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Foxp3⁺ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis

Shimpei Kawamoto,[1,6] Mikako Maruya,[1,6] Lucia M. Kato,[1,6] Wataru Suda,[4] Koji Atarashi,[2] Yasuko Doi,[1] Yumi Tsutsui,[1] Hongyan Qin,[1,5] Kenya Honda,[2] Takaharu Okada,[3] Masahira Hattori,[4] and Sidonia Fagarasan[1,*]
[1]Laboratory for Mucosal Immunity
[2]Laboratory for Gut Homeostasis
[3]Laboratory for Tissue Dynamics
Center for Integrative Medical Sciences (IMS-RCAI), RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi, Yokohama, 230-0045 Kanagawa, Japan
[4]Center for Omics and Bioinformatics, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwa-no-ha, Kashiwa, 277-8561 Chiba, Japan
[5]Department of Medical Genetics and Developmental Biology, 4th Military Medical University, Chang-Le Xi Street #17, Xi'an 710032, China
[6]Co-first author
*Correspondence: sidonia-f@rcai.riken.jp
http://dx.doi.org/10.1016/j.immuni.2014.05.016

SUMMARY

Foxp3⁺ T cells play a critical role for the maintenance of immune tolerance. Here we show that in mice, Foxp3⁺ T cells contributed to diversification of gut microbiota, particularly of species belonging to Firmicutes. The control of indigenous bacteria by Foxp3⁺ T cells involved regulatory functions both outside and inside germinal centers (GCs), consisting of suppression of inflammation and regulation of immunoglobulin A (IgA) selection in Peyer's patches, respectively. Diversified and selected IgAs contributed to maintenance of diversified and balanced microbiota, which in turn facilitated the expansion of Foxp3⁺ T cells, induction of GCs, and IgA responses in the gut through a symbiotic regulatory loop. Thus, the adaptive immune system, through cellular and molecular components that are required for immune tolerance and through the diversification as well as selection of antibody repertoire, mediates host-microbial symbiosis by controlling the richness and balance of bacterial communities required for homeostasis.

INTRODUCTION

The main function of the immune system is to protect the host against pathogens, such as bacteria or viruses. However, unlike the systemic immune system, the gut immune system does not eliminate microorganisms but instead nourishes rich bacterial communities and establishes advanced symbiotic relationships (Sutherland and Fagarasan, 2012). Not only are the gut bacteria essential for nutrient processing, production of vitamins, and protection against pathogens (through competition for space and nutrients), but the development and maturation of the immune system depends on these bacteria (Fagarasan et al., 2010; Geuking et al., 2011; Hooper et al., 2012; Sutherland and Fagarasan, 2012). The primary individual microbiota (Mb) composition probably reflects the maternal hand-over during or immediately after birth (Kau et al., 2011; Nicholson et al., 2012). However, the subsequent shaping of the microbial landscape is probably driven by complex interactions with the host immune system, through a network of regulatory components involving both the innate and adaptive immune system (Fagarasan et al., 2010; Hooper et al., 2012; Maynard et al., 2012).

Our previous studies demonstrated that the absence of immunoglobulin A (IgA) (the major effector molecule of the adaptive immunity in the gut) or the impaired IgA selection in germinal centers (GCs) due to deregulated T cell control severely affects the balance of gut bacterial communities, resulting in massive activation of the whole body immune system (Fagarasan et al., 2002; Kawamoto et al., 2012; Suzuki et al., 2004; Wei et al., 2011). The absence of a subset of Foxp3⁺ T cells induced by bacterial antigens also modifies the composition of gut Mb by evoking mucosal T helper 2 (Th2) cell-mediated inflammation (Josefowicz et al., 2012). Interestingly, the Foxp3⁺ T cells induce GC and IgA responses by generating GC T cells (Tsuji et al., 2009), and their depletion causes a rapid loss of specific IgA responses in the intestine (Cong et al., 2009). Together, all these observations point to the existence of a Foxp3-IgA axis in maintaining the balance of gut Mb. It remains unclear, however, how these specific arms of the adaptive immune system mediate host-microbial interactions in the gut.

We show that Foxp3⁺ T cells, by acting in both GC-independent and -dependent manners, repress inflammation and support IgA selection in the GCs of Peyer's patches (PPs), resulting in diversification of gut Mb. Balanced and diverse Mb stimulates, in turn, the host immune system by promoting the expansion of Foxp3⁺ T cells and induction of GC and IgA production in the gut through a symbiotic regulatory loop.

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RESULTS

Reduced Diversity of Gut Microbiota in Immunodeficient Mice

We evaluated the impact of acquired immunity on gut Mb by analyzing various mice that partially or completely lack cellular and structural components of the adaptive immune system in gut. We found that mice lacking both B and T cells (*Rag1*$^{-/-}$), and as such having just rudimentary PPs, have considerably less diverse bacterial communities compared with *Rag1*$^{+/-}$ littermates or with wild-type (WT) mice raised in the same facility (Figure S1A available online). Not only the *Rag1*$^{-/-}$ mice, but also mice lacking only B cells (*Ighm*$^{-/-}$) or T cells (*Cd3e*$^{-/-}$), and thus lacking GCs, also had reduced bacterial diversity and different phylogenetic structures of bacterial communities compared with their heterozygous littermates or WT mice (Figures S1A and S1B). The results indicate that the adaptive immune system and its functionally organized follicular structures (i.e., PPs with GCs) facilitated diversification and influenced the structures of bacterial communities in gut.

Foxp3^{+} T Cells Are Required for Maintenance of Diverse Microbial Communities in Gut

Given that the PPs are enriched in B cells that interact mainly with CD4^{+} T cells, we next asked which CD4^{+} T cell subset(s) contribute to diversity and composition of gut Mb. For this, we transferred distinct CD4^{+} T cell populations (naive CD4^{+} T cells and Foxp3^{+} T cells) into *Cd3e*$^{-/-}$ mice, and the Mb were assessed 10–12 weeks after the injection. The transfer of naive CD4^{+} T cells alone (isolated from the spleen and peripheral lymph nodes of WT mice) considerably decreased bacterial diversity even below that observed in *Cd3e*$^{-/-}$ mice (Figure 1A). The change was associated with gut inflammation caused by expansion of T cells with inflammatory properties (Figures 2A, 2B, and 2E). Therefore, in mice, similar to humans, the inflammatory environment due to deregulated T cell populations did not afford the maintenance of complex bacterial communities (Manichanh et al., 2006; Nishikawa et al., 2009; Ott et al., 2004). Furthermore, the transfer of naive CD4^{+} T cells failed to increase bacterial diversity even in the absence of overt inflammation (i.e., in mice treated with anti-IL-12p40 that had considerably reduced expansion of inflammatory T cells and no signs of wasting disease or colitis) (Figures S1C–S1G). The cotransfer of naive CD4^{+} T cells along with Foxp3^{+} T cells led to reconstitution of the microbial diversity to levels observed in WT mice (Figure 1A). Not only the diversity but also the phylogenetic structures of bacterial communities become more similar to WT mice when naive CD4^{+} T cells were transferred together with Foxp3^{+} T cells (Figure 1B). In fact, the transfer of Foxp3^{+} T cells alone increased bacterial diversity and modified the composition of Mb in *Cd3e*$^{-/-}$ mice almost to the degree found in WT mice (Figures 1A and 1B). Strikingly, the Foxp3^{+} T cells facilitated the diversification of Firmicutes, particularly of nonpathogenic Clostridia belonging to cluster IV and XIVa, which were recently reported to be effective inducers of Foxp3^{+} T cells in the gut (Figure 1C; Atarashi et al., 2011, 2013). Thus, not only can Firmicutes induce Foxp3 expression, but Foxp3^{+} T cells can in turn feedback to Mb facilitating the maintenance and diversification of these major spore-forming bacteria. Taken together, the presence of Foxp3^{+} T cells was required for the establishment of complex bacterial communities in both inflammatory and noninflammatory environments in the gut.

Requirement for Foxp3^{+} T Cells Acting as Tfr Cells for Gut Microbiota Regulation

Foxp3^{+} T cells could exert their regulatory effect on Mb in multiple ways. They could do it simply by preventing the expansion of Foxp3^{-} T cells and their excessive production of cytokines and therefore by controlling inflammation in a GC-independent manner (Izcue et al., 2006; Josefowicz et al., 2012). Alternatively, the Foxp3^{+} T cells could act through their regulatory roles on PP GCs and IgA synthesis, by becoming T follicular regulatory (Tfr) (CXCR5^{hi}PD1^{hi}Foxp3^{+}) and T follicular helper (Tfh) (CXCR5^{hi}PD1^{hi}Foxp3^{-}) cells, as previously reported (Chung et al., 2011; Cong et al., 2009; Linterman et al., 2011; Tsuji et al., 2009; Wollenberg et al., 2011). The transfer of Foxp3^{+} T cells into *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice (which lack both T cells and antibodies others than IgM), failed to increase Mb diversity even though the cells expanded well and generated considerably more GCs, including Tfh and Tfr cells in PPs (Figures 1D and 1E). This observation strongly suggests that the Foxp3^{+} T cells contributed to shape the Mb and supported the gut mutualism by regulation of IgA production in the intestine.

The generation of both Tfr and Tfh cells depends on activation and induction of Bcl6 expression (Chung et al., 2011; Johnston et al., 2009; Linterman et al., 2011; Nurieva et al., 2009; Yu et al., 2009). To discriminate between the regulatory functions outside and inside the GCs, we performed experiments with Foxp3^{+} T cells sufficient or deficient for Bcl6 expression isolated from WT mice and *Bcl6*$^{yfp/yfp}$ mice, respectively, in which the function of Bcl6 was inactivated by yellow fluorescent protein (YFP) insertion (Kitano et al., 2011). Thus, naive CD4^{+} T cells were cotransferred at a 1:1 ratio with CD4^{+}CD25^{+} T cells (more than 98% of which expressed Foxp3) from WT mice (hereafter CD25^{+}WT T cells) or from *Bcl6*$^{yfp/yfp}$ mice (hereafter CD25^{+} *Bcl6*$^{yfp/yfp}$ T cells) into *Cd3e*$^{-/-}$ mice. In the presence of naive CD4^{+} T cells, both CD25^{+} WT T cells and CD25^{+}*Bcl6*$^{yfp/yfp}$ T cells had a similar expansion and maintenance of Foxp3 expression (Figure S2A). Furthermore, both CD25^{+} WT T cells and CD25^{+}*Bcl6*$^{yfp/yfp}$ T cells prevented the expansion of naive T cells in the gut and there were no signs of mucosal inflammation or wasting disease in these transferred mice (Figures 2A–2D). Accordingly, the production of cytokines like IFN-γ and TNF-α was reduced whereas that of IL-10 was increased in the presence Foxp3^{+} T cells, regardless of their Bcl6 expression (Figure 2E). Both groups of mice remained protected from inflammation even 6 months after the transfer (Figures S3A–S3C). Therefore, in the gut, Foxp3^{+} T cells controlled the expansion of naive CD4^{+} T cells and their production of proinflammatory cytokines, independently of Bcl6 expression. However, CD25^{+}*Bcl6*$^{yfp/yfp}$ T cells failed to normalize the bacterial diversity, which remained considerably lower than that observed in the presence of CD25^{+} WT T cells (Figure 2F). The phylogenetic structures of bacterial communities in mice transferred with naive CD4^{+} T cells together with CD25^{+}*Bcl6*$^{yfp/yfp}$ T cells, although distinct from mice transferred with naive CD4^{+} T cells alone, remained different from those in mice cotransferred with

