Cell surface polysaccharides of Bifidobacterium bifidum induce the generation of Foxp3+ regulatory T cells

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Dysregulation of intestinal microflora is linked to inflammatory disorders associated with compromised immunosuppressive functions of Foxp3+ regulatory T (Treg) cells. Although mucosa-associated commensal microbiota has been implicated in Treg 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+ Treg 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 Treg induction. CSGG efficiently recapitulated the activity of whole bacteria and acted via regulatory dendritic cells through a partially Toll-like receptor 2–mediated mechanism. Treg 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 Treg-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 in complete germ-free (GF) settings display disorganized lymphoid tissues and abnormal immune responses; these deficits are corrected

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(CSGG) polysaccharides as the key effector component able to induce T<sub>reg</sub> cells with the capacity to ameliorate intestinal inflammation.

**RESULTS**

*Bb* monocolonization induces de novo generation of pT<sub>reg</sub> cells

To identify T<sub>reg</sub>-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 protein) (Foxp3<sup>GFP</sup>) reporter mice, thereby enabling detection of T<sub>reg</sub> 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>CD<sup>+</sup>GFP<sup>+</sup> T<sub>reg</sub> 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<sub>reg</sub>-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<sub>reg</sub> population in the cLP.](image-url)

(A) Localization analysis of Bb in the intestinal niches of GF mice by HISTO-FISH staining with DNA-Cys probes (EU8338, 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<sub>reg</sub> 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 ≥ 3 mice). All graph plots show means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (Student's t test). MFI, mean fluorescence intensity; ns, not significant.
cicum 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 Foxp3+ Treg 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 mono-colonized with the Bf was that previously reported to induce Treg cells (2, 16, 22) did not show any substantial increase in Foxp3+ Treg cells (Fig. 1B and fig. S1D). Treg cells in the cLP of Bb-monocolonized mice displayed a significantly higher proportion of CD103+ and memory-phenotype (CD62LloCD44hi) cells compared with Treg cells in unmanipulated GF mice, suggesting a higher state of Treg cell activation (Fig. 1, C and D, and fig. S1, G and H). Consistent with this idea, cLP Treg 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 CTLA4 and IL-10 were increased in Treg cells (fig. S2, A and B). A slight increase in IFNγ levels was observed in CD4+Foxp3− non-Treg cells in the Bb monocolonized mice (fig. S2, A and B).

Expression of the IKAROS family transcription factor Helios and neuropilin 1 (Nrp1) is widely used to distinguish HeliosloNrp1− thymically generated Treg (tTreg) cells from Helios+ Nrp1− peripherally generated Treg (pTreg) cells (37) when generated in vitro culture conditions (27–29). Whereas a small increase in Nrp1+ Treg cells was observed in Bb-colonized cLP, a much greater increase was observed for Nrp1+ Helioslo pTreg cells in all organs tested, indicating that Bb primarily promotes generation of pTreg cells (Fig. 2, A to D, and fig. S3, A and B). Furthermore, a large proportion of the newly generated pTreg cells expressed the transcription factor RORγt (RORγt Helioslo Foxp3+ and RORγt 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 Treg cells, we adoptively transferred allelically marked naïve phenotype CD4+CD45.1Foxp3+CD62LloCD44lo T cells sorted from Foxp3GFP mice into mice monocolonized with Bb (fig. S3E). Analysis of the donor cells 3 weeks later revealed emergence of a significant proportion of Foxp3+ Treg 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 pTreg cells by eliciting de novo up-regulation of Foxp3 in naïve CD4+ T cells, primarily at the sites of colonization. Similarly enhanced induction of pTreg 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 CD4+Foxp3+ cells, strongly suggesting that the Treg cell–inducing property of Bb is physiologically relevant (Fig. 2H).

