23). However, given that FMT has not yet been approved as a therapeutic modality due to safety issues, administration of probiotics could provide an alternative approach to achieve the same goal. Several studies have reported that consumption of safety-assured bacteria-like probiotics could ameliorate diverse immune disorders, such as allergies and inflammatory bowel disorders (24, 25). However, the functionality of probiotics might depend on the particular health disorder of the patients. For this approach to be successful, it is vital to identify beneficial microorganisms and to elucidate effector mechanisms that regulate their immunomodulatory activity.

Here, we have screened a large collection of probiotic strains for their T<sub>reg</sub>-inducing capacity and report a *Bifidobacterium bifidum* strain *PRI1* (*Bb*) to be the best candidate. Using GF mice, we have established that this strain facilitates efficient de novo generation of T<sub>reg</sub> cells with specificity for dietary antigens (Ags), commensal microbiota, and *Bb* itself. Further, we have identified a class of bacterial cell surface β-glucan/galactan

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(CSGG) polysaccharides as the key effector component able to induce  $T_{reg}$  cells with the capacity to ameliorate intestinal inflammation.

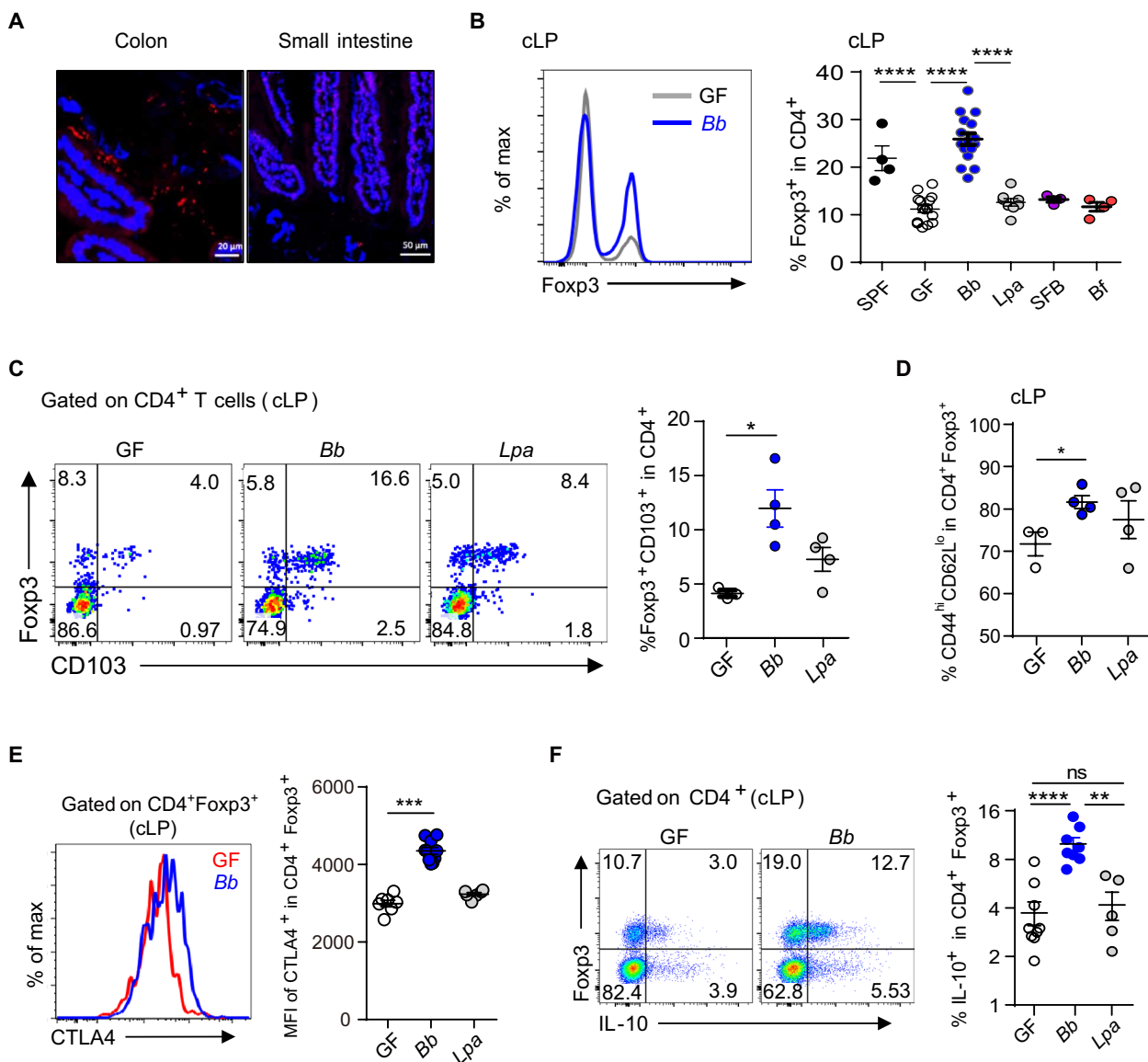
## RESULTS

### *Bb* monocolonization induces de novo generation of $pT_{reg}$ cells

To identify  $T_{reg}$ -inducing bacteria among the commensal microorganisms, we screened probiotic strains using our ex vivo screening protocol (20, 26). Briefly, individual bacterial strains were cultured in the presence of antibiotics with a suspension of total mesenteric lymph node (mLN) cells from Foxp3-GFP (green fluorescent pro-

tein) (Foxp3<sup>GFP</sup>) reporter mice, thereby enabling detection of  $T_{reg}$  cells (9). Bacterial strains that induced at least 10-fold greater production of IL-10 than IL-12 and also a > 50% higher frequency of CD4<sup>+</sup>GFP<sup>+</sup>  $T_{reg}$  cells than mock-treated cultures were selected. Among these, *Bb* was chosen as the best candidate (fig. S1, A and B). *Lactobacillus paracasei sub. Tolerans* 467 (*Lpa*), which displayed no noticeable immune response, was selected as a control inert strain (fig. S1A). *Bf* [American Type Culture Collection (ATCC) 25285] (2, 15–17) was also used to compare the  $T_{reg}$ -inducing capability among the bacterial species.

Single administration of *Bb* to GF mice led to its stable colonization primarily in the colon (Fig. 1A) and normalized the enlarged



**Fig. 1. *Bb* monocolonization enhances  $T_{reg}$  population in the cLP.** (A) Localization analysis of *Bb* in the intestinal niches of GF mice by Histo-FISH staining with DNA-Cy5 probes (EUB338, red) 3 weeks after colonization. (B) Representative flow cytometry plots and percentage analyses of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in cLP of GF mice colonized with indicated bacterial strains. (C to F) Representative flow cytometry plots and frequencies of CD103<sup>+</sup> and CD62L<sup>lo</sup>CD44<sup>hi</sup>, CTLA4<sup>+</sup>, and IL10<sup>+</sup> in  $T_{reg}$  cells in GF mice or those monocolonized with *Lpa* or *Bb*. Numbers in the quadrants represent cell percentage, and circles in the graph plots represent individual mouse corresponding to each parameter. Data are representative of three to five independent experiments with similar results ( $n \geq 3$  mice). All graph plots show means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (Student's  $t$  test). MFI, mean fluorescence intensity; ns, not significant.

cecum size of GF mice (fig. S1C). Three-week colonization with *Bb*, but not with *Lpa*, *SFB*, or *Bf*, led to a marked increase in the frequency of  $\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells in the colonic lamina propria (cLP; Fig. 1B and fig. S1, D and E) as well as in other organs, including mLN, spleen, and, to a lesser degree, small intestinal lamina propria (siLP) and peripheral LN (pLN) (fig. S1F). Compared with GF mice, mice monoclonalized with the *Bf* that was previously reported to induce  $\text{T}_{\text{reg}}$  cells (2, 16, 22) did not show any substantial increase in  $\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells (Fig. 1B and fig. S1D).  $\text{T}_{\text{reg}}$  cells in the cLP of *Bb*-monocolonized mice displayed a significantly higher proportion of  $\text{CD103}^+$  and memory-phenotype ( $\text{CD62L}^{\text{lo}}\text{CD44}^{\text{hi}}$ ) cells compared with  $\text{T}_{\text{reg}}$  cells in unmanipulated GF mice, suggesting a higher state of  $\text{T}_{\text{reg}}$  cell activation (Fig. 1, C and D, and fig. S1, G and H). Consistent with this idea, cLP  $\text{T}_{\text{reg}}$  cells induced by *Bb* colonization also expressed higher levels of CTLA4 and IL-10, two key molecules indicative of enhanced suppressive properties (Fig. 1, E and F). Although levels of interferon- $\gamma$  (IFN $\gamma$ ) and IL-13 were not altered, IL-17 levels were increased in  $\text{T}_{\text{reg}}$  cells (fig. S2, A and B). A slight increase in IFN $\gamma$  levels was observed in  $\text{CD4}^+\text{Foxp3}^-$  non- $\text{T}_{\text{reg}}$  cells in the *Bb* mono-associated mice (fig. S2, A and B).

Expression of the IKAROS family transcription factor Helios and neuropilin 1 (Nrp1) is widely used to distinguish Helios $^+$ Nrp1 $^+$  thymically generated  $\text{T}_{\text{reg}}$  ( $\text{tT}_{\text{reg}}$ ) cells from Helios $^-$ Nrp1 $^-$  peripherally generated  $\text{T}_{\text{reg}}$  ( $\text{pT}_{\text{reg}}$ ) cells (called induced “ $\text{iT}_{\text{reg}}$ ” when generated in in vitro culture conditions) (27–29). Whereas a small increase in Nrp1 $^+$   $\text{tT}_{\text{reg}}$  cells was observed in *Bb*-colonized cLP, a much greater increase was observed for Nrp1 $^-$  and Helios $^{\text{lo}}$   $\text{pT}_{\text{reg}}$  cells in all organs tested, indicating that *Bb* primarily promotes generation of  $\text{pT}_{\text{reg}}$  cells (Fig. 2, A to D, and fig. S3, A and B). Furthermore, a large proportion of the newly generated  $\text{pT}_{\text{reg}}$  cells expressed the transcription factor ROR $\gamma$ t (ROR $\gamma$ t $^+$ Helios $^-$   $\text{Foxp3}^+$  and ROR $\gamma$ t $^+$   $\text{Foxp3}^+$ ), which is up-regulated upon interactions with microbes in cLP and siLP (Fig. 2, E and F, and fig. S3, C and D) (30–32).

