Defined microbiota transplant restores Th17/RORγt+ regulatory T cell balance in mice colonized with inflammatory bowel disease microbiota

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The building evidence for the contribution of microbiota to human disease has spurred an effort to develop therapies that target the gut microbiota. In many chronic human diseases, this fact has driven an effort to understand how microbiota contribute to disease and if it represents a target for novel therapies. In the majority of conditions, no consistent association between the disease and any specific commensal species or strain has been made, and the distinction between the microbiota in the healthy and diseased states reflects broader differences in the community structure, diversity, or density (1, 2). In the rare cases where specific species or strains are hypothesized to contribute to disease—for example, adhesive-invasive Escherichia coli (AIEC) in Crohn’s disease (3)—targeted therapy using approaches such as phages may be possible (4). In other situations, rational therapeutic approaches are elusive, and the foremost strategy to modulate the microbiota has been fecal microbiota transplantation (FMT).

FMT is effective for treating recurrent Clostridioides difficile infection (rCDI) (5), but is also under investigation for the treatment of ulcerative colitis (UC), Crohn’s disease (CD), liver diseases, obesity and metabolic disease, food allergy, graft-versus-host disease, checkpoint colitis, and to improve the efficacy of cancer immunotherapy (6–8). Clinical trials of FMT in UC have suggested some potential for using microbiota-targeted therapy in...
this setting (9–12), but evidence for efficacy in other indications is currently extremely limited, and there is currently little or no scientific basis for the adoption of FMT as a mainstream therapeutic option outside of rCDI.

As seen in rCDI and some individuals with UC, FMT can restore health to the gut, but the mechanisms remain opaque. Studies in gnotobiotic mice have revealed defined gut microbiota manipulations that modulate the proportion or function of specific cells, and these data are aiding the development of candidate live biotherapeutic products designed to specifically target certain pathways or cells, for example, increasing the proportion of regulatory T cells (13, 14). However, to develop microbiota-targeted therapies for broad use, it is imperative to understand the specifics of how microbiota manipulations alter host processes and phenotypes in predictable ways. Only by doing this can we hope to achieve the maximum potential benefit of these approaches while exposing treatment recipients to the minimum of risk, as we demand of all other drugs and therapies in common use.

This is an extremely complex problem as numerous aspects of the immune system are shaped by the gut microbiota. This is evident in gnotobiotic mice, where the gut immune system is reproducibly altered by microbiotas of different composition (15, 16). However, even under tightly controlled conditions in gnotobiotic mice, predictably linking microbiota composition to host phenotype is challenging. Certain components of the intestinal microbiota have been suggested to influence immunomodulatory properties in the lamina propria when transferred to mice. For example, segmented filamentous bacteria (SFB) and some strains of E. coli induce T helper (Th)17 cells (17, 18), certain strains of Bacteroides ovatus induce high fecal IgA (20), and specific clostridial strains can induce regulatory T (Treg) cells (14, 16, 21). The context within which individual microbes colonize a host can alter their function. For example, immunomodulatory properties of individual strains can be altered in an inflamed host or when cocolonized with different organisms (20, 22). Thus, the immune landscape of the gut is influenced by the presence of specific strains and the compositional context of the broader microbiota, making the rational design of therapies targeting the microbiota challenging.

In ex-germ–free mice colonized with different complex human microbiotas from a diverse group of healthy donors, population-scale associations can be made between characteristics of the microbiota donors and the phenotype transferred to mice. For example, we have previously observed that microbiota from donors with inflammatory bowel disease induce more gut Th17 cells and fewer RORγt+ Treg cells than microbiotas from healthy donors (HDs) (15). These altered immune populations in wild-type mice are highly predictive of disease severity in a model of colitis using ex-germ–free mice colonized with the same IBD and non-IBD gut microbiotas, implying a strong functional relevance of these cells. These results suggest that different compositions of gut microbiota set stable immune landscapes in the gut lamina propria and alter disease susceptibility. Specifically, the inflammatory tissue immune program in ex-germ–free mice colonized by the gut microbiota of individuals with IBD and characterized by high Th17 cells and low RORγt+ Treg cells therefore represents a colitis-susceptible state that is potentially correctable by gut microbiota manipulation.

We established a gnotobiotic mouse experimental system to evaluate potential live bacterial therapy for IBD. This system allowed us to track recipient and donor strains during the procedure and monitor immunological changes in the host following treatment. Germ-free mice were initially colonized with a defined, cultured microbiota obtained from one of three donors with IBD, followed 3 wk later by a transplant with one of five defined consortia of HD-derived microbes. In testing 14 different IBD–HD combinations, we find that the inflammatory gut immune landscape induced by human IBD microbiotas is reversible and that transplantation with HD microbiotas can restore a tolerant immunological state. HD microbiota transplant consistently and reproducibly increased the proportion of gut RORγt+ Treg cells in mice initially colonized with IBD donor microbiotas. In mice colonized with an IBD donor that potently induces Th17 cells, transplants reduced the proportion of mucosal Th17 cells. We identify two distinct mechanisms associated with the recovery of gut immune health following transplant. Transplant-induced RORγt+ Treg cell induction was associated with increased fecal microbiota density, a measure previously associated with health (1, 23, 24). Where we observed reduced mucosal Th17 cells, this was associated with decreased colonization by a specific strain from the IBD microbiota. Further characterization revealed this strain was required for Th17 induction by this IBD microbiota. We demonstrate that the proinflammatory immune tone driven by IBD microbiotas is reversible by defined microbiota transplant.