**Bb colonization induces pTreg cells with a broad range of TCR specificity to diverse Ags**

Next, we analyzed the T cell receptor (TCR) specificity of pTreg cells generated in the presence of Bb. pTreg 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 pTreg cells to dietary Ags, we adoptively transferred Cell Trace Violet (CTV)–labeled ovalbumin (OVA)–specific naïve OT-II TCR transgenic CD4+ cells from OT-II. Thy1.1 Foxp3GFP 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 Foxp3 in the siLP and cLP. Notably, in Bb-monocolonized mice, but not in the Lpa-monocolonized mice, the efficiency of 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 pTreg cells specific to microbiota, we used CBir TCR transgenic mice that recognize bacterial flagellin (34). Naïve CD4+Foxp3− T cells sorted from CBir transgenic mice on a CD45.1Foxp3GFP 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 Treg cells (Fig. 3, E and F) but a high proportion of CD4+IFNγ+ and RORγtFoxp3+ 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 Treg cells along with RORγtFoxp3+Treg cells but few CD4+IFNγ+ cells (Fig. 3, C to G, and fig. S4C).

To test whether the pTreg cells induced by Bb monocolonization have specificity for Bb itself, we performed the following experiments. Total colonic CD4+ T cells containing Foxp3+ Treg cells from the GF mice and Bb-monocolonized mice were sorted and labeled with CTV and then cocultured with T cells–depleted splenic antigen-presenting cells (APCs) pretreated with fecal Ags from normal GF mice and mice monocolonized 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 Foxp3+ Treg cells from Bb-monocolonized mice displayed sustained Foxp3 expression and proliferation when stimulated with Bb fecal Ag-treated APC (Fig. 4A). By contrast, coculturing of total colonic CD4+ with APCs treated with fecal Ags from GF- or Lpa-monocolonized mice failed to sustain Treg 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 α and β chains of Foxp3+ Treg cells sorted from colon, mLN, and spleen. Although both Treg cells showed a similar pattern of diversity in TCR α and β chains in spleen and mLN, colonic Treg 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 Treg 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 CD4+Foxp3+ Treg cells with a broad range of TCR specificity to dietary Ags and/or commensal microbiota and Bb itself.

**Bb facilitates Treg cell induction through a dendritic cell–dependent mechanism**

Because intestinal induction of pTreg cells from naïve T cells requires an immunoregulatory cytokine milieu, we tested whether colonization
**Fig. 2.** *Bb* monocolonization facilitates de novo generation of pTreg 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+ and Nrp1− Treg cells. (B to F) Representative flow cytometry plots and frequencies for Nrp1+Foxp3− (B), Helios+Foxp3− (C), Helios−Nrp1−Foxp3− (D), RORγt+Helios−Foxp3− (E), and RORγt+Foxp3−, RORγt−Foxp3+ (F) T reg cells in the cLP of GF or *Bb*-or *Lpa*-monocolonized mice. (G) Naïve CD4+Foxp3− T cells sorted from CD45.1+Foxp3GFP reporter mice were transferred into GF mice. Animals were either left GF or monocolonized with *Bb* for 3 weeks. Foxp3+ Treg population was analyzed by GFP expression in the cLP. (H) Naïve CD4+Foxp3+ T cells sorted from CD45.1+Foxp3GFP reporter mice were transferred into SPF mice, and then mice were fed with mock (PBS) or *Bb* (5 × 10^8 CFU) every other day for 3 weeks and analyzed for Foxp3+ Treg 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 Treg cells. (A and B) CTV-labeled naïve allelically marked CD4+Thy1.1+Foxp3+ T cells from OT-IlThy1.1+Foxp3GFP 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+Foxp3+ population of the donor (Thy1+OT-Il) cells was analyzed. Representative fluorescence-activated cell sorting (FACS) plots (A) and frequencies of Treg cells generated in vivo (B) are shown. (C to E) Naïve CD4+CBir+CD45.1+Foxp3+ T cells sorted from CBir+CD45.1+Foxp3GFP mice were adoptively transferred into the SPF Rag1−/− 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+CD45.1+Foxp3+ Treg cells (F) and CD4+IFNγ+ 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 ≥ 3 mice). Graphs with error bars show means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 (Student's t test).
Fig. 4. *Bb* monocolonization induces *Bb*-reactive colonic T\(_{\text{reg}}\) cells with distinctive TCR repertoires. (A) Total colonic CD4\(^+\) T cells containing Foxp3\(^+\) T\(_{\text{reg}}\) 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\(_{\text{reg}}\) cells was analyzed by FACS. (B) Comparison of Shannon diversity from TCR \(\alpha\) and \(\beta\) chains of T\(_{\text{reg}}\) 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\(_{\text{reg}}\) 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+CD11c+CD11b+CD103+Foxp3+CD4+80+; isolated from the Bb-monocolonized mice displayed a significant increase in the expression of mRNA for a number of inhibitory molecules, namely, Il10, Csf2, Tgfβ1, 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 iTreg differentiation. We further analyzed the different subtypes of DCs affected by Bb monocolonization in the cLP, sLP, and mLN. In the cLP, we could observe a slight increase in CD103+CD11b+ and plasmacytoid DC (pDC) (Bst2+CD11c+) population but not in CX3CR1+CD11b+ population (Fig. 5D and fig. S6, B to D). In the mLN, but not in sLP, a significant increase in CD103+CD11b+ DCs and CX3CR1+CD11b+ population was observed (fig. S7, A and B). These results indicate that CD103+ cLP-DCs may play a key role in Bb-mediated iTreg induction in the colon. To examine this possibility, we performed an in vivo mimicking experiment by culturing sorted cLP-DCs (MHCII+CD11c+CD11b+CD103+Foxp3+CD4+80+) with Bb for 10 to 12 hours followed by washing and then coculture with naïve CD4+Foxp3+ T cells for 3 days in suboptimal iTreg-inducing conditions. Notably, pretreatment of cLP-DCs with Bb resulted in the significant induction and proliferation of iTreg cells relative to mock-treated group (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 iTreg or IL-10 production in these culture conditions (Fig. 5E).