To further confirm the peripheral origin of the *Bb*-induced  $\text{T}_{\text{reg}}$  cells, we adoptively transferred allelically marked naïve phenotype  $\text{CD4}^+\text{CD45.1}^+\text{Foxp3}^-$   $\text{CD62L}^{\text{hi}}\text{CD44}^{\text{lo}}$  T cells sorted from  $\text{Foxp3}^{\text{GFP}}$  mice into mice monoclonalized with *Bb* (fig. S3E). Analysis of the donor cells 3 weeks later revealed emergence of a significant proportion of  $\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells among donor cells in the intestine (cLP and siLP) of *Bb*-monocolonized mice, whereas such cells were sparse among the donor cells in mock-colonized control GF hosts (Fig. 2G and fig. S3, F and G).

Together, these data establish *Bb* as a potent inducer of  $\text{pT}_{\text{reg}}$  cells by eliciting de novo up-regulation of  $\text{Foxp3}$  in naïve  $\text{CD4}^+$  T cells, primarily at the sites of colonization. Similarly enhanced induction of  $\text{pT}_{\text{reg}}$  cells was observed in SPF mice. Here, SPF mice were adoptively transferred with allelically marked purified naïve T cells and then gavaged repeatedly with *Bb* for 3 weeks. These mice developed a high proportion of donor-derived  $\text{CD4}^+\text{Foxp3}^+$  cells, strongly suggesting that the  $\text{T}_{\text{reg}}$  cell-inducing property of *Bb* is physiologically relevant (Fig. 2H).

### ***Bb* colonization induces $\text{pT}_{\text{reg}}$ cells with a broad range of TCR specificity to diverse Ags**

Next, we analyzed the T cell receptor (TCR) specificity of  $\text{pT}_{\text{reg}}$  cells generated in the presence of *Bb*.  $\text{pT}_{\text{reg}}$  cells generated in the presence of *Bb* could be reactive to dietary Ags and/or reactive to a variety of species of commensal bacteria, including *Bb* itself. To test whether colonization of *Bb* promotes  $\text{pT}_{\text{reg}}$  cells to dietary Ags, we adoptively transferred Cell Trace Violet (CTV)-labeled ovalbumin (OVA)-

specific naïve OT-II TCR transgenic  $\text{CD4}^+$  cells from OT-II.  $\text{Thy1.1}^+\text{Foxp3}^{\text{GFP}}$  mice into normal GF or *Bb*-monocolonized mice that were then gavaged with OVA protein (fig. S4A). As previously observed (33), a small fraction of OT-II cells that underwent expansion in normal GF hosts up-regulated  $\text{Foxp3}$  in the siLP and cLP. Notably, in *Bb*-monocolonized mice, but not in the *Lpa*-monocolonized mice, the efficiency of  $\text{Foxp3}$  up-regulation by donor OT-II cells increased about two- to threefold in cLP (Fig. 3, A and B).

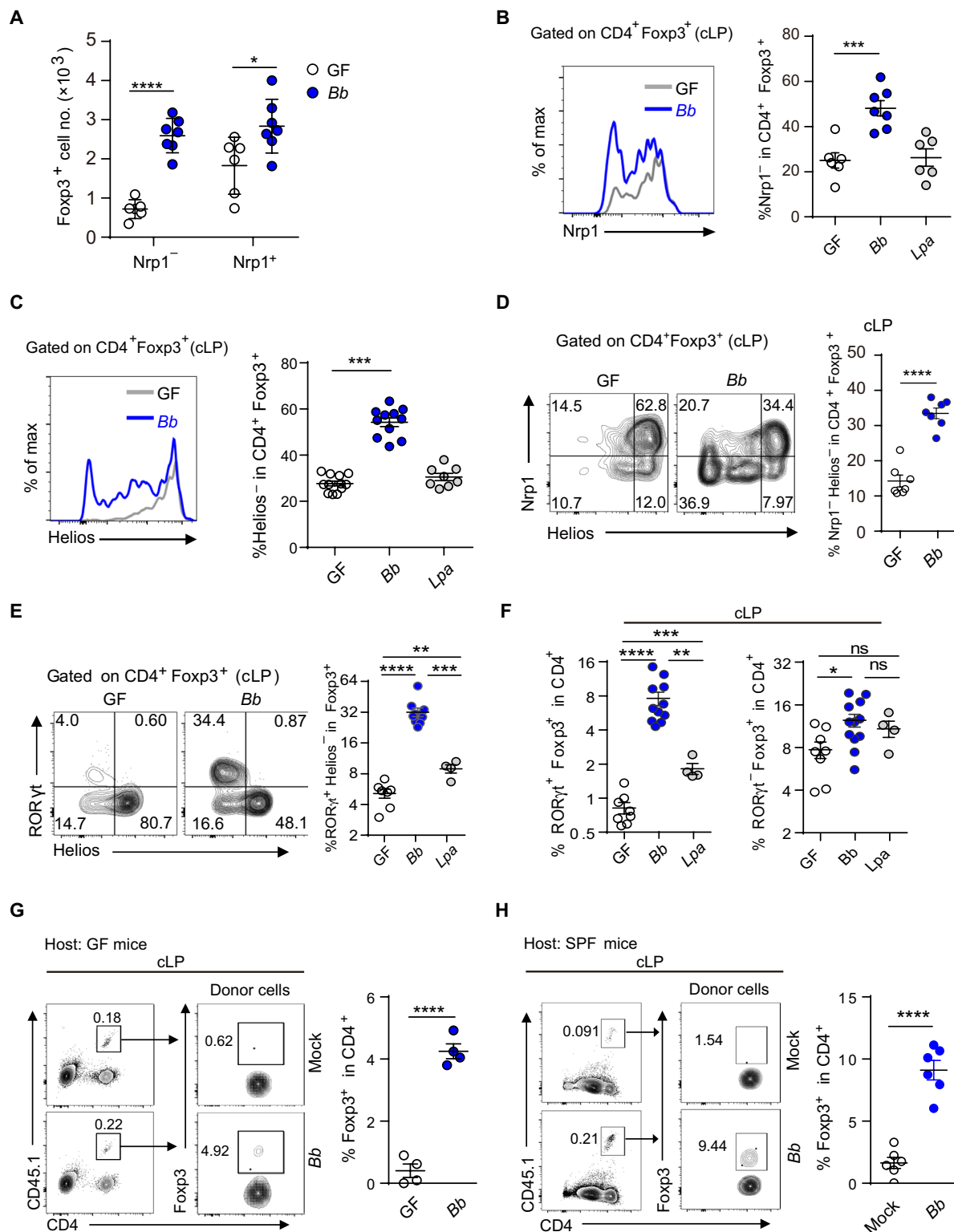
To determine whether the administration of *Bb* promotes generation of  $\text{pT}_{\text{reg}}$  cells specific to microbiota, we used CBir TCR transgenic mice that recognize bacterial flagellin (34). Naïve  $\text{CD4}^+\text{Foxp3}^-$  T cells sorted from CBir transgenic mice on a  $\text{CD45.1}^+\text{Foxp3}^{\text{GFP}}$  background were adoptively transferred into SPF *Rag1* $^{-/-}$  recipients, and then mock [phosphate-buffered saline (PBS)] or *Bb* was gavaged every other day until the end of the experiment (fig. S4B). Control SPF *Rag1* $^{-/-}$  recipients of CBir T cells alone showed prominent signs of colitis as indicated by progressive weight loss (Fig. 3C), a shortened colon with thickened mucosa (Fig. 3D), a change in colonic thickness, and increased infiltration of lymphocytes along with a high histological score that contained few  $\text{T}_{\text{reg}}$  cells (Fig. 3, E and F) but a high proportion of  $\text{CD4}^+\text{IFN}\gamma^+$  and  $\text{ROR}\gamma^+\text{Foxp3}^-$  T cells (Fig. 3G and fig. S4C). These various signs of pathology were absent or much less marked in hosts injected with CBir T cells plus *Bb* administration. In this situation, weight loss was minimal, the colon was normal with low histology score, and the cLP contained elevated numbers of  $\text{T}_{\text{reg}}$  cells along with  $\text{ROR}\gamma^+\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells but few  $\text{CD4}^+\text{IFN}\gamma^+$  cells (Fig. 3, C to G, and fig. S4C).

To test whether the  $\text{pT}_{\text{reg}}$  cells induced by *Bb* monoclonalization have specificity for *Bb* itself, we performed the following experiments. Total colonic  $\text{CD4}^+$  T cells containing  $\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells from the GF mice and *Bb*-monocolonized mice were sorted and labeled with CTV and then cocultured with T cell-depleted splenic antigen-presenting cells (APCs) pretreated with fecal Ags from normal GF mice and mice monoclonalized with *Bb* or irrelevant control bacteria *Lpa*; these cells were then analyzed for T cell proliferation (CTV dilution) 3 days later (fig. S5A). The results showed that  $\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells from *Bb*-monocolonized mice displayed sustained  $\text{Foxp3}$  expression and proliferation when stimulated with *Bb* fecal Ag-treated APC (Fig. 4A). By contrast, coculturing of total colonic  $\text{CD4}^+$  with APCs treated with fecal Ags from GF- or *Lpa*-monocolonized mice failed to sustain  $\text{T}_{\text{reg}}$  cell proliferation and stability under identical conditions (Fig. 4A).

We also compared the TCR specificity of GF and *Bb*-monocolonized mice by sequencing the CDR3 region of TCR  $\alpha$  and  $\beta$  chains of  $\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells sorted from colon, mLN, and spleen. Although both  $\text{T}_{\text{reg}}$  cells showed a similar pattern of diversity in TCR  $\alpha$  and  $\beta$  chains in spleen and mLN, colonic  $\text{T}_{\text{reg}}$  cells from *Bb*-monocolonized mice showed enhanced diversity with distinct TCR patterns that were not present in GF control (Fig. 4, B to E, and fig. S5B). Moreover, we observed that some of the colonic  $\text{T}_{\text{reg}}$  cells with specific TCR sequences were predominantly enriched (>0.1% frequency) compared with those of mLN and spleen (marked with asterisks in Fig. 4E; tables S1 and S2). Collectively, these results suggest that *Bb* colonization induces the generation of  $\text{CD4}^+\text{Foxp3}^+$   $\text{T}_{\text{reg}}$  cells with a broad range of TCR specificity to dietary Ags and/or commensal microbiota and *Bb* itself.