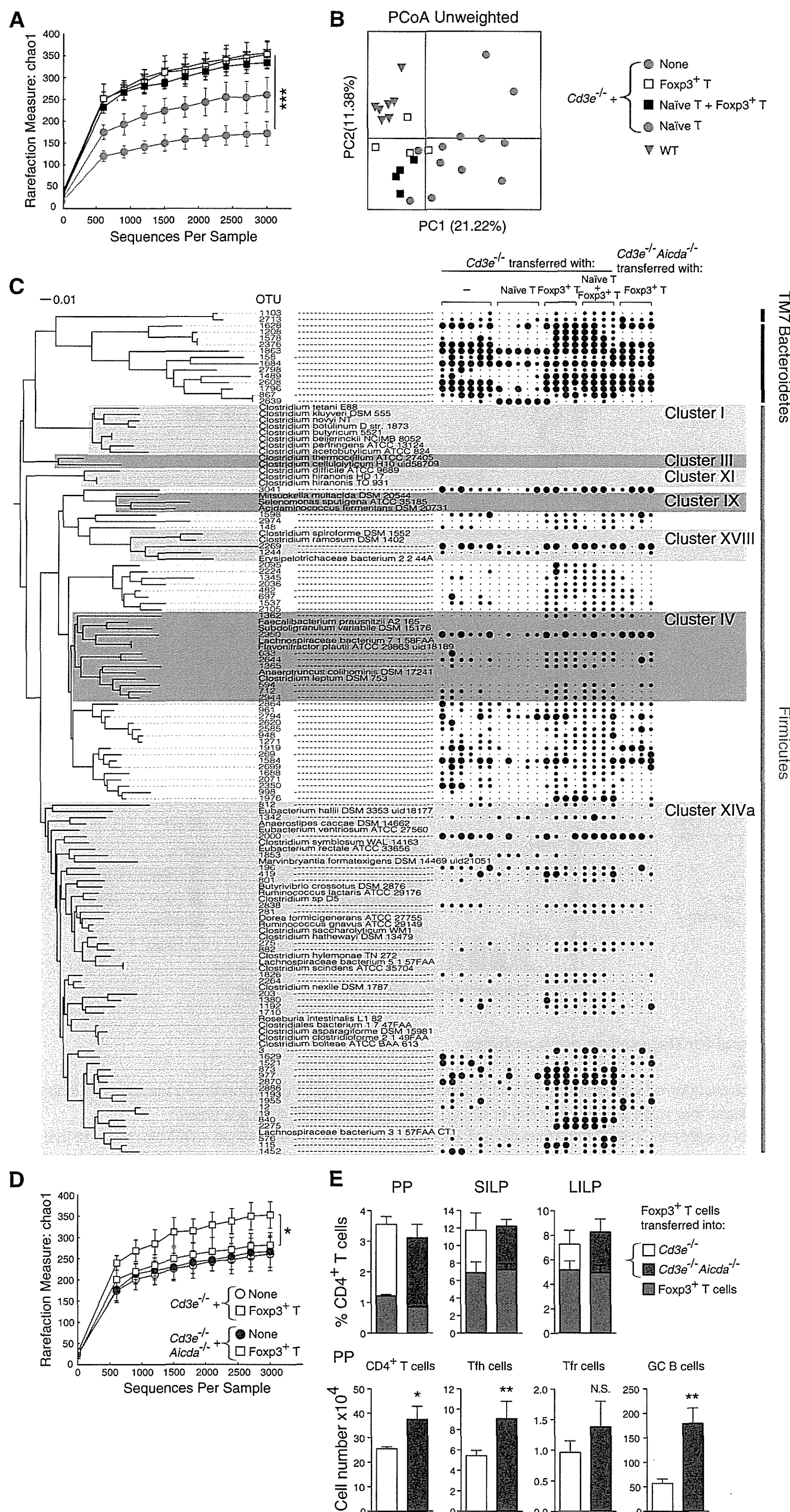


Figure 1. Increased Diversity of Firmicutes by Foxp3+ T Cells

(A and D) Diversity of bacterial species (>97% identity) as indicated by Chao1 rarefaction measure based on 1–3,000 sequences.

(B) Unweighted UniFrac plot, comparing phylogenetic differences between microbial communities, clustered by principal coordinate analysis (PCoA).

(C) Phylogenetic analysis of OTUs associated with the presence of Foxp3+ T cells. OTUs were defined and quantified by QIIME, followed by t test analyses between the Foxp3-deficient group (naive CD4+ T cells transferred mice) and the Foxp3-sufficient group (Foxp3+ T cells transferred alone or along with naive CD4+ T cells). OTUs with statistically significant difference ($p < 0.05$) are shown. The results of nontransferred *Cd3e*$^{-/-}$ or *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice transferred with Foxp3+ cells are shown in parallel. The 16S rRNA sequences of statistically different OTUs ($p < 0.05$) were used to construct the phylogenetic tree. The sequences of other Clostridia bacteria used for the tree were obtained from known genome sequences or ribosomal database project. The calculation was performed with the MEGA v.5.1 package and the neighbor-joining method with a bootstrap of 500 replicates. Circle size correlates with the relative abundance of each OTU. Large circles, medium circles, and small dots indicate ≥1%, <1%, and 0%, respectively.

(E) The percentage of CD4+ T cells in the gut and the numbers of indicated cell populations in *Cd3e*$^{-/-}$ and *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice transferred with Foxp3+ cells.

Mean ± SEM for four to seven mice per group. Two-tailed unpaired Student's t test was used to compare between (A) WT and *Cd3e*$^{-/-}$ + naive CD4+ T cells and between (D and E) *Cd3e*$^{-/-}$ and *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice transferred with Foxp3+ cells; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, N.S., no significant difference. See also Figure S1.

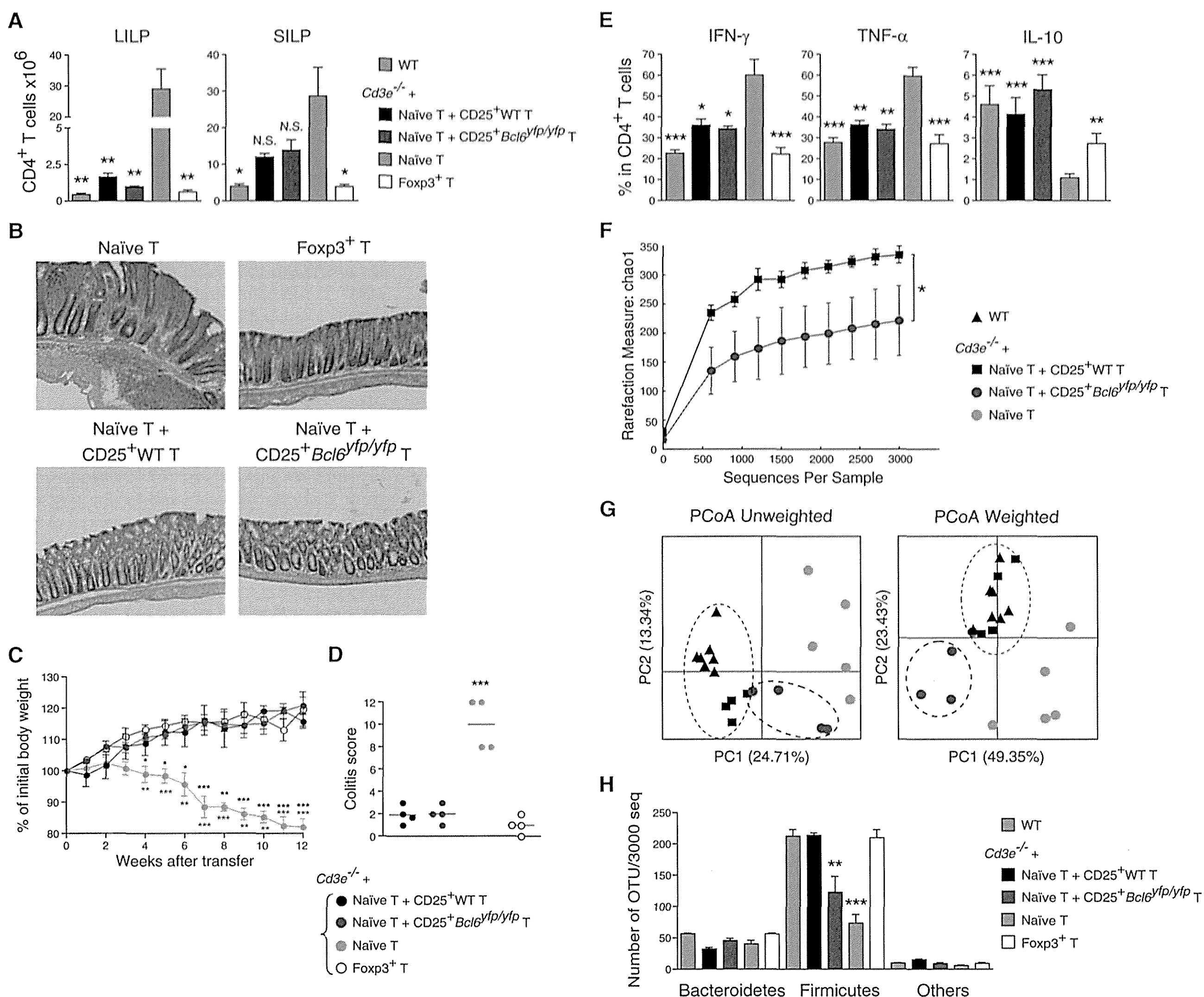


Figure 2. Foxp3+ T Cell Migration into PP GCs Is Critical for Regulation of Microbiota

(A) Total numbers of CD4+ T cells isolated from LILP and SILP.

(B) Hematoxylin-eosin staining sections of LI from *Cd3e*$^{-/-}$ mice transferred with indicated CD4+ T cells. Note that CD25+ T cells prevented the massive infiltration of inflammatory cells regardless of their Bcl6 expression. At least four mice per group were analyzed and representative data are shown.

(C) The change of body weight (presented as percent of original weight) of *Cd3e*$^{-/-}$ mice transferred with indicated CD4+ T cells. Mean ± SEM for three to five mice per group.

(D) Colitis score for *Cd3e*$^{-/-}$ mice transferred with indicated T cells. Each point represents an individual mouse.