**CSGG are the active components of Bb facilitating iTreg cell induction**

Using the above in vitro system, we performed experiments to identify Bb-derived effector molecules that facilitate iTreg differentiation. Because of limited availability of cLP-DCs, we used splenic DCs for these experiments in suboptimal iTreg-inducing conditions. Because Bb is strictly anaerobic and mostly nonviable during coculture with Bb, we tested whether some of its cellular components might act as effector molecules with iTreg-inducing activity. Among the subcellular fractions comprising cell surface, cell membrane, and cytosol components, only the cell surface extract effectively promoted iTreg generation (fig. S8A). Treatment of cell surface extract with ribonuclease (RNase), deoxyribonuclease (DNase), Pronase, or boiling at high temperature did not reduce the iTreg-inducing activity, suggesting that polysaccharides might be the effector molecules. Incubating DCs with total cell surface polysaccharide (tCSPS) extract effectively induced iTreg 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 their 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 phosphoglycerophosphate-lactofuran (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 iTreg cells in vitro. Significantly, only the neutral polysaccharides, and not the negatively charged PGβG, facilitated induction of iTreg 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 (CSGβG) might be the key effector molecule. Treatment of CSGG with β-1,6-glucanase reduced CSGG-induced iTreg 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 iTreg cells in vitro and in vivo. Naïve CD4+Foxp3+ T cells were cocultured with DCs pretreated with Bb, CSGG, Bf, or Lpa in suboptimal iTreg-inducing conditions. Significantly, the CSGG-treated DCs induced Foxp3+ iTreg cells as effectively as Bb-treated DCs, whereas Bf- or Lpa-treated DCs failed to induce iTreg cells (fig. S8D). Coculturing of naïve CD4+Foxp3+ T cells with DCs pretreated with Bf induced high levels of IFNγ and IL-10 mostly in Foxp3+ T cells (fig. S8E). Next, we tested whether CSGG could induce pTreg cells in vivo. Intraperitoneal injections of CSGG (100 μg per dose) into GF mice for 3 weeks induced CD4+Foxp3+ and Nrp1+ Rorγt+ Treg cells similar to the levels of Bb-monocolonized mice in the cLP and mLN (Fig. 5I and fig. S8F). These Treg cells displayed a higher proportion of CD103+ and activated CD44+CD62L+ cells than Treg cells from control GF mice (fig. S8G). Next, we tested whether CSGG treatment could induce Treg cells in the human CD4+ T cells. Coculturing of CSGG-treated human DCs and naïve CD4+ T cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors significantly induced CD25+Foxp3+ Treg cells in a dose-dependent manner (Fig. 5J).