### ***Bb* facilitates $\text{T}_{\text{reg}}$ cell induction through a dendritic cell-dependent mechanism**

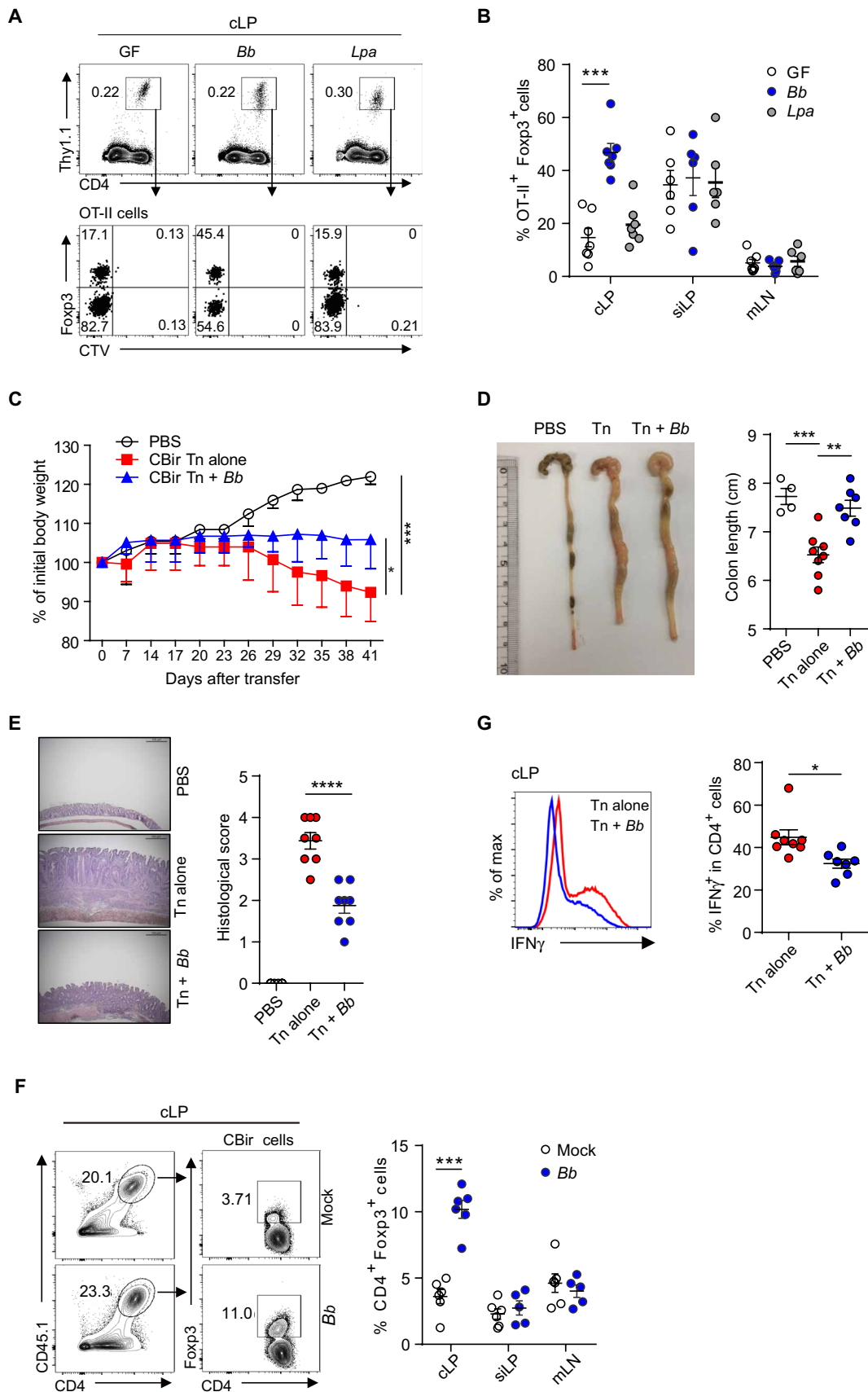
Because intestinal induction of  $\text{pT}_{\text{reg}}$  cells from naïve T cells requires an immunoregulatory cytokine milieu, we tested whether colonization

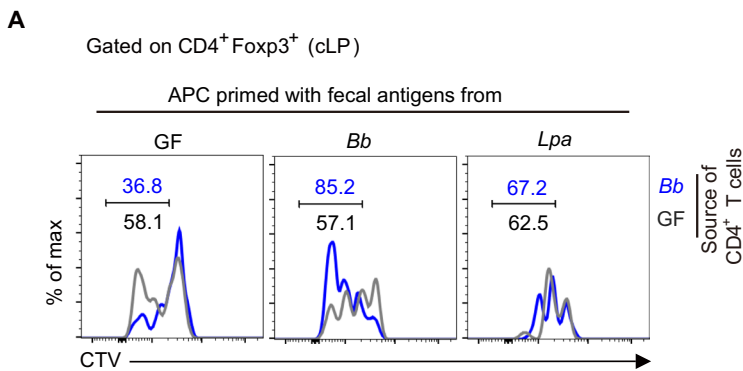


**Fig. 2. *Bb* monocolonization facilitates de novo generation of pT<sub>reg</sub> cells.** cLP cells were isolated from the GF mice or mice monocolonized with *Lpa* or *Bb* 3 weeks after colonization. (A) Absolute numbers of Nrp1<sup>+</sup> and Nrp1<sup>-</sup> T<sub>reg</sub> cells. (B–F) Representative flow cytometry plots and frequencies for Nrp1<sup>-</sup>Foxp3<sup>+</sup> (B), Helios<sup>-</sup>Foxp3<sup>+</sup> (C), Helios<sup>-</sup>Nrp1<sup>-</sup>Foxp3<sup>+</sup> (D), RORγt<sup>+</sup>Helios<sup>-</sup>Foxp3<sup>+</sup> (E), and RORγt<sup>+</sup>Foxp3<sup>+</sup>, RORγt<sup>-</sup>Foxp3<sup>+</sup> (F) T<sub>reg</sub> cells in the cLP of GF or *Bb*- or *Lpa*-monocolonized mice. (G) Naive CD4<sup>+</sup>Foxp3<sup>-</sup> T cells sorted from CD45.1<sup>+</sup>Foxp3<sup>GFP</sup> reporter mice were transferred into GF mice. Animals were either left GF or monocolonized with *Bb* for 3 weeks. Foxp3<sup>+</sup> T<sub>reg</sub> population was analyzed by GFP expression in the cLP. (H) Naive CD4<sup>+</sup>Foxp3<sup>-</sup> T cells sorted from CD45.1<sup>+</sup>Foxp3<sup>GFP</sup> reporter mice were transferred into SPF mice, and then mice were fed with mock (PBS) or *Bb* (5 × 10<sup>8</sup> CFU) every other day for 3 weeks and analyzed for Foxp3<sup>+</sup> T<sub>reg</sub> cells. Data are representative of at least five independent experiments with similar results (n ≥ 3 mice). All graph plots show means ± SEM. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 (Student's *t* test).

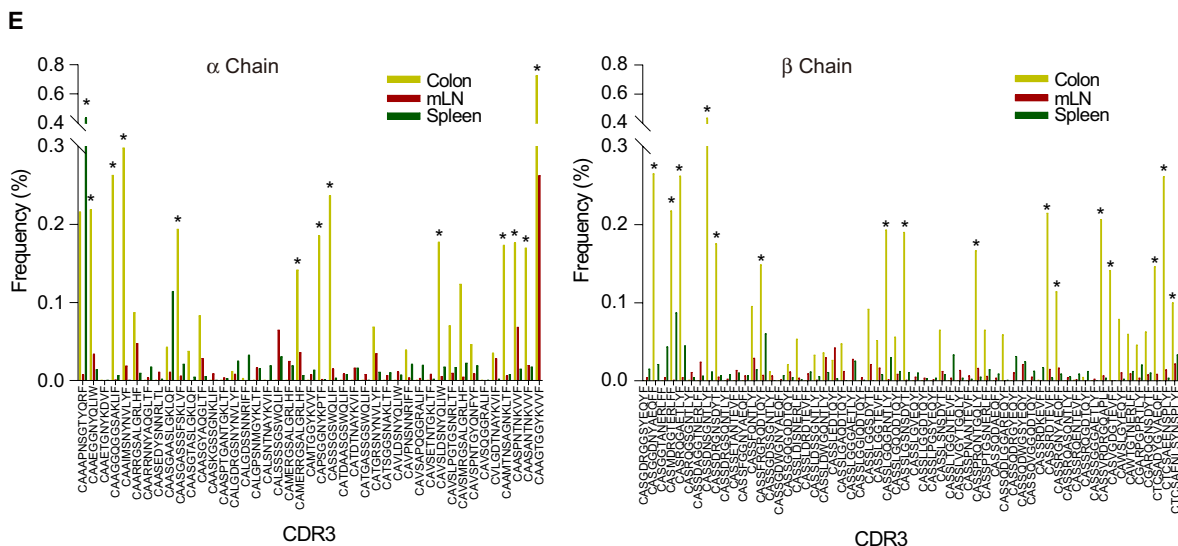
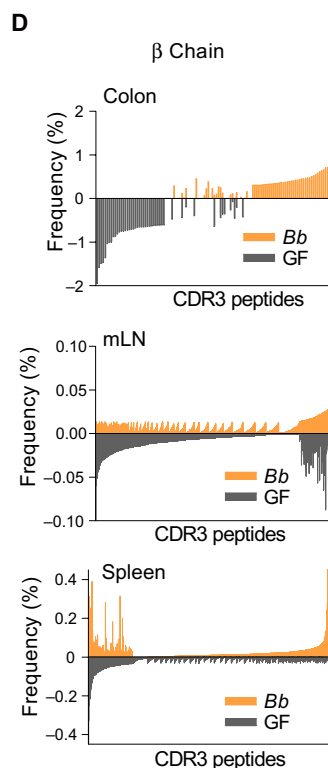
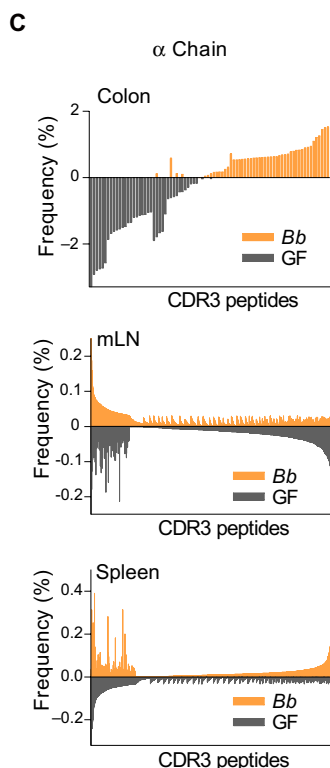
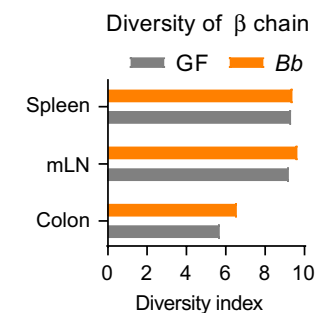
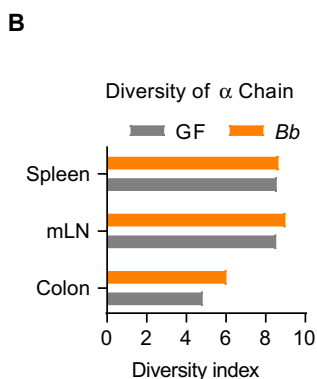
**Fig. 3. *Bb* colonization induces dietary Ag- and microbiota-reactive T<sub>reg</sub> cells.**