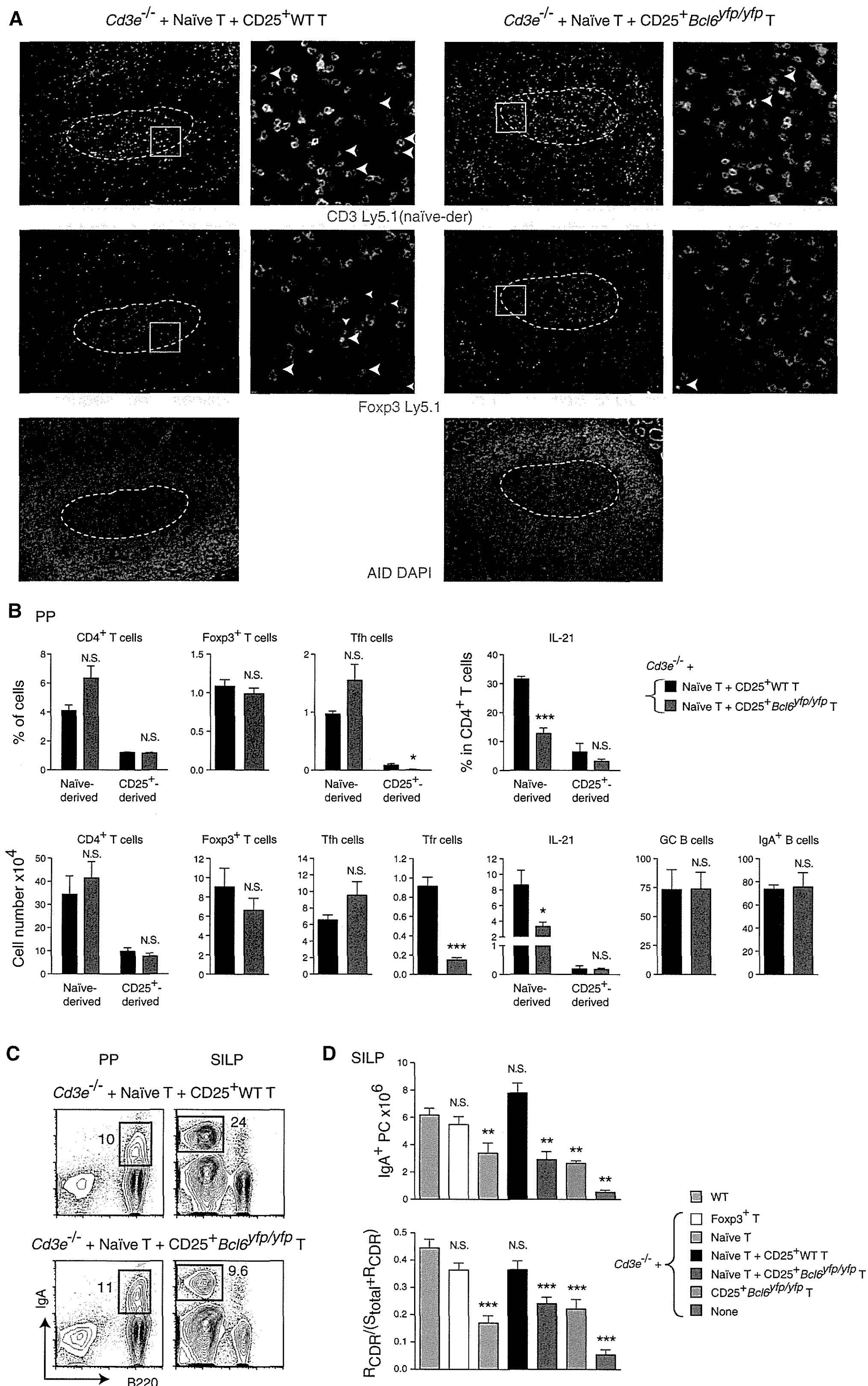
(E) Frequency of IFN-γ+, TNF-α+, and IL-10+CD4+ T cells from SILP of *Cd3e*$^{-/-}$ mice transferred with the indicated CD4+ T cell subsets or from WT mice. Means ± SEM for four to six mice per group.

(F) Diversity of bacterial species as indicated by Chao1 rarefaction measure based on 1–3,000 sequences in *Cd3e*$^{-/-}$ mice transferred with indicated CD4+ T cell subsets; four mice per group were analyzed.

(G) *Communities clustered by principal coordinates analyses of the unweighted and weighted UniFrac distances (which measure qualitative and quantitative* differences between microbial communities, respectively).

(H) Species diversity (numbers of OTUs/3,000 sequences) in cecal contents from WT or *Cd3e*$^{-/-}$ mice 10–12 weeks after the transfer of indicated CD4+ T cell subsets. Mean ± SEM from four to seven mice per group.

Two-tailed unpaired Student's t test was used to compare between the indicated mouse groups and (A and E) *Cd3e*$^{-/-}$ mice transferred with naive CD4+ T cells, (C) *Cd3e*$^{-/-}$ mice transferred with naive T cells along with CD25+ WT T cells or CD25+*Bcl6*$^{yfp/yfp}$ T cells, (D) other transferred mouse groups, (F) *Cd3e*$^{-/-}$ mice transferred with naive CD4+ T cells together with CD25+ WT T cells, or (H) WT mice; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; N.S., no significant difference. See also Figure S2.



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CD25$^+$ WT T cells (Figure 2G). Thus, we observed a pronounced reduction in diversity of Firmicutes in *Cd3e*$^{-/-}$ mice cotransferred with CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells compared with mice cotransferred with CD25$^+$ WT T cells or WT mice (Figure 2H). The decrease in Firmicutes diversity was mostly due to the excessive expansion of species belonging to *Lachnospiraceae*, which induced an overall shift in bacterial communities with an increase in the ratio of Firmicutes to Bacteroidetes (2.05 and 0.73 in mice cotransferred with CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells and CD25$^+$ WT T cells, respectively) (Figures S2B and S2C). Taken together, the results indicate that the maturation and differentiation of Foxp3$^+$ T cells into GC Tfr or Tfh cells, which depends on Bcl6 expression, was important for maintaining symbiosis with gut Mb.

Foxp3$^+$ T Cells Regulate Both Quantities and Qualities of IgAs

The cotransfer of naive T cells with either CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells or CD25$^+$ WT T cells equally induced GCs in PPs, but with very different features (Figure 3A). Thus, the mice that received naive CD4$^+$ T cells and CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells almost completely lacked Tfr cells yet contained many Tfh cells mostly derived from the naive CD4$^+$ T cells (Figures 3A and 3B). In contrast, the mice that received naive CD4$^+$ T cells with CD25$^+$ WT T cells had GCs with apparently more Tfr cells and fewer Tfh cells (some of them also generated from CD25$^+$ WT T cells upon downregulation of their Foxp3 expression) (Figures 3A, top and middle, and 3B). Interestingly, the production of IL-21 by PP T cells was considerably reduced in mice cotransferred with CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells, suggesting that the presence of Foxp3$^+$ T cells in GCs might regulate cytokine production by Tfh cells (Figure 3B).

Strikingly, although there were no obvious differences in IgA$^+$ B cells in PPs (Figures 3B and 3C), the frequencies and numbers of IgA plasma cells in the small intestine lamina propria (SILP) were much decreased in mice cotransferred with CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells (Figures 3C and 3D). More importantly, the IgAs produced in the absence of Bcl6 expression by Foxp3$^+$ T cells had a decreased affinity maturation index, suggesting defective selection in the GCs, in agreement with previous observations (Figure 3D; Linterman et al., 2011). In contrast, the CD25$^+$ WT T cells cotransferred with naive CD4$^+$ T cells reconstituted the SILP of *Cd3e*$^{-/-}$ mice with high numbers of apparently well-selected IgA plasma cells (Figures 3C and 3D). Thus, Foxp3$^+$ T cell presence in the GCs was critical for regulating both the qualities and frequencies of IgAs in the gut.

Quality of IgAs Regulates Diversity of Microbiota

Because GC and IgA regulation by Foxp3$^+$ T cells appeared to contribute considerably to shaping of gut Mb, we next evaluated the bacteria-coating properties of IgAs elicited in the presence or absence of Foxp3$^+$ T cells. Therefore, we stained fecal bacteria-bound IgA with antibodies recognizing both IgA heavy (V_H) and light ($V\kappa$) chains and established a setting and acquisition mode that allowed a clear separation of IgA-coated bacteria (Figures 4A, S4A, and S4B). In the absence of Foxp3$^+$ T cell regulation, the proportion of IgA-coated bacteria increased, as did the overall intensity of IgA staining (Figures 4A and S4B), a feature also observed in patients with inflammatory bowel diseases (IBD) (van der Waaij et al., 2004). This abundantly coated bacteria profile could reflect differences in the bacterial communities (i.e., large-size bacteria giving stronger signals) or differences in IgA qualities and their binding properties. To distinguish between these possibilities, we sequenced and analyzed bacteria that were sorted as IgA^{neg}, IgA^{int}, and IgA^{hi} from the feces of *Cd3e*$^{-/-}$ mice transferred with different populations of CD4$^+$ T cells or from control (WT) mice (Figure S4A). The identified species from noncoated or coated bacterial fractions (we consider one operational taxonomic unit [OTU] as one species) were used for principal coordinate analysis (PCoA) (Figure 4B). WT mice and *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells exhibited a similar profile in which all fecal bacteria or IgA^{neg} groups were substantially separated from IgA^{neg} or IgA$^{int/hi}$ groups or from cecal bacteria. In contrast, in the absence of Foxp3$^+$ T cell regulation (i.e., naive CD4$^+$ T cells transferred alone or along with CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells), there was no clear distinction among IgA^{neg}, IgA^{int}, and IgA^{hi} fractions (Figure 4B). This was confirmed by plotting in Venn graphs, in which the size of circles in the Venn graph reflects the diversity of bacterial species for each fraction (Figures 4C, S4C, and S4D). In mice with Foxp3$^+$ T cell regulation, a smaller percentage of bacterial species overlapped among all the IgA^{hi}, IgA^{int}, and IgA^{neg} fractions, and IgA^{hi} fraction exhibited higher diversity, compared with those in mice without Foxp3$^+$ T cell regulation (Figures 4C, 4D, and S4C).

The less-overlapping profiles correlated with a higher affinity selection index of IgAs, whereas the largely overlapping profiles associated with reduced affinity selection index of IgAs (Figure 3D). To test whether selected and nonselected IgAs differently coat the same bacteria, equal amounts of IgA elicited in the presence or absence of Foxp3$^+$ T cells were tested for binding in vitro against several defined anaerobic bacterial strains isolated from mouse gut. As shown in Figures S4E and S4F,

Figure 3. Foxp3$^+$ T Cell Migration into PP GCs Is Critical for IgA Selection

(A) Representative sections of PPs in *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells together with CD25$^+$ WT T cells or CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells stained as indicated and revealing GC B cells and T cells. T cells derived from naive CD4$^+$ T cells appeared in yellow, and the CD25$^+$-derived T cells are stained in green only. The arrows in upper panels indicate the presence of Foxp3-derived Tfh cells, while the arrows in middle panels indicate Tfr cells. Smaller arrows indicate reduced levels of Foxp3 by some Tfr cells.

(B) The percentage and total numbers of the indicated cells in PPs of *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells together with CD25$^+$WT T cells or CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells. Mean ± SEM for three to five mice per group.

(C) Flow cytometric profiles of cells from PPs and SILP. B220$^-$IgA$^+$ gate represent plasma cells. Numbers on plots indicate the frequency of cells in the gate.

(D) Total numbers and affinity maturation index of IgA-producing cells from the SILP of WT, *Cd3e*$^{-/-}$, and *Cd3e*$^{-/-}$ transferred with indicated CD4$^+$ T cell subsets. Two to three mice per group, around 50 sequences per mouse were analyzed. R_{CDR}, replacement in CDR1 and CDR2; S_{total}, silent mutations in both CDRs and in framework regions 1 to 3 (FWR1–3).

Two-tailed unpaired Student's t test was used to compare between the indicated mouse groups and (B) *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells together with CD25$^+$ WT T cells or (D) WT; ***$p < 0.001$; N.S., no significant difference. See also Figure S3.