**CSGG induces Treg cells through generation of regulatory DCs**

Treatment of naïve CD4+ T cells with CSGG in the absence of DCs failed to induce Foxp3+ Treg cells, implying a DC-dependent mechanism for iTreg induction (fig. S9A). We therefore tested the role of different DC subtypes for generation of CSGG-induced iTreg cells (fig. S9B). pDCs, cDCs, and CX3CR1+ and CX3CR1− DCs were sorted from the spleen; treated with mock, Bb, or CSGG; and then cocultured with naïve CD4+Foxp3+ T cells in suboptimal iTreg-inducing conditions. Although all the tested DC subtypes were able to induce iTreg cells upon Bb or CSGG treatment, cDCs, pDCs, and CX3CR1− cells induced high levels of iTreg cells (fig. S9, C and D). We also tested whether pretreatment of DCs with CSGG could induce Ag-specific iTreg generation because Bb administration induced pTreg cells with a broad range of TCR specificity to diverse Ags in vivo (Figs. 3 and 4). Sorted total splenic DCs (MHCII+CD11c+), as well as either of the two main types of DCs (CD8α−; MHCII+CD11c+CD11b+ and CD8α+; MHCII+CD11c−CD11b+), were pretreated with CSGG, and then naïve OT-II TCR transgenic CD4+ cells from OT-II.Thy1.1Foxp3+GFP mice were cultured in the presence of OVA Ag (fig. S9E). CSGG treatment potentiated Ag-specific iTreg generation (fig. S9F). 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+ splenic DCs treated for 4 hours with CSGG. Compared with mock control treatment, CSGG treatment significantly elevated levels of rDC-associated markers such as Ifna2, Pdcd1, Tgfb1, Csf2, Ptgs2, Iil10, and...
Fig. 5. Cell surface CSGG of Bb mediates T<sub>reg</sub> 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 (MHC<sup>I</sup>/CD11c<sup>+</sup>CD11b<sup>−</sup>CD103<sup>−</sup>/CD103<sup>+</sup>) of GF or Bb- or Lpa-monocolonized mice. Data are representative of three independent experiments with similar results. (D) Graph shows absolute number of cLP-DCs from GF mice or monocolonized with Lpa or Bb for CD103<sup>−</sup>, CD11b<sup>−</sup>, CX3CR1, and pDCs (Bst2<sup>−</sup>/CD11c<sup>−</sup>). Numbers represent the frequency of each population from the total CD11c<sup>+</sup>MHC<sup>I</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<sub> cells</sub> in suboptimal T<sub>reg</sub>-inducing conditions for 3 days, after which Foxp3<sup>+</sup> T<sub>reg</sub> cells were analyzed within live cells. Representative flow cytometric analysis and mean frequencies of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> 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 (PG<sub>G</sub>) isolated from the tCSPS of Bb, Glcp, glucopyranosyl, Galp, lactopyranosyl, Galf, glycuronol, Gro, glycerol. n = repeating unit. Relative abundances of each polysaccharide are shown as % (mol/mol). (G) Comparison of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>-inducing activity between the neutral (CSGG, 50 µg/ml) and negatively charged polysaccharides (PG<sub>G</sub>, 100 µg/ml) compared with tCSPS (100 µg/ml). (H) Effect of β-1,6-glucanase treatment on CSGG-induced CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells. (I) Populations and phenotypes of T<sub>reg</sub> cells in GF mice or mice monoclonized 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>Foxp3<sup>+</sup> T<sub>reg</sub> cells by coculturing of DCs pretreated with CSGG with naïve CD4<sup>+</sup> T<sub> cells</sub> 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-β1 and decreased IFNγ levels measured from culture supernatants (Fig. 6B). Moreover, compared with IL-10, TGF-β played a pivotal role in iTreg generation because addition of anti–TGF-β–neutralizing antibody (Ab) almost completely abolished CSGG-mediated iTreg induction (Fig. 6C). IL-10–deficient DCs also displayed a tendency toward reduced iTreg 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+ DCs isolated from mouse strain lacking TLR2 (TLR2−/−) displayed a significant reduction in iTreg-inducing activity compared with WT DCs (Fig. 6F and fig. S10A). TLR2-deficient DCs elicited reduced levels of IL-10 and TGF-β1 in the cultures (Fig. 6, G and H) but no change in IFNγ 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, Treg induction was unaffected when TLR3−/−TLR7−/−TLR9−/− triple knockout DCs were used (fig. 10C). To test potential involvement of C-type lectin receptors, we cultured Dectin1−/− and Dectin2−/− DCs or CD11c+ DCs pretreated with blocking Ab for DC-SIGN and mannose receptor with naïve CD4+ T cells in the presence of CSGG. However, no significant reduction was observed in the level of CSGG-induced iTreg 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 iTreg cells. However, provided that the TLR2/−DCs still retained low degree of Treg induction activity upon CSGG treatment (Fig. 6F), it seems possible that additional signaling pathways may also be involved.