(A and B) CTV-labeled naïve allelically marked CD4<sup>+</sup>Thy1.1<sup>+</sup>Foxp3<sup>-</sup> T cells from OT-II.Thy1.1<sup>+</sup>Foxp3<sup>GFP</sup> mice were adoptively transferred into GF mice or mice premonocolonized with *Bb* or *Lpa* for 14 days. The mice were fed with OVA (20 mg) every other day for 7 days. The cLP CD4<sup>+</sup>Foxp3<sup>+</sup> population of the donor (Thy1<sup>+</sup>OT-II) cells was analyzed. Representative fluorescence-activated cell sorting (FACS) plots (A) and frequencies of T<sub>reg</sub> cells generated in vivo (B) are shown. (C to E) Naïve CD4<sup>+</sup>CBir<sup>+</sup>CD45.1<sup>+</sup>Foxp3<sup>-</sup> T cells sorted from CBir<sup>Tg</sup>CD45.1<sup>+</sup>Foxp3<sup>GFP</sup> mice were adoptively transferred into the SPF *Rag1*<sup>-/-</sup> recipients. The recipient mice were fed with either *Bb* or PBS every other day until the end of the experiment. Changes in the body weight (C), colon length (D), and histopathology and histological score (E) in colonic tissue were measured and analyzed. (F and G) Representative FACS plots and frequencies of CBir<sup>+</sup>CD45.1<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells (F) and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells (G) within cLP of mock- or *Bb*-administered mice analyzed at the end of experiment. Numbers indicate cell percentages in the quadrants, and circles in the graph plots represent individual mouse corresponding to each parameter, respectively. Data are representative of at least three independent experiments with similar results ( $n \geq 3$  mice). Graphs with error bars show means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (Student's *t* test).





**Fig. 4. *Bb* monoclonization induces *Bb*-reactive colonic T<sub>reg</sub> cells with distinctive TCR repertoires.** (A) Total colonic CD4<sup>+</sup> T cells containing Foxp3<sup>+</sup> T<sub>reg</sub> cells from the GF mice or *Bb*-monocolonized mice were sorted and labeled with CTV and then cocultured with T cell-depleted splenic APC in the presence of indicated fecal Ags from GF or *Bb*- or *Lpa*-monocolonized mice. Three days later, relative proliferation of T<sub>reg</sub> cells was analyzed by FACS. (B) Comparison of Shannon diversity from TCR  $\alpha$  and  $\beta$  chains of T<sub>reg</sub> cell repertoires obtained from different lymphoid organs of GF or *Bb*-monocolonized mice. (C and D) Frequency distribution two-way bar plot representation of common TCR-CDR3  $\alpha$  regions (85 from colon, 1469 from mLN, and 1381 from spleen) (C) and  $\beta$  regions (118 from colon, 1724 from mLN, and 1381 from spleen) (D). (E) Comparative enrichment frequency distribution of unique  $\alpha$  and  $\beta$  chains of TCR-CDR3 regions of T<sub>reg</sub> cells isolated from colon, mLN, and spleen of *Bb*-monocolonized mice compared with GF mice.



with *Bb* induces a tolerogenic microenvironment in the colon. Compared with normal GF mice, total colonic cells (fig. S6A) as well as cLP-DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>CD103<sup>+</sup>F4/80<sup>-</sup>) isolated from the *Bb*-monocolonized mice displayed a significant increase in the expression of mRNA for a number of inhibitory molecules, namely, *il10*, *Csf2*, *Tgfb1*, *ido*, *Ptgs2*, and *Pdcd1*, as well as costimulatory molecules *Cd86* and *Cd40* (Fig. 5, A to C, and fig. S6A). These findings suggest that colonization with *Bb* induces a population of regulatory dendritic cells (rDCs), which, in turn, may enhance de novo pT<sub>reg</sub> differentiation. We further analyzed the different subtypes of DCs affected by *Bb* monocolonization in the cLP, siLP, and mLN. In the cLP, we could observe a slight increase in CD103<sup>+</sup>CD11b<sup>+</sup> and plasmacytoid DC (pDC) (Bst2<sup>+</sup>CD11c<sup>lo</sup>) population but not in CX3CR1<sup>+</sup>CD11b<sup>+</sup> population (Fig. 5D and fig. S6, B to D). In the mLN, but not in siLP, a significant increase in CD103<sup>+</sup>CD11b<sup>+</sup> DCs and CX3CR1<sup>+</sup>CD11b<sup>+</sup> population was observed (fig. S7, A and B). These results indicate that CD103<sup>+</sup> cLP-DCs may play a key role in *Bb*-mediated T<sub>reg</sub> induction in the colon. To examine this possibility, we performed an in vivo mimicking experiment by culturing sorted cLP-DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>CD103<sup>+</sup>F4/80<sup>-</sup>) with *Bb* for 10 to 12 hours followed by washing and then coculture with naïve CD4<sup>+</sup>Foxp3<sup>-</sup> T cells for 3 days in suboptimal T<sub>reg</sub>-inducing conditions. Notably, pretreatment of cLP-DCs with *Bb* resulted in the significant induction and proliferation of iT<sub>reg</sub> cells relative to mock treatment or exposure to control *Lpa* (Fig. 5E). IL-10 secretion in the *Bb*-treated culture was also increased compared with mock-treated group (Fig. 5E, bottom panel). By contrast, cLP-DC-treated *Lpa* did not induce iT<sub>reg</sub> or IL-10 production in these culture conditions (Fig. 5E).

### CSGG are the active components of *Bb* facilitating T<sub>reg</sub> cell induction

Using the above in vitro system, we performed experiments to identify *Bb*-derived effector molecules that facilitate iT<sub>reg</sub> differentiation. Because of limited availability of cLP-DCs, we used splenic DCs for these experiments in suboptimal T<sub>reg</sub>-inducing conditions. Because *Bb* is strictly anaerobic and mostly nonviable during coculture with DCs, we tested whether some of its cellular components might act as effector molecules with iT<sub>reg</sub>-inducing activity. Among the subcellular fractionates comprising cell surface, cell membrane, and cytosol components, only the cell surface extract effectively promoted iT<sub>reg</sub> generation (fig. S8A). Treatment of cell surface extract with ribonuclease (RNase), deoxyribonuclease (DNase), Pronase, or boiling at high temperature did not reduce the T<sub>reg</sub>-inducing activity, suggesting that polysaccharides might be the effector molecules. Incubating DCs with total cell surface polysaccharide (tCSPS) extract effectively induced iT<sub>reg</sub> cells in a dose-dependent manner under similar culture conditions (fig. S8B).

We further purified polysaccharides by two successive chromatography steps. On the basis of its molecular weight features, tCSPS was further separated by ion exclusion chromatography, followed by in-depth nuclear magnetic resonance (NMR) analysis of the fractions eluted at low ionic strength. Cell surface polysaccharides of *Bb* consisted of at least five different polysaccharides. Among these, the 8000-Da (average molecular weight), negatively charged polysaccharide phosphoglycero-β-galactofuran (PGβG), with 64% abundance, was the most prevalent (Fig. 5F). The remaining four polysaccharides (β-1-6-glucan, β-1-4-galactan, β-1-6-galactan, and β-galactofuranan) had similar molecular weights (average ~4000 Da) without charge differences (neutral), precluding their further separation.