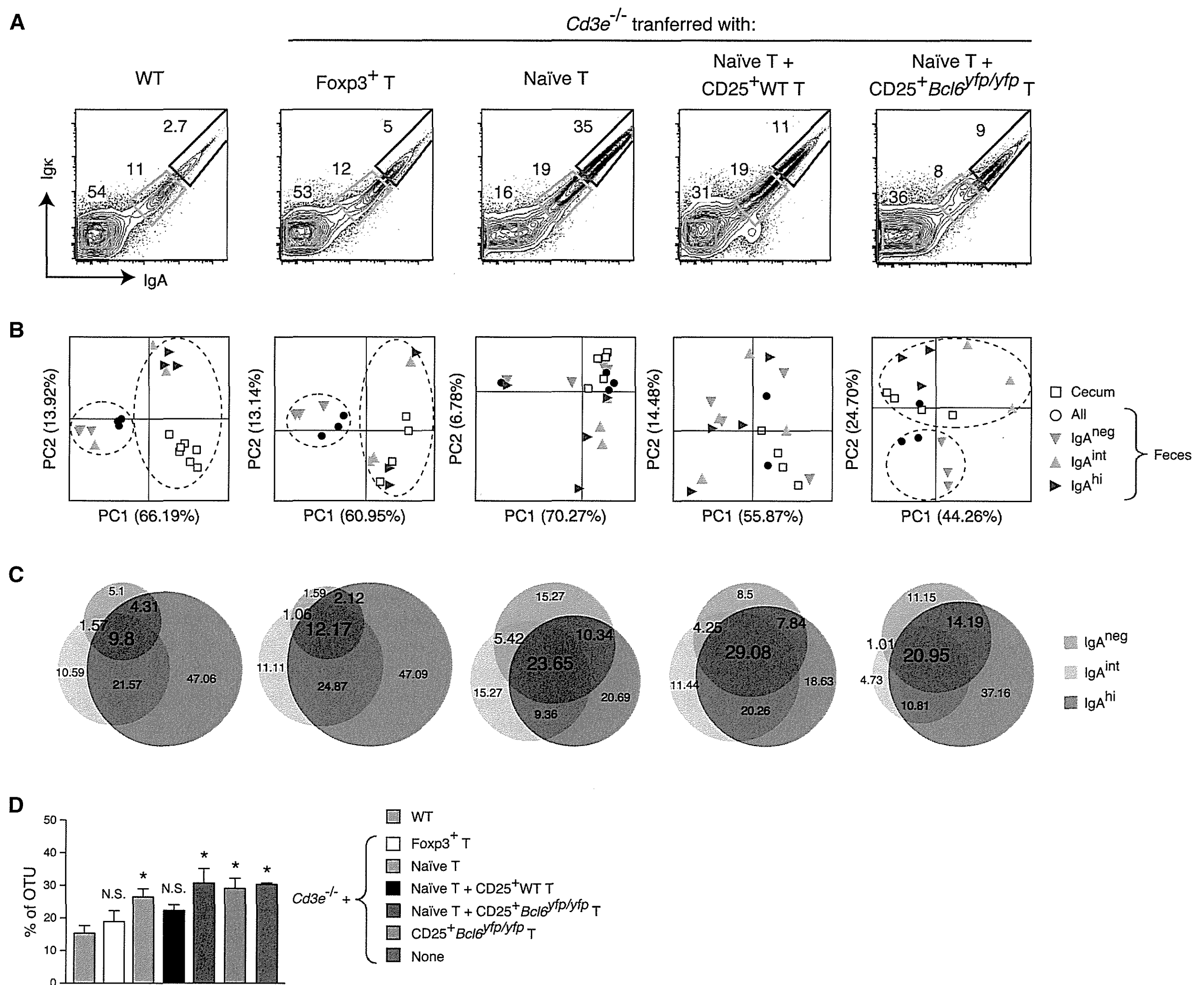

Figure 4. Specific IgA Coating Contributes Maintenance of Microbiota

(A) Representative flow cytometric profiles of fecal bacteria stained as indicated from WT or *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cell subsets. Sorting gates for IgA^{neg} (IgA$^-$Igκ$^-$), IgA^{int} (IgA^{int}Igκ^{int}), or IgA^{hi} (IgA^{hi}Igκ^{hi}) are indicated.

(B) Communities clustered by principal coordinate analyses of weighted UniFrac distance of 16S rRNA sequences from total cecal and fecal bacteria, and the IgA^{neg}, IgA^{int}, and IgA^{hi} sorted bacterial fractions from WT or *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cell subsets.

(C) Venn diagrams showing the frequencies of bacterial species (OTUs) from the IgA^{neg}, IgA^{int}, and IgA^{hi} sorted bacterial fractions from WT or *Cd3e*$^{-/-}$ mice 10–12 weeks after the transfer of the indicated CD4$^+$ T cell subsets. Data represent one of three to four experiments with consistent results. Numbers represent percentages.

(D) The frequency of OTU overlap among IgA^{neg}, IgA^{int}, and IgA^{hi} sorted bacterial fractions from WT or *Cd3e*$^{-/-}$ mice 10–12 weeks after the transfer of the indicated CD4$^+$ T cell subsets. Mean ± SEM from three to four mice per group. Two-tailed unpaired Student's t test was used to compare between WT and the indicated mouse groups; $^*p < 0.05$; N.S., no significant difference.

See also Figure S4.

the nonselected IgAs from mice transferred with naive CD4$^+$ T cells or CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells had higher coating capacity than the IgAs from mice transferred with Foxp3$^+$ T cells. These results suggest that the IgAs generated and selected in the presence of GC Foxp3$^+$ T cells coated moderately and rather specifically a large diversity of bacterial species. This coating might contribute to maintenance rather than elimination of indigenous bacteria to keep the diversity.

Regulation of Immune System by Microbiota

We hypothesized that the presence of very diverse bacterial species would facilitate the perpetual induction of GCs and IgA and the maintenance of Foxp3$^+$ T cell pool in the gut. To test this hypothesis, we performed Mb transplantation experiments. Thus, germ-free (GF) mice were gavaged with Mb harvested from feces of *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells (hereafter Foxp3Mb) or naive CD4$^+$ T cells (hereafter Naive4Mb). Mb

harvested from nontransferred *Cd3e*$^{-/-}$ mice (CD3Mb) served as control. The mice were analyzed 2 weeks later. In the PPs, colonization with Foxp3Mb resulted in increase of B cells and T cells, and their activation and differentiation into GC B cells and Tfh cells, respectively (Figures 5A, 5C, S5A, and S5B). Interestingly, the Foxp3Mb induced preferential switching of GC B cells from IgM to IgA, the characteristic gut GC signature. In contrast, Naive4Mb or CD3Mb induced fewer GCs and Tfh cells, with the Naive4Mb supporting B cell class switching to IgG1 rather than to IgA, because of increased IL-4 production by CD4$^+$ T cells located in the PPs of these mice (Figures 5A and 5C). In the SILP, Foxp3Mb generated many more IgA-producing cells and Foxp3$^+$ T cells compared with CD3Mb or Naive4Mb (the latter inducing not Foxp3 but rather T cells secreting IL-4, IL-17, or TNF-α) (Figures 5B, 5D, and S5C). Importantly, the induction of these two characteristic gut homeostatic responses (IgA and Foxp3) by Foxp3Mb associated with a considerably higher diversity of bacterial species compared with mice transplanted with NaiveMb or CD3Mb (Figures 5E and 5F). Because most species correlating with the induction of IgA and Foxp3$^+$ T cells were Firmicutes (Figure S5D) and this phylum contains many spore-forming bacteria, we next performed gavage experiments with the spore fraction from Foxp3Mb (Spore^{Foxp3Mb}). Colonization of GF mice with Spore^{Foxp3Mb} induced GC B cells and Tfh cells that facilitated preferential switching to IgA in the PPs (Figures 5A, 5C, and S5B) and increased the IgA plasma cells compartment and favored the generation or expansion of Foxp3$^+$ T cells in the LP (Figures 5B, 5D, and S5C).

To further evaluate the link between Mb and gut Foxp3$^+$ T cells, we changed the experimental strategy slightly. We first inoculated GF *Cd3e*$^{-/-}$ mice with Foxp3Mb or Naive4Mb and 1 week later transferred Foxp3$^+$ T cells into these mice (Figure 6A). The T cell expansion and B cell responses were assessed 2 weeks after the cell transfer. As shown in Figures S6A and S6B, Foxp3Mb helped the expansion of Foxp3$^+$ T cells in MLN and LP of the SI and LI more vigorously than Naive4Mb. In the PPs, Foxp3Mb but not Naive4Mb facilitated the activation and differentiation of Foxp3$^+$ T cells into Tfh cells with GC and IgA-inducing properties (Figures 6A and 6B; Tsuji et al., 2009). Thus, Foxp3Mb promoted maturation of the gut immune system and exhibited robust Foxp3 and IgA-supportive properties in gut.

Dominant Immune-Regulatory Role of Foxp3Mb

To further evaluate the features of Foxp3Mb in more competitive settings, we performed experiments with specific-pathogen-free (SPF) young mice. Thus, 3-week-old WT mice were colonized by fur painting with Foxp3Mb. Adult WTMb, CD3Mb, or Naive4Mb mice were used for comparison. Strikingly, even in competitive situations, the Foxp3Mb (or a similarly complex and balanced adult WTMb) had IgA-inducing properties (Figure 7A). Indeed, compared with noninfected mice, the Foxp3Mb induced a substantial increase in frequencies and numbers of GCs and IgAs in PPs (Figures 7A and 7B). Naive4Mb or CD3Mb induced activation and IgA differentiation in PPs, but to a much more limited degree than Foxp3Mb or WTMb. Yet, the IgA production was increased when Naive4Mb was mixed with Foxp3Mb (Figures 7A and 7B). The results clearly demonstrated the prevalent IgA-inducing properties of Foxp3Mb. They also confirmed the potential of Mb selected and maintained by the immunocompetant host to dominantly regulate postnatal maturation of the immune system.

DISCUSSION

In this manuscript we have revealed that (1) differentiation of Foxp3$^+$ T cells into Tfr cells is required for the IgA's selection in GCs; (2) the amount and quality of IgAs directly influence the diversity and phylogenetic structure of bacterial communities; (3) rich and balanced Mb induce maturation of the gut immune system by promoting Foxp3$^+$ T cells and IgAs; and (4) in turn, the Foxp3$^+$ T cells and IgAs, through controlled diversification of stimulatory bacterial species, establish a self-regulatory loop mediating host-bacterial mutualism. Thus, it appears that the adaptive immune system contributes to the maintenance, rather than elimination, of complex microbial communities that probably enrich the genomic and metabolic capacity of the host, which is required for gut homeostasis and health.

Multiple studies revealed the importance of balanced Mb for the maintenance of gut barrier and immune homeostasis. Biased expansion of certain bacterial species impairs epithelial barrier and induces excessive activation of the immune system and generation of T cell subsets with inflammatory properties (Fagarasan et al., 2010; Kamada et al., 2013; Kawamoto et al., 2012; Littman and Pamer, 2011). Furthermore, reduced microbiome richness when accompanied by inflammatory phenotypes also associates with obesity, insulin resistance, and dyslipidemia (Karlsson et al., 2013; Le Chatelier et al., 2013; Qin et al., 2012). We demonstrated that the acquired arm of the immune system impacts considerably the diversity and phylogenetic structure of microbial communities in the gut.

We showed that Foxp3$^+$ T cell migration and differentiation into Tfr cells in the GCs is critical for IgA selection. The lack of Tfr cells associates with increased number of Tfh cells that license not only the mutated, presumably high-affinity, but also the germline and less mutated (and presumably poly- and/or self-reactive) B cells to emerge from the GCs, as previously reported (Baumjohann et al., 2013; Good-Jacobson et al., 2010; Kawamoto et al., 2012; Linterman et al., 2011; Vinuesa et al., 2013). In the absence of Tfr cell regulation, Tfh cells had skewed helper properties (e.g., due to production of different cytokines). The Tfh cell phenotype in GCs lacking Tfr cells may reflect not only the lack of direct suppressive effects of Tfr cells on Tfh cells, but also the downstream events resulting from deregulation of GCs.