CSGG-induced iTreg cells are capable of suppressing intestinal inflammation

The identification of CSGG as the major Treg-inducing component of Bb suggested that identification of CSGG might be effective in generating and boosting Treg cell function and thereby suppressing inflammatory conditions. Under in vitro conditions, sorted iTreg cells generated in the presence of CSGG-treated DCs displayed significantly enhanced suppressive capacity relative to iTreg cells induced with mock-treated DCs (fig. S11A). For in vivo studies, we used the T cell transfer model of colitis induction. Naïve CD4+ T cells were transferred into Rag1−/− lymphopoenic hosts, either alone or together with various types of sorted alellically marked (CD45.1+) iTreg cells. nTreg cells sorted from the Foxp3GFP+ 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 iTreg cells, co-injection of control mock-induced iTreg cells was ineffective and failed to inhibit signs of colitis (Fig. 7A). In marked contrast, Bb-induced iTreg cells were highly efficient in suppressing colitis, as too were nTreg cells (Fig. 7A). Equivalent suppression of colitis was mediated by CSGG-induced iTreg cells (Fig. 7A). Like Bb-induced iTreg cells, CSGG-induced iTreg 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, nTreg cells are more stable than the iTreg 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+Foxp3+ T cells sorted from CD45.1+Foxp3GFP+ mice into SPF Rag1−/− hosts, followed by intraperitoneal administration of PBS or CSGG (100 μg 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+ Treg cells (fig. S11D) and reduced frequency of IFNγ-producing effector T cells (Fig. 7I). Together, these data suggest that CD4+ Foxp3+ Treg 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+ iTreg 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 Treg cells.

Previous work has shown that the Treg-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 Treg function (21, 35). In parallel, butyrate negatively affects proinflammatory cytokine production from DCs, thereby facilitating de novo Treg 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+ iTreg induction is mediated by the cell surface polysaccharides CSGG. Although previous reports suggest that PSA or outer membrane vesicles from the Bf can induce Treg cells, the types of Treg cells might be quite different compared with CSGG-induced cells. PSA mainly generated Foxp3+ population of Treg cells with an IL-10highIFNγlow phenotype (2, 6, 15–17, 36), whereas CSGG-induced Treg cells are mostly Foxp3+IL-10+IFNγ−low phenotype. Bf monocolonization mainly generated IFNγ+IL-10+high cells with a slight increase in Foxp3+ Treg 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 beding), and analysis time point after monoclonization of the bacteria (3 weeks versus 60 days).