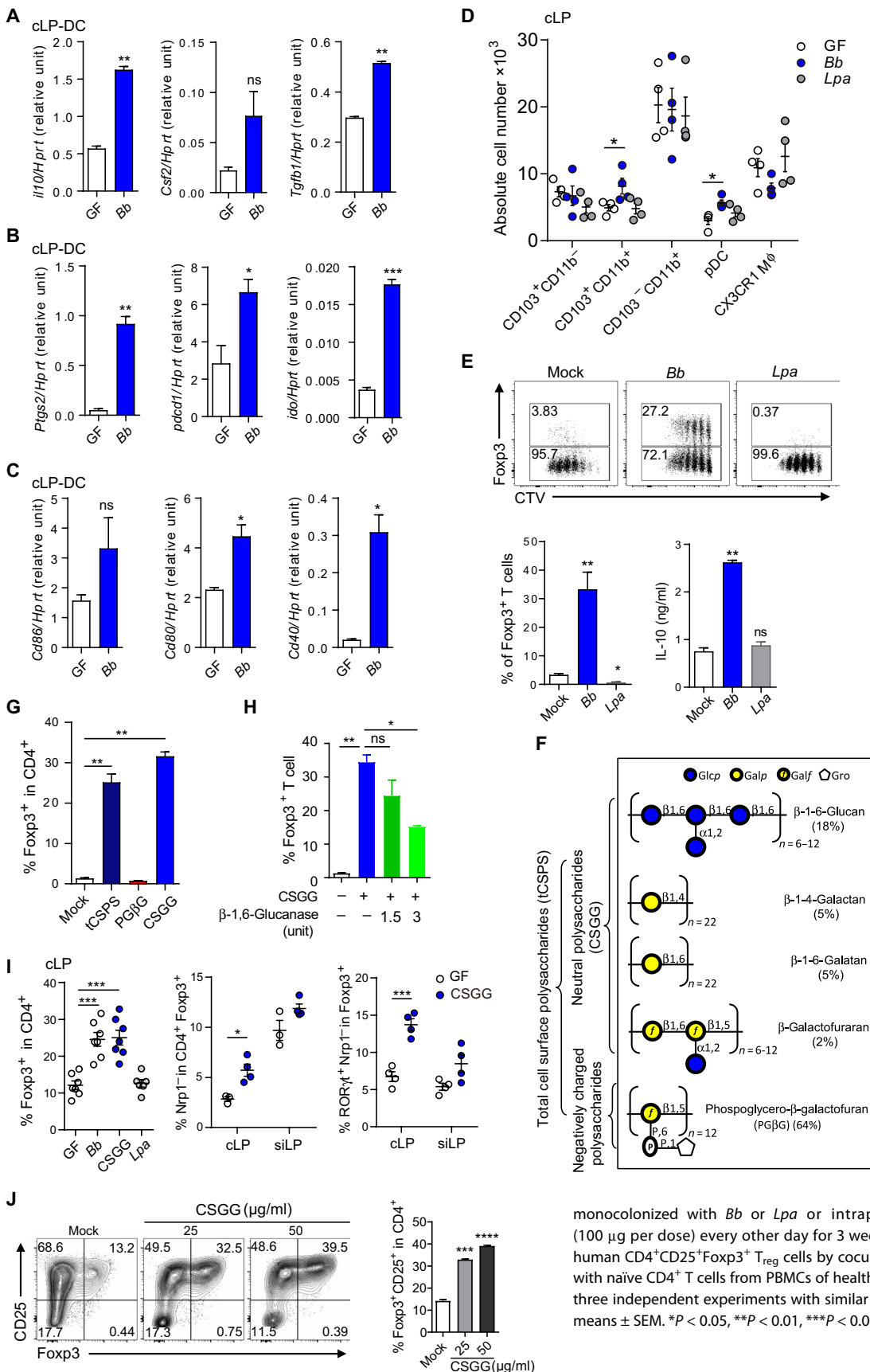
We tested which of the polysaccharides, the neutral mixture or PGβG, have the activity to induce T<sub>reg</sub> cells in vitro. Significantly, only the neutral polysaccharides, and not the negatively charged PGβG, facilitated induction of T<sub>reg</sub> cells (Fig. 5G). We named this mixture of neutral polysaccharides derived from the *Bb* as CSGG. Among the CSGG, cell surface β-1-6-glucan (CSβG) might be the key effector molecule. Treatment of CSGG with β-1,6-glucanase reduced CSGG-induced iT<sub>reg</sub> cell levels in a dose-dependent manner, whereas other enzyme treatments such as β-1,4-galactanase or β-1,6-galactanase displayed no significant reduction (Fig. 5H and fig. S8C).

Next, we tested whether CSGG could recapitulate the capacity of whole bacteria to induce T<sub>reg</sub> cells in vitro and in vivo. Naïve CD4<sup>+</sup>Foxp3<sup>-</sup> T cells were cocultured with DCs pretreated with *Bb*, CSGG, *Bf*, or *Lpa* in suboptimal T<sub>reg</sub>-inducing conditions. Significantly, the CSGG-treated DCs induced Foxp3<sup>+</sup> iT<sub>reg</sub> cells as effectively as *Bb*-treated DCs, whereas *Bf*- or *Lpa*-treated DCs failed to induce iT<sub>reg</sub> cells (fig. S8D). Coculturing of naïve CD4<sup>+</sup>Foxp3<sup>-</sup> T cells with DCs pretreated with *Bf* induced high levels of IFNγ<sup>+</sup>IL-10<sup>+</sup> mostly in Foxp3<sup>-</sup> T cells (fig. S8E). Next, we tested whether CSGG could induce pT<sub>reg</sub> cells in vivo. Intraperitoneal injections of CSGG (100 μg per dose) into GF mice for 3 weeks induced CD4<sup>+</sup>Foxp3<sup>+</sup> and Nr1<sup>-</sup>Roryt<sup>+</sup> T<sub>reg</sub> cells similar to the levels of *Bb*-monocolonized mice in the cLP and mLN (Fig. 5I and fig. S8F). These T<sub>reg</sub> cells displayed a higher proportion of CD103<sup>+</sup> and activated CD44<sup>hi</sup>CD62L<sup>lo</sup> cells than T<sub>reg</sub> cells from control GF mice (fig. S8G). Next, we tested whether CSGG treatment could induce T<sub>reg</sub> cells in the human CD4<sup>+</sup> T cells. Coculturing of CSGG-treated human DCs and naïve CD4<sup>+</sup> T cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors significantly induced CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in a dose-dependent manner (Fig. 5J).

### CSGG induces T<sub>reg</sub> cells through generation of regulatory DCs

Treatment of naïve CD4<sup>+</sup> T cells with CSGG in the absence of DCs failed to induce Foxp3<sup>+</sup> T<sub>reg</sub> cells, implying a DC-dependent mechanism for iT<sub>reg</sub> induction (fig. S9A). We therefore tested the role of different DC subtypes for generation of CSGG-induced iT<sub>reg</sub> cells (fig. S9B). pDCs, cDCs, and CX3CR1<sup>+</sup> and CX3CR1<sup>-</sup> DCs were sorted from the spleen; treated with mock, *Bb*, or CSGG; and then cocultured with naïve CD4<sup>+</sup>Foxp3<sup>-</sup> T cells in suboptimal T<sub>reg</sub>-inducing conditions. Although all the tested DC subtypes were able to induce iT<sub>reg</sub> cells upon *Bb* or CSGG treatment, cDCs, pDCs, and CX3CR1<sup>-</sup> cells induced high levels of iT<sub>reg</sub> (fig. S9, C and D). We also tested whether pretreatment of DCs with CSGG could induce Ag-specific iT<sub>reg</sub> generation because *Bb* administration induced pT<sub>reg</sub> cells with a broad range of TCR specificity to diverse Ags in vivo (Figs. 3 and 4). Sorted total splenic DCs (MHCII<sup>+</sup>CD11c<sup>+</sup>), as well as either of the two main types of DCs (CD8α<sup>+</sup>; MHCII<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>-</sup> and CD8α<sup>-</sup>; MHCII<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>), were pretreated with CSGG, and then naïve OT-II TCR transgenic CD4<sup>+</sup> cells from OT-II.Thy1.1<sup>+</sup>Foxp3<sup>GFP</sup> mice were cultured in the presence of OVA Ag (fig. S9E). CSGG treatment potentiated Ag-specific iT<sub>reg</sub> generation (fig. S9E). These results suggest that the CSGG treatment acts by converting conventional DCs into rDCs, similar to the above findings for the cLP-DC populations of *Bb*-colonized mice (Fig. 5, A to D). To test this possibility, we performed RNA sequencing (RNA-seq) analysis of the CD11c<sup>+</sup> splenic DCs treated for 4 hours with CSGG. Compared with mock control treatment, CSGG treatment significantly enhanced levels of rDC-associated markers such as *Ifna2*, *Pdcd1*, *Tgfb1*, *Csf2*, *Ptgs2*, *il10*, and





**Fig. 5. Cell surface CSGG of *Bb* mediates Treg cell induction.**

(A to C) Quantitative polymerase chain reaction analysis of mRNA for cytokines and inhibitory and costimulatory molecules plotted as fold change in the cLP-DCs (MHCI<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>CD103<sup>+</sup>F4/80<sup>-</sup>) of GF or *Bb*-monocolonized mice. Data are representative of three independent experiments with similar results. (D) Graph shows absolute number of cLP-DCs from GF mice or monoclonalized mice with *Lpa* or *Bb* for CD103, CD11b, CX3CR1, and pDCs (Bst2<sup>+</sup>CD11c<sup>+</sup>). Numbers represent the frequency of each population from the total CD11c<sup>+</sup>MHCI<sup>+</sup> cells. Data are representative of two independent experiments with similar results (*n* = 4 mice). (E) CD11c<sup>+</sup>DCs pretreated with *Bb* or *Lpa* were cocultured with naïve CD4<sup>+</sup> T cells in suboptimal Treg-inducing conditions for 3 days, after which Fopx3<sup>+</sup> Treg cells were analyzed within live cells. Representative flow cytometric analysis and mean frequencies of CD4<sup>+</sup>Fopx3<sup>+</sup> Treg cells are shown. From culture supernatant, IL-10 secretion was determined by enzyme-linked immunosorbent assay. Data are representative of five independent experiments with similar results. (F) Structures of neutral (CSGG) or negatively charged polysaccharides (PGβG) isolated from the tCSPS of *Bb*. Glcp, glucopyranose; Galp, galactopyranose; Galf, glucofuranose; Gro, glycerol. *n* = repeating unit. Relative abundances of each polysaccharide are shown as % (mol/mol). (G) Comparison of CD4<sup>+</sup>Fopx3<sup>+</sup> Treg-inducing activity between the neutral (CSGG, 50 μg/ml) and negatively charged polysaccharides (PGβG, 100 μg/ml) compared with tCSPS (100 μg/ml). (H) Effect of β-1,6-glucanase treatment on CSGG-induced iTreg cells. (I) Populations and phenotypes of Treg cells in GF mice or mice

monocolonized with *Bb* or *Lpa* or intraperitoneally injected with CSGG (100 μg per dose) every other day for 3 weeks. (J) Dose-dependent induction of human CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells by coculturing of DCs pretreated with CSGG with naïve CD4<sup>+</sup> T cells from PBMCs of healthy donors. Data are representative of three independent experiments with similar results. Graphs with error bars show means ± SEM. \**P* < 0.05, \*\*\**P* < 0.01, \*\*\*\**P* < 0.001, \*\*\*\*\**P* < 0.0001 (Student's *t* test).

*il27* (Fig. 6A). CSGG treatment also significantly increased the protein levels of IL-10 and TGF- $\beta$ 1 and decreased IFN $\gamma$  levels measured from culture supernatants (Fig. 6B). Moreover, compared with IL-10, TGF- $\beta$  played a pivotal role in  $iT_{reg}$  generation because addition of anti-TGF- $\beta$ -neutralizing antibody (Ab) almost completely abolished CSGG-mediated  $iT_{reg}$  induction (Fig. 6C). IL-10-deficient DCs also displayed a tendency toward reduced  $iT_{reg}$  generation compared with wild-type (WT) DCs, although statistical significance was not achieved (Fig. 6D).