Thus, it is highly likely that defective selection of IgAs leading to microbial changes would affect the generation of bacterial metabolic products that are required for induction of gut effector T cell subsets, like Foxp3$^+$, RORγt$^+$, or Foxp3$^+$RORγt$^+$ T cells (Atarashi et al., 2011, 2013; Ivanov et al., 2009; Lochner et al., 2008; Zhou et al., 2008). For example, short-chain fatty acids (SCFAs), especially butyric acid, derived from fermentation of dietary fibers by certain bacterial species (e.g., *Clostridia*), appear to facilitate the induction and expansion of Foxp3$^+$ T cells in gut through epigenetic changes (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). We also observed that a reduced bacterial diversity in *Cd3e*$^{-/-}$ mice coincided with lower intestinal amounts of SCFAs (including acetate, propionate, or butyrate)

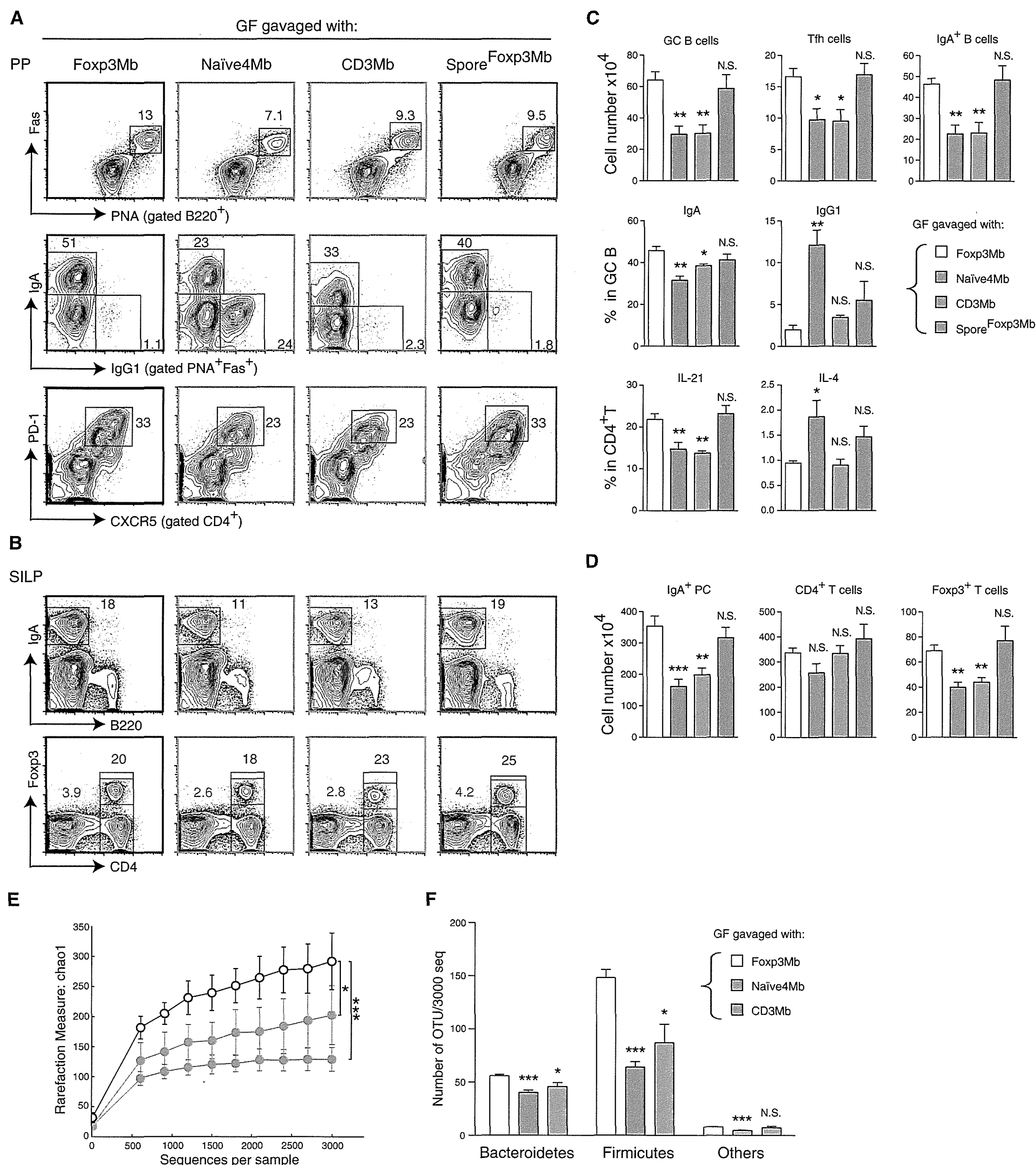

Figure 5. Foxp3-Regulated Microbiota Induces Maturation of Gut Immune System
(A–D) Flow cytometric profiles of (A) PP cells and (B) SILP stained as indicated and total numbers of indicated cell populations from (C) PPs and (D) SILP of GF mice gavaged with fresh microbiota obtained from *Cd3e*$^{-/-}$ mice transferred with naive CD4^{+} T cells (Naive4Mb) or Foxp3^{+} T cells (Foxp3Mb), or nontransferred *Cd3e*$^{-/-}$ mice (CD3Mb) as control. Gavage with spore fraction obtained from Foxp3Mb is also shown. Feces were obtained 10–12 weeks after T cell transfer. Mice were analyzed 2 weeks after bacterial transplantation. Data represent one of the three experiments with consistent results. At least four mice per group were analyzed.

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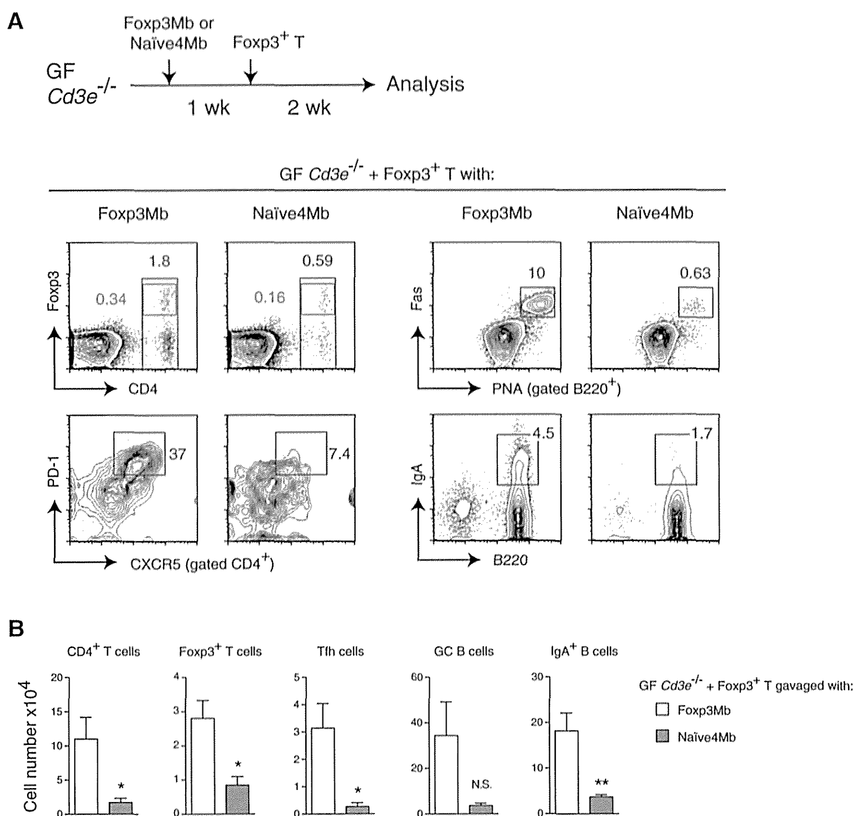

Figure 6. Foxp3-Regulated Microbiota Has Foxp3-Supportive Properties

(A) Scheme of the transfer experiment with GF $Cd3e^{-/-}$ mice and flow cytometric profiles of cells isolated from PPs of GF $Cd3e^{-/-}$ mice gavaged with fresh microbiota obtained from transferred mice and injected with Foxp3$^+$ T cells as indicated in scheme and stained for the indicated markers. Numbers indicate the frequency of cells in the gate. Data represent one of the two experiments with consistent results. At least four mice per group were analyzed.

(B) Total numbers of CD4$^+$ T, Foxp3$^+$ T, Tfh, GC B, and IgA$^+$ B cells in PP obtained from the indicated mice. Mean ± SEM in bar graphs for four mice per group. Two-tailed unpaired Student's t test was used to compare between these groups; $^{**}p < 0.01$; $^{*}p < 0.05$; N.S., no significant difference. See also Figure S6.

and that these levels recovered upon reconstitution of mice with Foxp3$^+$ T cells and normalization of Mb (data not shown). Additional microbial-derived metabolites are probably modulating other subsets of T cells, which upon activation and interaction with B cells could convert into GC Tfh cells with distinct helper characteristics (Hirota et al., 2013; Takahashi et al., 2012; Tsuji et al., 2009).

The Foxp3-IgA module probably involves complex feedback and feed-forward loops between Mb and immune cells that extend well beyond mucosal immune system (Fagarasan et al., 2002; Suzuki et al., 2004; Wei et al., 2011). For example, when Foxp3 control of GCs was missing (i.e., $Cd3e^{-/-}$ mice cotransferred with naive and CD25$^+$$Bcl6^{yfp/yfp}$ T cells), we observed a higher ratio of Firmicutes to Bacteroidetes even in the absence of overt inflammation. This shift associates with a considerable increased body weight, suggesting alterations in energy harvest and metabolism (Turnbaugh et al., 2006). In contrast, in the complete absence of Foxp3$^+$ T cells, there was a pronounced reduction of Firmicutes, which together with expansion of Proteobacteria could account for the decreased weight observed in such mice with overt inflammation (Elson and Cong, 2012).

We revealed that reduced diversification and affinity maturation of IgAs in the GCs associated with abundant coating of bacteria with largely nonspecific IgAs and reduced diversity and skewed gut Mb. Conversely, diversified and well-selected IgA repertoires in GCs associated not only with specific bacteria coating but also with rich and balanced bacterial communities. These observations suggest that bacteria coating by highly diversified and selected IgAs contributes to maintenance rather than elimination of indigenous bacteria, thus increasing the diversity and stability of Mb. It is accepted that IgA can control infection by coating pathogenic bacteria and preventing their contact to the gut epithelium, a process called immune exclusion (Strugnell and Wijburg, 2010). However, IgA coating of commensal bacteria might promote changes in the bacteria itself, such as modification in the bacterial gene expression (Peterson et al., 2007), influencing their metabolic processes as well as their biogeography, proliferation, and survival within the gut. There might be multiple mechanisms (which may work differently depending on the bacteria type, its growth stage and location, and available dietary components) by which IgA binding controls the Mb, but these remain to be elucidated in future studies.

Our study raises the question as to whether different types of Mb are "seen" differently by the immune system and, if so, whether they trigger distinct type of immune responses. We found that a complex and balanced Mb promptly elicits immune responses with typical mucosal characteristics, namely induction of GCs with IgA-supporting properties, and induction or expansion of CD4$^+$ T cells, especially of Foxp3$^+$ T cells. In contrast, a poor and skewed Mb provokes responses with mixed mucosal and systemic characteristics (i.e., GC

(E and F) Diversity of bacterial species (>97% identity) (E) and numbers of OTUs/3,000 sequences (F) in cecal contents from GF mice gavaged with the indicated microbiota; mean ± SEM from four to six mice per group. Two-tailed unpaired Student's t test was used to compare between the GF mice gavaged with Foxp3Mb and the indicated mouse groups; $^{***}p < 0.001$; $^{*}p < 0.05$; N.S., no significant difference.
See also Figure S5.