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Fig. 6. CSGG facilitates iTreg induction through TLR2-dependent generation of regulatory DCs. (A) RNA-seq analysis of CD11c+ splenic DCs treated with mock or CSGG for 4 hours. (B) Cytokine levels in the culture supernatants after coculturing naïve CD4+ T cells with CD11c+ DCs pretreated with mock or CSGG. Data are representative of four independent experiments with similar results. nd, not detected. (C) Effect of anti–TGF-β Ab treatment on in vitro CD4+Foxp3+ iTreg induction after Bb or CSGG treatment. (D) CD11c+ DCs from the WT or IL-10−/− mice pretreated with CSGG were cocultured with WT naïve CD4+ T cells, and iTreg 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 CSGG treatment. (F) Naïve CD4+ T cells and CD11c+ DCs derived from indicated mice, pretreated with mock or CSGG, were cocultured in suboptimal iTreg generation condition. CD4+Foxp3+ iTreg population was determined by flow cytometry. Data are representative of five independent experiments with similar results. (G and H) IL-10 and TGF-β1 cytokine levels in the culture supernatants after naïve CD4+ T cells were cocultured with WT or TLR2-deficient CD11c+ DCs pretreated with mock or CSGG. Data are representative of at least three independent experiments with similar results. All bar graphs show means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t test).
Fig. 7. CSGG-induced iTreg cells are capable of suppressing intestinal inflammation. (A to D) Naïve CD4+Foxp3− T cells sorted from Thy1.1+Foxp3GFP reporter mice were adoptively cotransferred into Rag1−/− recipients in indicated combinations of iTreg cells or nTreg cells. Changes in body weight, colon length, histopathology, and histological score of colonic tissue were measured. (E) Analysis of the Foxp3 stability of transferred Treg cells in the cLP at the end of the experiment. At the time of transfer, the purity of the sorted CD4+Foxp3GFP+ cells was more than 98%. (F to H) Naïve CD4+Foxp3− T cells sorted from CD45.1+Foxp3GFP reporter mice were adoptively transferred into Rag1−/− 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 IFNγ-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 ≥ 3 mice). Graphs with error bars show means ± 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 Treg 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-β1 (4) and IL-10 (37). Bb monocolonization also enriches the number of CD103+CD11b+ DCs in cLP, which potentiates conversion of naive CD4 T cells into Treg cells with TCR specificity to diverse Ags. We also provide evidence that TLR2 expression on DCs, and not on CD4+ T cells, is largely responsible for the sensing and delivery of CSGG-induced tolerance signals for secretion of IL-10 and TGF-β1, which induce Treg cell generation. Previous studies have suggested that bacterial components could induce IL-10–producing Treg cells via activation of DCs in a TLR2-dependent manner (16, 17, 38). PSA could directly convert naive CD4+ T cells into Treg cells in a TLR2-dependent manner (6, 39). However, it is still unclear how TLR2 signaling is involved in the generation of rDCs or Treg cells. Previous studies reported that treatment of splenic DCs with TLR2 ligand induces IL-10 and a retinoic acid–metabolizing enzyme, Raldh, thereby inducing iTreg 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 Th1 responses (41). Although TLR2 and MyD88 are important for Bb/CSGG–dependent induction of Treg 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 Treg cells. In addition, further investigations are required to understand how Bb/CSGG could induce Treg 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 Treg cells elsewhere; (ii) Treg 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 Treg cells at these sites in monoclonized mice.

Bb-induced pTreg cells that display potent suppressive capacity against intestinal inflammation. Colonic Treg 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 Treg cell clones may be originated from thymic selection (42), Bb administration could induce the conversion of naive CD4+Foxp3+ cells into the CD4+Foxp3+ 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 Treg–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 Treg 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 Biological Clean Ltd., 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−/−, Tlr4−/−, Tlr6−/−, and MyD88−/− animals were obtained from the Jackson Laboratory. C57BL/6-CD45a(Ly5a)-Rag1−/− TCR OT-1 (Rag1−/− OT-1 TCR transgenic) and Rag1−/− mice were obtained from Taconic. Dectin1−/− and Dectin2−/− 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 monocolonization, lyophilized bacteria [5 × 10⁸ colony-forming units (CFU)/200 μl] were administered once orally to GF mice, and their colonization was confirmed by HISTO-FISH or DNA sequencing. Bf monocolonization 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+ DCs stimulated with mock or CSGG (100 μg/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 dia lyzed 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).

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Cell surface polysaccharides of *Bifidobacterium bifidum* induce the generation of Foxp3+ regulatory T cells

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**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 (Treg) cells in the intestine. They have identified β-glucan/galactan polysaccharides derived from the cell wall of *B. bifidum* to be responsible for promoting Treg 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.