To determine the role of pattern recognition receptors (PRRs) on DCs, we reanalyzed the RNA-seq data for the expression of genes encoding all the Toll-like receptor (TLR) subtypes upon CSGG treatment. We observed a prominent selective increase in *TLR2* expression, suggesting CSGG recognition via TLR2 (Fig. 6E). When cocultured with CSGG and assessed in vitro, CD11c<sup>+</sup> DCs isolated from mouse strain lacking TLR2 (*TLR2*<sup>-/-</sup>) displayed a significant reduction in  $iT_{reg}$ -inducing activity compared with WT DCs (Fig. 6F and fig. S10A). TLR2-deficient DCs elicited reduced levels of IL-10 and TGF- $\beta$ 1 in the cultures (Fig. 6, G and H) but no change in IFN $\gamma$  levels (fig. S10D). The defect seen with TLR2-deficient DCs was not further enhanced by additional blocking of TLR6 signaling (fig. 10B). A similar defect was also observed when DCs were isolated from mice lacking the downstream adaptor protein MyD88 (Fig. 6F and fig. S10A). TLR4- or TLR6-deficient DCs displayed no significant defect (Fig. 6F and fig. S10A). Also,  $T_{reg}$  induction was unaffected when TLR3<sup>-/-</sup>TLR7<sup>-/-</sup>TLR9<sup>-/-</sup> triple knockout DCs were used (fig. 10C). To test potential involvement of C-type lectin receptors, we cultured Dectin1<sup>-/-</sup> and Dectin2<sup>-/-</sup> DCs or CD11c<sup>+</sup> DCs pre-treated with blocking Ab for DC-SIGN and mannose receptor with naïve CD4<sup>+</sup> T cell in the presence of CSGG. However, no significant reduction was observed in the level of CSGG-induced  $iT_{reg}$  cell generation among the tested experimental groups (fig. S10, E and F). Together, these data strongly suggested that the TLR2/MyD88 signaling pathway is required for CSGG-mediated induction of  $iT_{reg}$  cells. However, provided that the TLR2<sup>-/-</sup> DCs still retained low degree of  $T_{reg}$  induction activity upon CSGG treatment (Fig. 6F), it seems possible that additional signaling pathways may also be involved.

### CSGG-induced $iT_{reg}$ cells are capable of suppressing intestinal inflammation

The identification of CSGG as the major  $T_{reg}$ -inducing component of *Bb* suggested that administration of CSGG might be effective in generating and boosting  $T_{reg}$  cell function and thereby suppressing inflammatory conditions. Under in vitro conditions, sorted  $iT_{reg}$  cells generated in the presence of CSGG-treated DCs displayed significantly enhanced suppressive capacity relative to  $iT_{reg}$  cells induced with mock-treated DCs (fig. S11A). For in vivo studies, we used the T cell transfer model of colitis induction. Naïve CD4<sup>+</sup> T cells were transferred into *Rag1*<sup>-/-</sup> lymphopenic hosts, either alone or together with various types of sorted allelically marked (CD45.1<sup>+</sup>)  $iT_{reg}$  cells.  $nT_{reg}$  cells sorted from the *Foxp3*<sup>GFP</sup> mice were used as a positive control. As expected, mice injected with naïve T cells alone developed severe signs of colitis, as indicated by weight loss (Fig. 7A), shortening of the colon (Fig. 7B and fig. S11B), observed by histological staining, changes in colonic thickness, and tissue histology with high histology score (Fig. 7, C and D). For  $iT_{reg}$  cells, co-injection of control mock-induced  $iT_{reg}$  cells was ineffective and failed to inhibit signs of colitis (Fig. 7A). In marked contrast, *Bb*-induced  $iT_{reg}$  cells were highly efficient in suppressing colitis, as too were  $nT_{reg}$  cells (Fig. 7A).

Equivalent suppression of colitis was mediated by CSGG-induced  $iT_{reg}$  cells (Fig. 7A). Like *Bb*-induced  $iT_{reg}$  cells, CSGG-induced  $iT_{reg}$  analysis from cLP at the end of the experiment present at a much higher proportion than in the mock-treated control group, indicating enhanced stability of the *Foxp3* locus (Fig. 7E). However, as expected,  $nT_{reg}$  cells are more stable than the  $iT_{reg}$  cells induced in vitro by *Bb* or CSGG treatment (Fig. 7E).

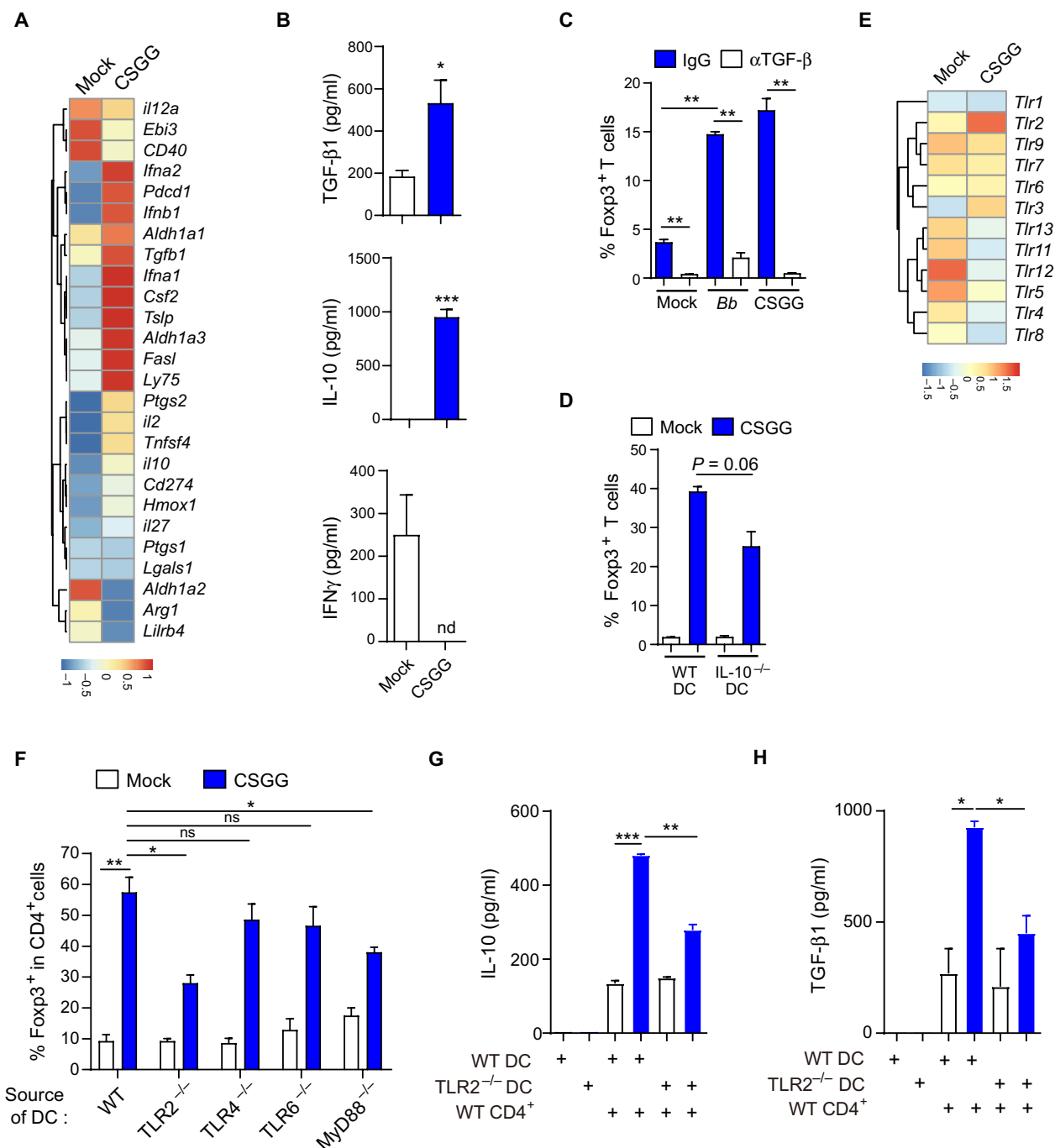
To test whether administration of CSGG itself could suppress colitis development, we adoptively transferred naïve CD4<sup>+</sup>*Foxp3*<sup>-</sup> T cells sorted from CD45.1<sup>+</sup>*Foxp3*<sup>GFP</sup> mice into SPF *Rag1*<sup>-/-</sup> hosts, followed by intraperitoneal administration of PBS or CSGG (100  $\mu$ g per dose) three times a week until the end of the experiment. CSGG administration suppressed colitis development (Fig. 7, F to H, and fig. S11C). Thus, whereas the PBS-treated animals developed colitis and displayed progressive weight loss and enhanced histopathology with high histology score, CSGG treatment largely ameliorated the disease progression (Fig. 7, F to H, and fig. S11C). The protective effect of CSGG treatment was well correlated with an increase in total *Foxp3*<sup>+</sup>  $T_{reg}$  cells (fig. S11D) and reduced frequency of IFN $\gamma$ -producing effector T cells (Fig. 7I). Together, these data suggest that CD4<sup>+</sup>*Foxp3*<sup>+</sup>  $T_{reg}$  cells induced by CSGG treatment are functionally active to suppress the progression of inflammatory colitis.