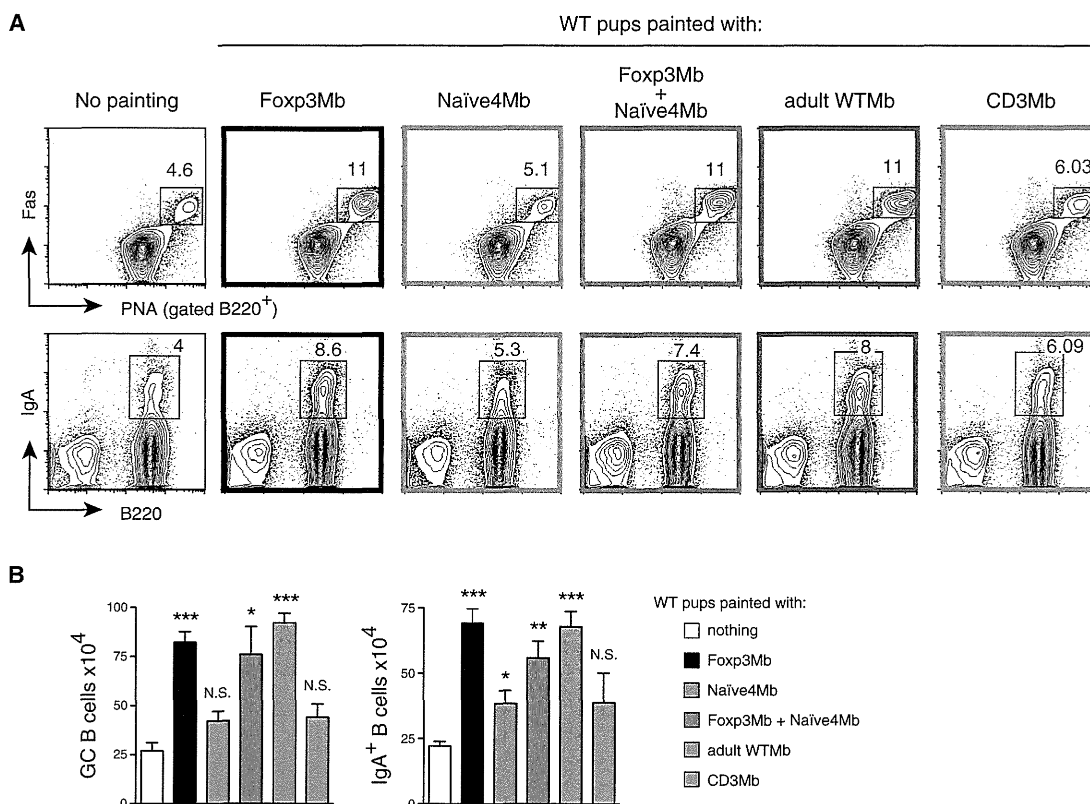

Figure 7. Dominant Effect of Foxp3-Regulated Microbiota

(A) Representative flow cytometric profiles of cells from PPs of WT pups "painted" with fecal extracts from adult WT mice (adult WTMb), nontransferred *Cd3e*$^{-/-}$ mice (CD3Mb), or the *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells (Naive4Mb) or Foxp3$^+$ T cells (Foxp3Mb). Fecal extracts from two to four mice per group were pooled 10–12 weeks after T cell transfer, and host mice were analyzed 2 weeks after the painting. Numbers indicate the frequency of cells in the gates. Data represent one of the three experiments with consistent results. At least four mice per group were analyzed.

(B) Total numbers of GC B cells and IgA$^+$ B cells from the PPs of indicated mice. Mean ± SEM for three to five mice per group. Two-tailed unpaired Student's t test was used to compare between nonpainted and the indicated mouse groups; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; N.S., no significant difference.

B cells switching not only to IgA but also to IgG1, and possibly to IgE). These observations suggest that the immune system recognizes complex and balanced microbial communities as "gut Mb signature" and respond by adaptations that foster the maintenance of such complex bacterial structures. An increased diversity probably enhances the stability of Mb potentiating its metabolic capacity, in parallel with exerting a constant yet controllable pressure for diversification and fitness of the immune system. In contrast, poor and skewed bacterial communities might be recognized as having pathogenic treats and as such elicit systemic type of responses that in certain conditions could lead to autoimmune diseases or allergies. Thus, the acquisition of Foxp3, IgA, and its secretory mechanisms, and the development of complex GALT structures that facilitate coordinated and controlled immune-receptor diversification seem adaptations that allowed vertebrates to establish symbiotic relationships with Mb and to become an evolutionary success.

The results presented here should be useful when considering strategies to reestablish symbiosis in intestinal pathologies caused by various immunodeficiencies and associated with gut inflammation.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 germ-free (GF) mice were initially purchased from Sankyo Laboratories Japan and were bred and maintained in vinyl isolators in the animal facility at IMS-RCAI, RIKEN Yokohama. 5-week-old GF mice were used for microbiota transplantation experiments. Other mice, like wild-type (WT), *Cd3e*$^{-/-}$ (Malissen et al., 1995), *Ighm*$^{-/-}$, *Rag1*$^{-/-}$, *Foxp3*^{EGFP} (Ly5.1 or 5.2) (Wang et al., 2008), *Bcl6*$^{yfp/yfp}$ (Kitano et al., 2011), and *Aicda*$^{-/-}$, were on a C57BL/6 background, bred and maintained in SPF facility at IMS-RCAI. All animal experiments were performed in accordance with approved protocols from the Institutional Animal Care at RIKEN. Littermate information of immunodeficient mice is described in Supplemental Experimental Procedures.

IgA$^+$ Cell Sorting, IgA Heavy Chain Gene Sequencing, and Mutational Analyses

Single B220$^-$IgA$^+$ plasma cells from lamina propria of small intestine (SILP) were sorted into 96-well PCR plates containing 10 μl of 50 μg/ml yeast tRNA as carrier, using FACS Aria cell sorter (Becton Dickinson). The method for IgA heavy chain gene sequence analyses was previously described (Hershberg et al., 2008; Kawamoto et al., 2012).

Histological Analysis

For immunohistochemical analysis, small intestine samples were fixed and stained as previously described (Kawamoto et al., 2012). Before Foxp3

staining, formaldehyde-fixed sections were treated with HistoVT One (Nacalai Tesque) at 70°C for 20 min for antigen retrieval. For hematoxylin-eosin (HE) staining, large intestine samples were fixed and stained with HE (Muto Pure Chemicals) according to the manufacturer's protocol. The stained slides were examined with a Zeiss Axioplan 2 fluorescence microscope.

Assessment of Intestinal Inflammation

Mice were sacrificed usually around 10–12 weeks after the transfer of T cells. Distal colons were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin. The degree of intestinal inflammation was graded from 0 to 3 for the four following criteria: degree of epithelial hyperplasia and goblet cell depletion; leukocyte infiltration in the lamina propria; area of tissue affected; and the presence of markers of severe inflammation such as crypt abscesses and submucosal inflammation (Izcue et al., 2008). Scores for each of the criteria were added to give a total score of 0 to 12 for each sample. The total colonic score was calculated as the average of the individual scores from several sections per mouse.

Fecal Suspension for Microbiota Reconstitution

Fecal pellets were collected from C57BL/6 or from T-cell-transferred *Cd3e*$^{-/-}$ mice and suspended with sterile PBS (5–7 feces/3 ml PBS). Bacteria number in fecal suspension was counted by Flow-Check Fluorospheres (Beckman Coulter) with FACS CantoII (Becton Dickinson) and adjusted to same number in each sample. The spore fraction was prepared as described previously (Atarashi et al., 2013).

Germ-free WT Mice Experiments

Germ-free C57BL/6 mice were transferred into autoclaved sterile cages and gavaged with 100 μl of fecal suspension from the *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells, naive CD4$^+$ T cells, or nontransferred *Cd3e*$^{-/-}$ mice. At 2 weeks after gavage, mice were analyzed and cecum contents were collected for bacterial DNA sequencing.

Germ-free *Cd3e*$^{-/-}$ Mice Experiments

Germ-free *Cd3e*$^{-/-}$ mice were gavaged with fecal suspension from the *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells or naive CD4$^+$ T cells. After 1 week, 2×10^5 CD4$^+$GFP$^+$ (Foxp3$^+$) T cells sorted from spleen and LNs of Foxp3^{EGFP} mice were injected intravenously. The recipient mice were analyzed 2 weeks after the Foxp3$^+$ T cell transfer.

Fur-Painting of SPF Mice

For painting experiments, 3-week-old female C57BL/6 mice purchased from CLEA Japan had fecal suspension from different mice painted on their fur. Mice were analyzed 2 weeks after the painting.

Evaluation of IgA-Coated Bacteria by Flow Cytometry

Flow cytometric analysis of bacteria was performed as described (van der Waaij et al., 1996) with some modifications. Detailed procedure is described in Supplemental Experimental Procedures.

Sorting of IgA-Coated Fecal Bacteria

Fecal bacteria were stained as described above and purified on a FACSAria (Becton Dickinson) cell sorter as IgA^{neg} (IgA$^-$Igκ$^-$), IgA^{int} (IgA^{int}Igκ^{int}), or IgA^{hi} (IgA^{hi}Igκ^{hi}) cells. Sorted bacteria were centrifuged at 12,000 × *g* for 10 min and bacterial pellet was stored at −80°C until use. Bacterial genomic DNA was purified with conventional phenol:chloroform extraction followed by ethanol precipitation. 16*S* rRNA genes were amplified and analyzed as described below.

Preparation of DNA and Pyrosequencing

Cecum and stool samples were stored at −80°C until use. DNA was purified with the QIAamp DNA stool mini kit (QIAGEN) according with manufacturer's instructions with a high-temperature incubation option. DNA samples were amplified using V1–V2 region primers targeting bacterial 16*S* rRNA genes with Roche 454 Lib-L pyrosequencing adaptor and barcode sequence as previously described (Kawamoto et al., 2012). PCR products were cleaned by Wizard SV Gel and PCR Clean-up system (Promega) and sequencing was carried out by with a 454 GS Junior pyrosequencer (Roche).

16*S* rRNA Data Processing and Analysis

Sequences were processed and analyzed with QIIME pipeline (Caporaso et al., 2010). Detailed procedure is described in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The bacterial 16S rRNA amplicon sequence data are available in DNA Data Bank of Japan (DDBJ) under the accession number PRJDB2881.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.05.016.

AUTHOR CONTRIBUTIONS

S.K. conducted all mice experiments and analysis. M.M. conducted the bacterial 16S rRNA pyrosequencing, data processing, and analysis. L.M.K. conducted IgA mutational analyses, evaluation, and sorting of IgA-coated fecal bacteria. W.S. and M.H. provided help for 16S rRNA data analysis. K.A. and K.H. provided help for isolation and culture of bacterial strains from mouse cecum. Y.D., Y.T., and H.Q. provided help for mice experiment and Y.D. provided help with IgA heavy chain gene sequencing. T.O. provided *Bcl6*$^{yfp/yfp}$ mice. S.K., M.M., L.M.K., and S.F. interpreted the data and wrote the manuscript. S.F. designed and supervised the study.

ACKNOWLEDGMENTS

We thank T. Honjo, O. Kanagawa, I. Taniuchi, and D. Littman for inspiring discussions, suggestions, and critical comments and M. Miyajima, K. Suzuki, K. Moro, A. Hijikata, H. Fujimoto, Y. Hachiman, Y. Murahashi, C. Shindo, K. Komiya, H. Kuroyanagi, E. Iioka, Y. Takayama, E. Ohmori, M. Kiuchi, and Y. Hattori for technical assistance. The data reported in this paper are tabulated in the main paper and the Supplemental Data. This work was supported in part by Grants-in-Aid for Scientific Research (25293118) (S.F.) and for Young Scientist (25860375), the Naito Foundation, RIKEN special Postdoctoral Researchers Program (S.K.), the global COE project "Genome Information Big Bang" from the MEXT of Japan (M.H.), and JSPS Postdoctoral Fellowship for Foreign Researchers (L.M.K.).

Received: December 30, 2013
Accepted: May 9, 2014
Published: July 10, 2014

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ARTICLE ADDENDUM

Gut Microbes 5:3, 333–339; May/June 2014; © 2014 Landes Bioscience

Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia

Seiko Narushima[1,†], Yuki Sugiura[2,5,†], Kenshiro Oshima[3,4], Koji Atarashi[1,5], Masahira Hattori[3], Makoto Suematsu[2,6], and Kenya Honda[1,4,*]

[1]RIKEN Center for Integrative Medical Sciences (IMS-RCAI); Yokohama, Japan; [2]Department of Biochemistry; School of Medicine; Keio University; Tokyo, Japan; [3]Graduate School of Frontier Sciences; The University of Tokyo; Kashiwa, Japan; [4]Japan Science and Technology Agency; Core Research for Evolutional Science and Technology (CREST); Kawaguchi, Japan; [5]Japan Science and Technology Agency; Precursory Research for Embryonic Science and Technology (PRESTO); Kawaguchi, Japan; [6]Japan Science and Technology Agency; Exploratory Research for Advanced Technology (ERATO); Suematsu Gas Biology Project; Tokyo, Japan

[†]These authors contributed equally to this work.