### DISCUSSION

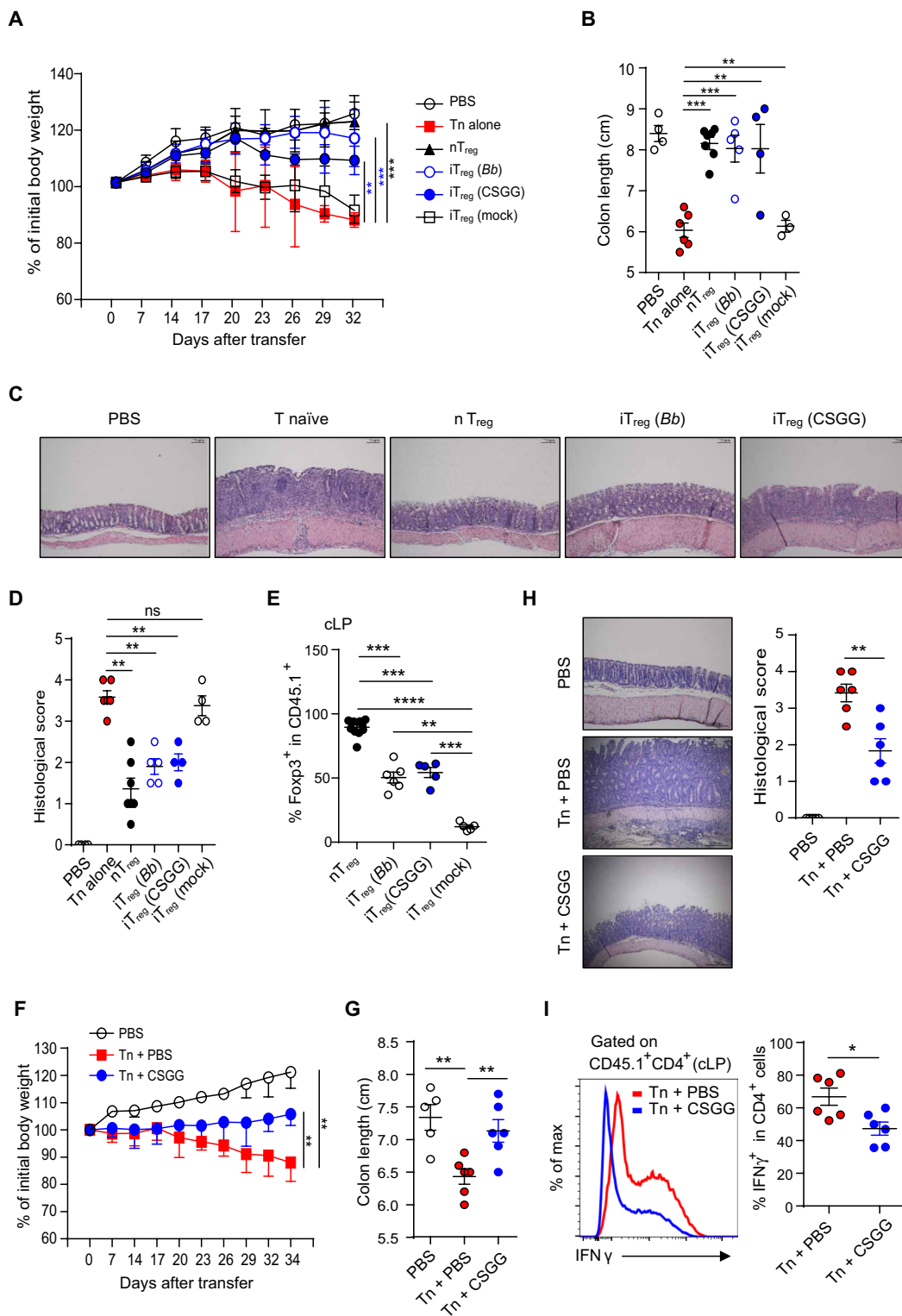
Here, we have demonstrated that *Bb*, a component of the human gut microbiome, can induce the generation of *Foxp3*<sup>+</sup>  $pT_{reg}$  cells that have diverse TCR specificity to dietary Ags, commensal bacteria, and *Bb* itself and that these T cells can potentially suppress intestinal inflammation. Furthermore, we have identified *Bb*-derived CSGG as a key effector component that promotes the *Bb*-dependent induction of  $T_{reg}$  cells.

Previous work has shown that the  $T_{reg}$ -inducing activity of bacterial metabolites involves both T cell intrinsic and extrinsic molecular events that affect generation and function of various immune cells. For example, by virtue of histone deacetylase inhibitory activity, short-chain fatty acids (SCFAs) like butyrate and propionate enhance histone acetylation of the *Foxp3* locus and the acetylated state of the *Foxp3* protein itself, a modification that correlates with enhanced  $T_{reg}$  function (21, 35). In parallel, butyrate negatively affects proinflammatory cytokine production from DCs, thereby facilitating de novo  $T_{reg}$  generation (35). Although a number of studies have established the ability of distinct microbes to drive intestinal T cells to specific fates, identification of the microbial components responsible has not always been feasible.

Here, we have established that *Bb*-dependent *Foxp3*<sup>+</sup>  $pT_{reg}$  induction is mediated by the cell surface polysaccharides CSGG. Although previous reports suggest that PSA or outer membrane vesicles from the *Bf* can induce  $T_{reg}$  cells, the types of  $T_{reg}$  cells might be quite different compared with CSGG-induced cells. PSA mainly generated a *Foxp3*<sup>-</sup> population of  $T_{reg}$  cells with an IL-10<sup>high</sup>IFN $\gamma$ <sup>high</sup> phenotype (2, 6, 15–17, 36), whereas CSGG-induced  $T_{reg}$  cells are mostly *Foxp3*<sup>+</sup>IL-10<sup>high</sup>IFN $\gamma$ <sup>low</sup> phenotype. *Bf* monocolonization mainly generated IFN $\gamma$ <sup>+</sup>IL-10<sup>high</sup> cells with a slight increase in *Foxp3*<sup>+</sup>  $T_{reg}$  cells (2). This discrepancy may be attributed to the differences in the experimental systems such as mouse strain (B6 mice versus Swiss-Webster mice), route of bacterial administration (oral gavage versus spread on food and bedding), and analysis time point after mono-association of the bacteria (3 weeks versus 60 days).



**Fig. 6. CS GG facilitates iT<sub>reg</sub> induction through TLR2-dependent generation of regulatory DCs.** (A) RNA-seq analysis of CD11c<sup>+</sup> splenic DCs treated with mock or CS GG for 4 hours. (B) Cytokine levels in the culture supernatants after coculturing naïve CD4<sup>+</sup> T cells with CD11c<sup>+</sup> DCs pretreated with mock or CS GG. Data are representative of four independent experiments with similar results. nd, not detected. (C) Effect of anti-TGF- $\beta$  Ab treatment on in vitro CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> induction after Bb or CS GG treatment. (D) CD11c<sup>+</sup> DCs from the WT or IL-10<sup>-/-</sup> mice pretreated with CS GG were cocultured with WT naïve CD4<sup>+</sup> T cells, and iT<sub>reg</sub> induction was determined after 3 days. Data are representative of three independent experiments with similar results (C and D). (E) RNA-seq data were analyzed to determine the expression of genes encoding the TLR subtypes upon CS GG treatment. (F) Naïve CD4<sup>+</sup> T cells and CD11c<sup>+</sup> DCs derived from indicated mice, pretreated with mock or CS GG, were cocultured in sub-optimal iT<sub>reg</sub> generation condition. CD4<sup>+</sup>Foxp3<sup>+</sup> iT<sub>reg</sub> population was determined by flow cytometry. Data are representative of five independent experiments with similar results. (G and H) IL-10 and TGF- $\beta$ 1 cytokine levels in the culture supernatants after naïve CD4<sup>+</sup> T cells were cocultured with WT or TLR2-deficient CD11c<sup>+</sup> DCs pretreated with mock or CS GG. Data are representative of at least three independent experiments with similar results. All bar graphs show means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Student's *t* test).



**Fig. 7. CSGG-induced iT<sub>reg</sub> cells are capable of suppressing intestinal inflammation.** (A to D) Naïve CD4<sup>+</sup>Foxp3<sup>-</sup> T cells sorted from Thy1.1<sup>+</sup>Foxp3<sup>GFP</sup> reporter mice were adoptively cotransferred into *Rag1*<sup>-/-</sup> recipients in indicated combinations of iT<sub>reg</sub> cells or nT<sub>reg</sub> cells. Changes in body weight, colon length, histopathology, and histological score of colonic tissue were measured. (E) Analysis of the Foxp3 stability of transferred T<sub>reg</sub> cells in the cLP at the end of the experiment. At the time of transfer, the purity of the sorted CD4<sup>+</sup>Foxp3<sup>GFP</sup> cells was more than 98%. (F to H) Naïve CD4<sup>+</sup>Foxp3<sup>GFP</sup> reporter mice were adoptively transferred into *Rag1*<sup>-/-</sup> mice, followed by intraperitoneal administration of PBS or CSGG (100 µg/ml). Changes in body weight, colon length, histopathology, and histological score in colonic tissue were measured. (I) Analysis of the IFN $\gamma$ -producing effector T cells in cLP at the end of the experiment. Data are representative of at least three independent experiments with similar results ( $n \geq 3$  mice). Graphs with error bars show means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (Student's  $t$  test).

Our data also suggest that *Bb*/CSGG induces T<sub>reg</sub> cells via a DC-dependent mechanism, which involves phenotypic conversion of conventional DCs into a population of rDCs that produce abundant levels of inhibitory cytokines, namely, TGF- $\beta$ 1 (4) and IL-10 (37). *Bb* monoclonization also enriches the number of CD103<sup>+</sup>CD11b<sup>+</sup> DCs in cLP, which potentiates conversion of naïve CD4 T cells into T<sub>reg</sub> cells with TCR specificity to diverse Ags. We also provide evidence that TLR2 expression on DCs, and not on CD4<sup>+</sup> T cells, is largely responsible for the sensing and delivery of CSGG-induced tolerance signals for secretion of IL-10 and TGF- $\beta$ 1, which induce T<sub>reg</sub> cell generation. Previous studies have suggested that bacterial components could induce IL-10-producing T<sub>reg</sub> cells via activation of DCs in a TLR2-dependent manner (16, 17, 38). PSA could directly convert naïve CD4<sup>+</sup> T cells into T<sub>reg</sub> cells in a TLR2-dependent manner (6, 39). However, it is still unclear how TLR2 signaling is involved in the generation of rDCs or T<sub>reg</sub> cells. Previous studies reported that treatment of splenic DCs with TLR2 ligand induces IL-10 and a retinoic acid–metabolizing enzyme, Raldh, thereby inducing iT<sub>reg</sub> cells (38, 40). In addition, it is also reported that, depending on the types of TLR agonists, they differentially modulate extracellular signal–regulated kinase signaling, c-Fos activity, and cytokine responses in DCs to induce different T<sub>H</sub> responses (41). Although TLR2 and MyD88 are important for *Bb*/CSGG-dependent induction of T<sub>reg</sub> cells, other PRRs might also be involved. In the future, we will direct our studies to identify these molecules and to understand the relative contributions of these pathways to *Bb*/CSGG-dependent induction of T<sub>reg</sub> cells. In addition, further investigations are required to understand how *Bb*/CSGG could induce T<sub>reg</sub> cells in the spleen and pLN in addition to the mucosal lymphocytes. These could be exerted by several possibilities: (i) CSGG may circulate systemically, thereby inducing T<sub>reg</sub> cells elsewhere; (ii) T<sub>reg</sub> cells generated at the mucosal sites (colon, siLP, and mLN) may emigrate to spleen and pLN; and/or (iii) alteration of intestinal permeability may account for the observed induction of T<sub>reg</sub> cells at these sites in monoclonized mice.

*Bb* induced pT<sub>reg</sub> cells that display potent suppressive capacity against intestinal inflammation. Colonic T<sub>reg</sub> cells isolated from *Bb*-monoclonized mice have distinct TCR repertoire and could proliferate in response to *Bb*-derived Ags. These findings suggest that, although dominant colonic T<sub>reg</sub> cell clones may be originated from thymic selection (42), *Bb* administration could induce the conversion of naïve CD4<sup>+</sup>Foxp3<sup>-</sup> cells into the CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the colon, which could suppress colitis mediated by commensal-induced inflammatory T cells. Patients with inflammatory bowel disease were reported to have specific reduction in *Bifidobacterium* as compared with healthy controls (43). *Bifidobacterium* are members of bacterial species known to colonize the gut of breastfed infants early in life (44). Administration of *Bifidobacterium* to the allergic infants with aberrant composition of gut microbiota alleviated allergic inflammation (45). The identification of CSGG as an active component of T<sub>reg</sub>-inducing *Bb* able to suppress colitis thus emerges as a potentially important mediator for establishing immune homeostasis in the gut. Moreover, because *Bb* could induce food-reactive T<sub>reg</sub> cells, administration of CSGG-producing *Bb* could be considered for the treatment of allergic diseases.

## MATERIALS AND METHODS

### Mice

Mice were maintained in the animal facility of POSTECH Biotech Center, and all the experimental procedures were approved by the

POSTECH Institutional Animal Care and Use Committee. A colony of GF C57BL/6 (B6) mice was established at POSTECH from breeders obtained from A. Macpherson (Bern University, Switzerland) and D. Artis (then at University of Pennsylvania, currently at Cornell University, USA) and maintained in sterile flexible film isolators (Class Biological Clean Ltd., USA). GF status was monitored monthly by culture of cecal contents. *Foxp3*-eGFP, *Tlr2*<sup>-/-</sup>, *Tlr4*<sup>-/-</sup>, *Tlr6*<sup>-/-</sup>, and *MyD88*<sup>-/-</sup> animals were obtained from the Jackson Laboratory. C57BL/6-CD45a(*Ly5a*)-*Rag1*<sup>-/-</sup> TCR OT-II (*Rag1*<sup>-/-</sup> OT-II TCR transgenic) and *Rag1*<sup>-/-</sup> mice were obtained from Taconic. *Dectin1*<sup>-/-</sup> and *Dectin2*<sup>-/-</sup> mice were provided by Y. Iwakura (Tokyo University of Science, Japan). The CBir mouse was a gift from C. O. Elson (University of Alabama at Birmingham). Gender- and age-matched mice between 6 and 12 weeks old were used.

### Bacterial strain and analysis of bacterial colonization

*Bb* was anaerobically cultured in MRS media (BD Difco) supplemented with 0.1% L-cysteine. *Lpa* was cultured in MRS media. *Bf* (ATCC 25285; NCTC 9343) was obtained from ATCC and anaerobically cultured in GAM Broth (code: 05422; Nissui, Japan) (46). For monoclonization, lyophilized bacteria [ $5 \times 10^8$  colony-forming units (CFU)/200  $\mu$ l] were administered once orally to GF mice, and their colonization was confirmed by HISTO-FISH or DNA sequencing. *Bf* monoclonization was confirmed by DNA sequencing. National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) analysis confirmed that nucleotide sequences correspond to the *Bf*NCTC 9343 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (fig. S12).

### Data availability for RNA-seq

Total RNA was extracted from the splenic CD11c<sup>+</sup> DCs stimulated with mock or CSGG (100  $\mu$ g/ml) for 4 hours and purified with Ribospin II (GeneAll Biotechnology). RNA quantitation and quality control were performed using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). Library preparation was performed using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA), and RNA-seq was performed using the NextSeq 500 Sequencing System.

### Purification of cell surface polysaccharides from *Bb*

Cultured *Bb* was harvested and washed by PBS two times. Purification of cell surface polysaccharide was performed as previously described (47) with minor modifications. Briefly, acidic phenol (Sigma-Aldrich) treatment was performed at 68°C to extract capsular polysaccharides, and residual phenol was removed by ether treatment followed by dialysis against distilled water for 3 days. To remove nucleic acids and proteins, we performed DNase I (Roche) and RNase (Sigma-Aldrich) digestion overnight at 37°C, followed by Pronase (protease from *Streptomyces griseus*; Sigma-Aldrich) digestion at 37°C overnight. After acetic acid treatment, centrifugation was performed to remove precipitates. Chilled ethanol was added to precipitate polysaccharides and then dialyzed against distilled water for 3 days and freeze dried. Purified polysaccharides were dissolved in water, and gel filtration was performed by high-performance liquid chromatography (HPLC) column (TSKgel G5000PWXL, Tosho). Anion exchange chromatography was performed (HiPrep Q FF 16/10, GE Healthcare) to further separate neutral and negative charge polysaccharides. The concentration of polysaccharide was determined by acid phenol assay (48).

## SUPPLEMENTARY MATERIALS

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## Materials and Methods

Fig. S1. Identification of *Bb* as T<sub>reg</sub>-inducing bacteria.

Fig. S2. Effect of *Bb* monocolonization on cytokine levels in T<sub>reg</sub> and non-T<sub>reg</sub> cells.

Fig. S3. *Bb* monocolonization facilitates de novo generation of pT<sub>reg</sub> cells.

Fig. S4. *Bb* monocolonization induces dietary Ag- and/or microbiota-reactive T<sub>reg</sub> cells.

Fig. S5. Effect of *Bb* monocolonization on the TCR repertoire of T<sub>reg</sub> cells.

Fig. S6. Effect of *Bb* monocolonization on phenotypes and population of cLP-DC subtypes.

Fig. S7. Effect of *Bb* monocolonization on phenotypes and population of DC subtypes in mLN and sILP.

Fig. S8. CSGG of the *Bb* enhances T<sub>reg</sub> cell induction.

Fig. S9. Role of DC subtypes in inducing *Bb*/CSGG-mediated iT<sub>reg</sub> cells.

Fig. S10. CSGG facilitates iT<sub>reg</sub> induction through TLR2-mediated generation of regulatory DCs.

Fig. S11. CSGG-induced iT<sub>reg</sub> cells are capable of suppressing intestinal inflammation.

Fig. S12. Confirmation of *Bb* monocolonization by DNA sequencing.

Table S1. Peptide sequences of  $\alpha$ -chain CDR3 region of T<sub>reg</sub> cells sorted from colon, mLN, and spleen of *Bb*-monocolonized mice compared with GF mice.

Table S2. Peptide sequences of  $\beta$ -chain CDR3 region of T<sub>reg</sub> cells sorted from colon, mLN, and spleen of *Bb*-monocolonized mice compared with GF mice.

Table S3. Raw data (Excel file).

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**Acknowledgments:** We thank H. Jung for technical assistance for cell sorting, Y. S. Gho for HPLC analysis, K. S. Lee for mouse husbandry, and S.-W. Lee and Y.-M. Kim for helpful discussions and comments on this study. **Funding:** This work was supported by a grant from the Institute for Basic Science, Korea (IBS-R005). **Author contributions:** R.V. and S.-H.I. designed the studies. R.V., D.R., and S.-H.I. wrote the manuscript. R.V. performed most of the experiments and analyzed the data. R.V., C.-D.J., and S.-H.I. participated in data acquisition and analysis and provided the specialized technical and intellectual support to the project. C.L., S.B., and E.-J.J. participated by performing the experiments. R.V., C.L., G.-C.K., and C.-G.L. performed RNA-seq data analysis. J.Y. and K.S.K. helped in GF experiments and provided intellectual contributions. C.D.C. and A.M. performed NMR and data analysis. C.-G.L. and H.-J.K. performed SCFA analysis. C.-H.S. and J.-Y.J. provided human samples. G.J. performed the fractionation of *Bb*. D.R. provided intellectual suggestions during the course of the study and edited the manuscript along with S.-H.I., J.S., and C.D.S. S.-H.I. has full access to all data in the study and takes responsibility for the integrity of the data, as well as for the manuscript. All authors have read and approved the final manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** The RNA-seq data were deposited in the Gene Expression Omnibus (NCBI) data repository under accession number GSE98947.

Submitted 5 April 2018

Accepted 14 August 2018

Published 19 October 2018

10.1126/sciimmunol.aat6975

**Citation:** R. Verma, C. Lee, E.-J. Jeun, J. Yi, K. S. Kim, A. Ghosh, S. Byun, C.-G. Lee, H.-J. Kang, G.-C. Kim, C.-D. Jun, G. Jan, C.-H. Suh, J.-Y. Jung, J. Sprent, D. Rudra, C. De Castro, A. Molinaro, C. D. Surh, S.-H. Im, Cell surface polysaccharides of *Bifidobacterium bifidum* induce the generation of Foxp3<sup>+</sup> regulatory T cells. *Sci. Immunol.* **3**, eaat6975 (2018).

## Cell surface polysaccharides of *Bifidobacterium bifidum* induce the generation of Foxp3<sup>+</sup> regulatory T cells

Ravi Verma, Changhon Lee, Eun-Ji Jeun, Jaew Yi, Kwang Soon Kim, Ambarnil Ghosh, Seohyun Byun, Choong-Gu Lee, Hye-Ji Kang, Gi-Cheon Kim, Chang-Duk Jun, Gwenaël Jan, Chang-Hee Suh, Ju-Yang Jung, Jonathan Sprent, Dipayan Rudra, Cristina De Castro, Antonio Molinaro, Charles D. Surh and Sin-Hyeog Im

*Sci. Immunol.* **3**, eaat6975.

DOI: 10.1126/sciimmunol.aat6975

### Deconstructing probiotics

Besides supporting host metabolism, our intestinal microbiota also play a vital role in modulating functions of immune cells in the gut. Here, Verma *et al.* have examined how a particular probiotic strain, *Bifidobacterium bifidum*, promotes the generation of T regulatory (T<sub>reg</sub>) cells in the intestine. They have identified β-glucan/galactan polysaccharides derived from the cell wall of *B. bifidum* to be responsible for promoting T<sub>reg</sub> cell induction in the intestine. Further, they report this process to be dependent on intestinal dendritic cells that express Toll-like receptor 2. Studies such as this open up the possibility of using microbial components rather than live microbes to treat microbial dysbiosis associated with gastrointestinal disorders including colitis and Crohn's disease.

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