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Keywords: Clostridia, Treg, gut microbiota

*Correspondence to: Kenya Honda; Email: kenya@rcai.riken.jp

Submitted: 28/01/2014; Accepted: 17/03/2014; Published Online: 18/03/2014

http://dx.doi.org/10.4161/gmic.28572

Addendum to: Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 2013; 500:232-6; PMID:23842501; http://dx.doi.org/10.1038/nature12331

The gut microbiota plays important roles in the development of the host immune system. We have previously shown that a combination of 46 strains of commensal Clostridia isolated from conventionally reared mice can induce the accumulation of CD4^{+}Foxp3^{+} regulatory T (Treg) cells in the mouse colonic lamina propria. Subsequently, we succeeded in isolating and selecting 17 strains of Clostridia from a healthy human fecal sample that can significantly increase the number and function of colonic Treg cells in colonized rodents, thereby attenuating symptoms of experimental allergic diarrhea and colitis. Here we characterize each of the 17 strains of human-derived Clostridia in terms of sensitivity to antibiotics and ability to produce short chain fatty acids and other metabolites, and discuss their potential as biotherapeutics to correct dysbiosis and treat immune-inflammatory diseases.

Seventeen Strains of Treg-Inducing Clostridia Isolated From the Human Intestine

The intestinal microbiota is composed of ~100 trillion commensal bacteria and has co-evolved with the host by participating in many essential physiologic and metabolic functions. There is also abundant evidence that the gut microbiota affects the host immune status. Immunological effects of the microbiota are not simply due to the presence of innocuous bacteria, but to the biological activities of the gut microbiota consortium.[1] In many cases, regulation of development and/or effector functions of different immune cell populations (such as Treg cells vs Th17 cells) depends on the activity of different members of the commensal community. The relative abundance of these different immunomodulatory members can direct the general nature of host mucosal and systemic immunity. Previously, we referred to such immunomodulatory members of the microbiota as "autobionts".[2] However, currently there are relatively few specific examples of autobionts.

We and other groups have shown that segmented filamentous bacteria can potently induce interleukin-17-producing CD4^{+} T cells (Th17 cells) in the small intestine of mice.[3] We have also shown that a combination of 46 strains of Clostridia indigenous to conventionally reared mice can induce Treg cells in the mouse colonic lamina propria and thereby contribute to protecting mice against colitis and allergic responses.[4] In the most recent publication by our group, we identified 17 strains of human-derived Clostridia as potent inducers of Treg cells.[5] Starting from a complete healthy human fecal sample, a sequence of selection steps was applied to obtain Treg cell-inducing

Cluster	strain#	Closest species/strain		Similarity (%)	Benzylpenicillin	Ampicillin	Ampicillin /sulbactam	Amoxicillin /clavulanate	Tazobactam /piperacillin	Cefmetazole	Vancomycin	Imipenem	Meropenem	Metronidazole	Clindamycin
Cluster XVIII	St.1	*Clostridium*	*saccharogumia*	99.46											
	St.8	Clostridiaceae	JC13	99.16											
	St.18	*Clostridium*	*ramosum*	100.00											
Cluster IV	St.3	Lachnospiraceae	7_1_58FAA	100.00											
	St.13	*Anaerotruncus*	*colihominis*	100.00											
Cluster XIVa	St.4	*Clostridium*	*hathewayi*	99.06											
	St.6	*Blautia*	*producta*	99.79											
	St.7	*Clostridium*	*bolteae*	99.53											
	St.9	*Clostridium*	*indolis*	99.26											
	St.14	*Ruminococcus*	*sp.* ID8	98.59											
	St.15	*Clostridium*	*asparagiforme*	99.73											
	St.16	*Clostridium*	*sp. 7_3_54FAA*	100.00											
	St.21	*Eubacterium*	*contortum*	99.58											
	St.26	*Clostridium*	*scindens*	99.72											
	St.27	Lachnospiraceae	3_1_57FAA_CT1	97.54											
	St.28	Clostridiales	1_7_47FAA	99.73											
	St.29	Lachnospiraceae	3_1_57FAA_CT1	99.60											
CLSI break points (μg/ml)				Susceptible	0.25	≤0.25	≤4/8	≤4/2	≤4/32	≤16		≤4	≤4	≤8	≤2
				Intermediate	1	1	8/16	8/4	4/64	32		8	8	16	4
				Resistant	≥2	≥2	≥16/32	≥16/8	≥4/128	≥64		≥16	≥16	≥32	≥8

Figure 1. Antibiotic sensitivity of the 17 strains. The closest known species/strains for each of the 17 strains are shown. Antibiotic sensitivities are categorized as susceptible (white), intermediate (light orange) or resistant (dark orange). Antimicrobial susceptibility testing was performed using the broth microdilution method with dry plates (Eiken Chemical, Japan) according to the Clinical and Laboratory Standards institute (CLSI) guidelines M11-A8 and M100-S23. Briefly, each strain grown on Eggerth-Gagnon agar was harvested and suspended in ABCM broth (Eiken Chemical, Tokyo, Japan). The number of colony-forming units (CFU) was adjusted to 1×10^5 CFU/mL and an aliquot (100 μL) of the suspension was inoculated into each well of the plates. After incubation at 37 °C for 48 h, bacterial growth was visually assessed to determine the minimal inhibitory concentrations (MICs). The Etest (Sysmex-bioMérieux, Japan) was also employed to test for susceptibility to metronidazole and vancomycin.

human-derived bacterial strains using gnotobiotic techniques.[5] We first observed full Treg cell induction in the colon of ex-germ-free mice orally inoculated with a chloroform-treated human fecal sample. Then the cecal contents from these mice were treated with chloroform, diluted, and serially transplanted into other germ-free mice, while monitoring Treg induction capability. We succeeded in obtaining colonized mice in which the complexity of the gut microbiota was greatly decreased without sacrificing Treg-inducing potency. From these mice, we cultured and selected 17 strains which, when mixed together and orally administered to gem-free mice and rats, were able to induce a significant accumulation of $CD4^+Foxp3^+$ Treg cells in the colon. Furthermore, repeated oral ingestion of the mixture of 17 strains rendered specific-pathogen-free mice resistant to experimental allergic diarrhea and trinitrobenzene sulfonic acid (TNBS)-induced colitis.[5] Therefore, the 17 strains have at least a prophylactic effect in mouse colitis models.

Characterization of the 17 Strains

The 17 Treg-inducing strains isolated in our study all belong to the class Clostridia.[5] Clostridia species are gram-positive anaerobic rods and typically can form endospores. Some Clostridia species, including *Clostridium tetani*, *C. botulinum*, and *C. perfringens*, are well-known pathogens that are often isolated as a singular cause of infectious disease, and *C. difficile* is responsible for antibiotic-associated diarrhea and colitis. On the other hand, Clostridia species are extremely heterogeneous and many of them inhabit the large intestine of human and animals as predominant symbiotic microbes. Clostridia species can be classified into 19 clusters (I to XIX),[6] and many of the pathogenic species listed above belong to

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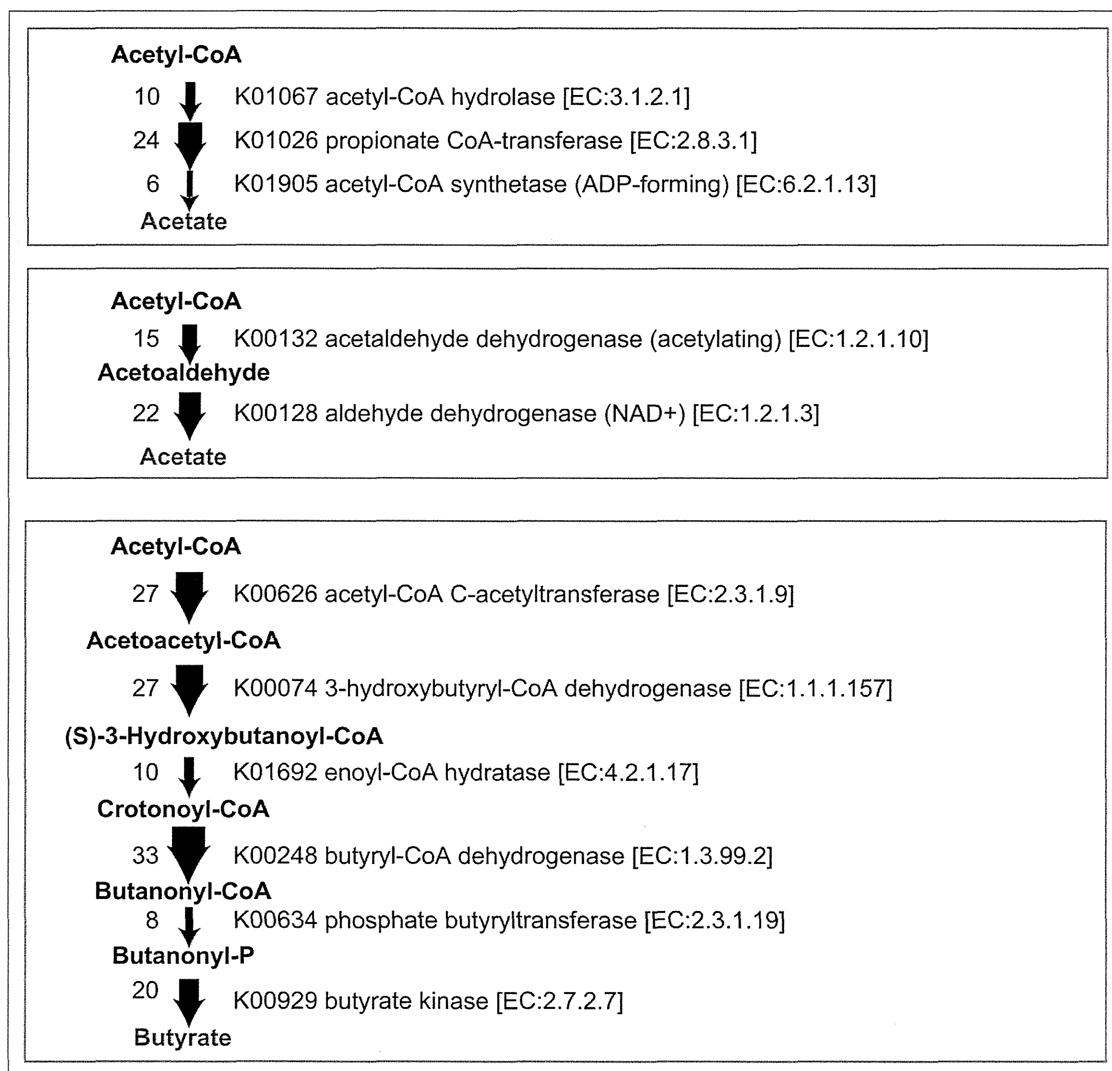

Figure 2. SCFA biosynthesis pathways and corresponding gene copy numbers in the 17 strains. Potential metabolic pathways leading to the production of acetate and butyrate from acetyl-CoA are shown. The thickness of the arrow and the number on the left of the arrow indicates the copy number of genes identified in the genomes of the 17 strains.

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cluster I. The 17 Treg-inducing strains fall within clusters IV, XIVa, and XVIII of Clostridia (**Fig. 1**). It has been reported that species within clusters XIVa and IV are indispensable for various physiological host functions. For instance, colonization with Clostridia clusters XIVa and IV normalizes the enlarged cecum found in germ-free mice and supports epithelial growth and turnover.[7] It was also shown that colonization with Clostridia cluster XIVa strains renders mice resistant to *C. difficile* colonization.[8]

It should be noted that there have been several reports of Clostridia clusters IV, XIVa, and XVIII species (*Clostridium clostridioforme*, *Clostridium innocuum*, and *Clostridium ramosum*, in particular) in clinical specimens of opportunistic infections.[9] However they are rarely single isolates, but rather a fraction of the multiple organisms in these clinical samples. Considering that commensal Clostridia species colonize in close proximity to the gut epithelial surface,[10] they may translocate when the barrier is compromised, not necessarily because of specific pathogenic properties, but simply because of their local abundance. Sequencing of the genomes of the 17 strains revealed that they lack known toxins and virulence factors.[5] Some of the 17 strains possess genes encoding putative sialidase, hyaluronidase, flagella-related protein, and fibronectin binding protein, but with low similarity to genes found in pathogenic Clostridia species. Furthermore, ingestion of a mixture of the 17 strains of Clostridia was effective in preventing colitis induced by TNBS[5] and even by dextran sulfate sodium (DSS) (data not shown), which chemically disrupts the epithelial barrier. Given the lack of major virulence factors and toxins in their genomes and the lack of any toxicity upon dosing to animals,

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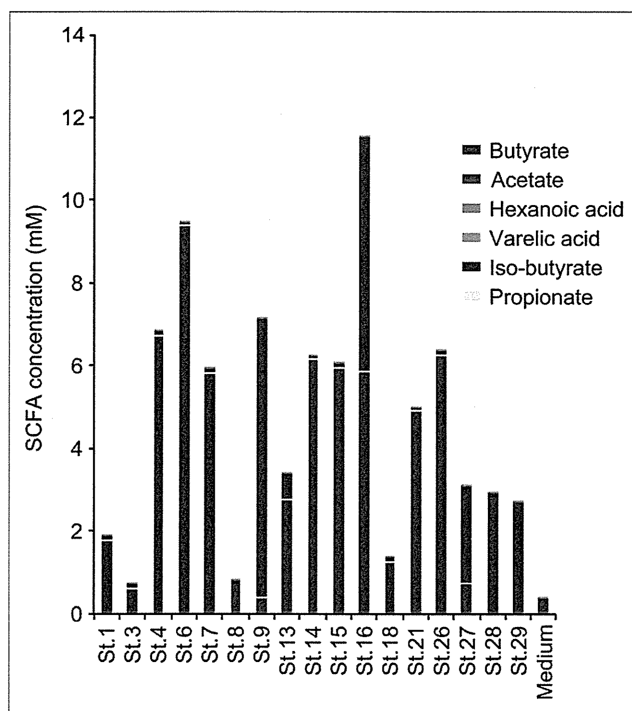

Figure 3. SCFA production by each of the 17 strains. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) analysis of short chain fatty acid metabolites was performed on 48 h cultures of peptone-yeast extract-Fildes solution supplemented with glucose at a concentration of 1% (w/v). Fatty acids in the culture supernatant were derivatized with 2-nitrophenylhydrazine and purified by liquid/liquid extraction.[28] Short chain fatty acid peaks were identified by both their specific MS/MS ion-transitions and comparison of the retention times with those of known short chain fatty acids in a standard solution.

the Clostridia strains isolated in our study appear to be safe for clinical use.

To further confirm that the isolated Clostridia strains are not harmful, antibiotic sensitivity of each strain was tested. All strains were susceptible to ampicillin-sulbactam, piperacillin-tazobactam, amoxicillin-clavulanate, metronidazole, and chloramphenicole, although some strains were resistant to penicillin G and ampicillin alone, and strains 1, 3, 8, and 18 showed low level resistance to vancomycin with MIC values of 4 to 8 μg/ml (**Fig. 1**). Since *C. ramosum* and *C. innocuum* are reported to have intrinsic resistance to glycopeptides and lipopeptides (vancomycin),[9] and genes identical to VanB2 ligase of *Enterococcus* spp. can be found in *C. hathewayi*, *C. boltae*, and *C. inocuum*-like bacteria,[11] it is not surprising that strains 1, 3, 8 and 18, which have 16S sequence similarities with those species, showed low level resistance to vancomycin. Overall, none of the 17 strains have high-level resistance to antibiotics tested; rather they show a benign safety profile. Further careful characterization of the strains and pre-clinical studies will be required before clinical translation.

Mechanisms of Treg Accumulation by the 17 Strains

The precise mechanism underlying how the 17 strains of Clostridia stimulate the induction of colonic Treg cells remains to be further elucidated. One suggested mechanism is the production of short chain fatty acids (SCFAs), which have multiple metabolic and immune functions.[12] In the context of Treg induction, SCFAs can elicit a TGF-β1 response in epithelial cells, which can contribute to de novo induction of peripheral Treg cells (pTreg).[5] SCFAs, particularly butyrate, can suppress dendritic cell activation through suppression of expression of the NFκB component RelB.[13] It has also been shown that butyrate activates signaling pathways through GPR109a to induce anti-inflammatory genes in dendritic cells.[14] In addition to its effects on dendritic cells, butyrate can directly stimulate thymic Treg cell (tTreg) proliferation through activation of GPR43[15] and the differentiation of naïve CD4⁺ T cells into pTreg cells through histone H3 acetylation of the *Foxp3* gene intronic enhancer by inhibition of histone deacetylase (HDAC).[13,16]

The genomes of the 17 strains contain abundant genes predicted to be involved in the biosynthesis of acetate and butyrate (**Fig. 2**). SCFA production by each of the 17 strains when cultured in vitro in glucose-supplemented medium was analyzed by a validated liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) system[17] (**Fig. 3**). Among SCFAs, acetate and butyrate were detected at high concentrations in most of the culture supernatants except those of strains 1, 3, 8, and 18. Notably, strains 9, 13, 16, 27, and 29 showed very high butyrate production (**Fig. 3**). These findings are consistent with previous reports showing that bacterial strains belonging to Clostridia cluster IV and XIVa were positive for butyryl-CoA:acetate CoA transferase, an enzyme responsible for butyrate production.[18] Importantly, mono-colonization of GF mice with one of each of the 17 strains was insufficient to induce Treg cells in vivo[5]; however, the mixture of 17 strains is effective, suggesting synergistic effects in a microbial community-dependent manner.

We also examined enzymatic properties of the 17 strains using API ZYM, Rapid ID 32A, and API 20A systems in vitro (**Fig. 4**). Enzyme activities related to virulence such as trypsin, α-chymotrypsin, and gelatin hydrolase, which have been implicated in infective endocarditis, and β-glucuronidase, which may release toxic substances by

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Figure 4. Enzymatic activity of the 17 strains. APIZYM and Rapid API 32A tests (Sysmex BioMérieux) were used for determination of enzymatic activities of each of the 17 strains. Cells from cultures grown on Eggerth-Gagnon blood agar plates for 48 h at 37 °C in an anaerobic chamber were suspended in saline and the turbidity was adjusted to 5–6 in the McFarland scale (approx. 1.5–1.9 × 10^9 CFU/ml). Aliquots of 65 μl of the suspensions were inoculated into cupules in the strip. In the case of Rapid API 32A, the turbidity was matched to 4 in the McFarland scale and aliquots of 55 μl were applied to the cupules. The strips were incubated at 37 °C for 4 h and the reactions were determined according to the manufacturer's instructions.

cleaving glucuronic acid-conjugation in the intestine, were very low or absent in the 17 strains. Of note, 17 strains showed variety of enzyme activities. Some strains were positive for the activity of principal mucin degrading enzymes, such as β-galactosidase and N-acetyl-β-glucosaminidase, and many strains exhibited strong activity of α-glucosidase, which is an intestinal enzyme that breaks down a variety of carbohydrates. Strain 9 and 13 could convert tryptophan into indole, which is reported to enhance epithelial barrier function and contribute to suppression of colitis.[19] Strain 14 was positive for proline arylamidase and pyroglutamic acid arylamidase activities. Together with SCFA production profiles, the 17 strains are diverse and heterogeneous in terms of enzymatic and metabolic properties. Considering that the dysbiotic microbiota associated with inflammatory bowel disease (IBD) is frequently accompanied by significantly decreased diversity,[20] the use of a functionally diverse mixture like the 17 strains of Clostridia described here may be reasonable for clinical applications to correct dysbiosis.

Future Directions

Imbalance in the gut microbiota, termed dysbiosis, significantly contributes to various human diseases. Several studies have reported decreased levels of Clostridia and Bacteroidetes in fecal samples from IBD patients.[21] Marked

decreases in the prevalence of Clostridia have been associated with increased risk of airway hypersensitivity and atopic dermatitis.[22,23] Animal studies have also shown that bacterial dysbiosis helps perpetuate the cycle of chronic inflammation characteristic of IBD.[24] Therefore, manipulation of the gut microbiota as a therapeutic strategy holds great promise for immuno-inflammatory diseases including IBD and allergy.

Alteration of the gut microbiome via fecal microbiota transplantation (FMT), which involves placing stool from a healthy donor via duodenal tubing, colonoscopy, or enema, has been shown effective in patients with pseudomembranous colitis induced by *Clostridium difficile* infection,[25] and researchers and patients are interested in testing the potential of FMT for the treatment of other disease associated with disruption of the intestinal microbiota, including insulin resistance, multiple sclerosis, and IBD.[26] While FMT has been established as a proof of principle for the feasibility of manipulating the human microbiome as a therapeutic strategy, the development of commercial products based on fecal transplants faces a number of hurdles from manufacturing, quality assurance, pathogen contamination risk, donor selection, and patient acceptance perspectives. Therefore, treatment with a composite of well-characterized benign microorganisms is more desirable from many perspectives for medical purposes.

There are many probiotics with a history of safe use in humans for medicinal purposes. However, probiotics currently in use have generally been selected based on properties such as ease of culture and tolerance to acid and oxygen and are not among the major colonizers of the human gut.[27] In other words, they have not been isolated based on their ability to correct microbiome dysbiosis associated with human disease or to boost specific arms of the host immune system. Presumably as a result, the dysbiotic microbiota are refractory to treatment with currently available individual probiotic strains, and most probiotics tested to date have demonstrated, at best, mediocre effects in the clinic. Thus, there is a compelling need to identify more robust therapeutic organism compositions that are compatible and symbiotic to the host and, ideally, able to induce broader changes to the microbial ecosystem to correct dysbiosis and drive the immune system to normal homeostasis.

In this context, we believe that the 17 strains of Clostridia isolated in our study can form the basis for a future live biotherapeutic product to treat certain forms of IBD, allergy, and other immune-inflammatory diseases. Compositions based on these strains could (1) help correct microbiome imbalances; (2) be easily administered orally and not necessarily frequently, providing patients with a convenient therapeutic option; (3) be safe for human use, since they are commensal strains that are long-term colonizers of the healthy human gut, do not have prominent virulence factors, and are sensitive to antibiotics; (4) be viewed favorably by patients given that they are natural compositions; and (5) be manufactured with traditional fermentation methods and developed under the live biotherapeutics route outlined by the FDA, thus circumventing the manufacturing, regulatory, and patient acceptance hurdles that hamper commercialization of fecal transplants.

Our next step should be optimization of the therapeutic mixture by elimination of nonessential components of the 17 strains for Treg cell induction. In addition to this, we need to identify and isolate other bacterial strains from human intestine that can regulate differentiation and activation of other immune cell subsets, such as Th17 cells. Our long-term goal is to offer a diverse but minimal consortium of microbes that can colonize the human gut and restore and maintain immune homeostasis to prevent and cure immune-inflammatory diseases.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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