**Defined Microbiota Transplant Enables Donor Strain Engraftment**

To establish a recipient-specific baseline immune tone, we first colonized germ-free C57BL/6 mice with defined, cultured fecal microbiotas (15) isolated from one of three different individuals with IBD (Fig. 1A and Dataset S1). Three weeks later, we introduced one of five defined cultured HD fecal microbiota via a single gavage (i.e., a defined microbiota transplant [DMT]) whereupon mice were individually housed to prevent mouse-to-mouse transmission of the DMT. We tested combinations of three IBD and five HDs in a minimum of six mice (14 total combinations) (Fig. 1A, Dataset S1, and Methods). We focused these experiments on cultured consortia where each species has a complete genome sequence, as this facilitates strain-level tracking of engraftment and downstream identification of immunomodulatory strains.

We performed metagenomic sequencing of DNA isolated from feces of mice before DMT, 3 wk after DMT, and from mice colonized with only the healthy microbiotas used in the DMTs. The HD microbiotas were composed of between 9 and 22 strains (mean 15.6). An average of 59.3% (range: 31.2 to 100%) of the he IBD strains were detected in the mice 3 wk after DMT (Fig. 1B and C). The proportion of strains that were engrafted from each HD was not significantly different (P = 0.42, ANOVA; Fig. 1D), and there was no difference in the mean proportion of strains that were engrafted from each HD among different combinations of IBD donor microbiota (P = 0.18, ANOVA; Fig. 1C). The three IBD microbiotas comprised 17, 13, and 20 strains, respectively. An average of 58.0% (range: 15.0 to 87.5%) of the strains from the IBD donors remained detectable in the mice following DMT (Fig. 1E). No one donor induced a greater loss of IBD microbiota strains than any other (P = 0.98, ANOVA), but the proportion of strains from donor IBD C that persisted following transplant was lower than microbiotas from donors IBD A and IBD B, although it is unclear if this was due to compositional differences between the IBD microbiotas or due to variation in the number of strains in each consortium (Fig. 1E). On average, the number of strains lost from the microbiotas was balanced by the engraftment of HD-derived strains, and the total number of strains comprising the posttransplant microbiotas was not significantly different from the number of strains detected in the IBD microbiotas before DMT (Fig. 1F).

**Modulation of Intestinal Th17 Cells by DMT**

Three weeks after DMT, we assessed the impact of the transplanted microbes on the host intestine tissue resident immune populations in comparison to the impact on mice receiving no transplant that were colonized with either recipient IBD microbiota or HD microbiota alone. We used flow cytometry to profile CD4 T cell populations in the colon and ileum for each donor, recipient, and donor+recipient combination. A hallmark of mice colonized with IBD donor microbiotas is an increase in the...
A proportion of gut Th17 cells, relative to mice colonized with HD microbiotas (15, 18, 25). As demonstrated in multiple animal models, increased Th17 cells are associated with susceptibility to inflammatory disease (26). Therefore, we hypothesize that a rational aim of microbiota-targeted therapy for IBD is to develop interventions that reduce these potentially pathogenic microbiota-induced Th17 cells. With this in mind, we selected as DMT donors HD-derived microbiotas that consistently induced a low proportion of LP Th17 cells (Fig. 2B). We measured the proportion of gut Th17 cells in mice colonized with IBD microbiotas alone, HD mice alone, and in mice with IBD microbiotas that had received healthy DMT; IBD A, from a donor with Crohn’s disease, was notable for inducing a particularly high proportion of colonic Th17 cells in gnotobiotic mice (Fig. 2B) (15). In mice colonized with microbiota IBD A, DMT with all of the healthy microbiotas reduced the proportion of Th17 cells in the colon (Fig. 2B and C). In mice colonized with IBD B or IBD C where baseline Th17 cells was lower, DMT did not significantly reduce the proportion of colon Th17 cells. A similar effect was observed in the ileum where the proportion of Th17 cells was reduced following four of the five DMTs in mice colonized with IBD A, but not significantly altered in mice colonized with IBD B or C (SI Appendix, Fig. S1).

**Identification of a Th17-Inducing Strain from a Donor with Crohn’s Disease**

Intestinal Th17 cells can be induced by specific microbes, for example, SFB (17), a strain of *Bifidobacterium adolescentis* (27), and specific strains of *Escherichia coli* (18). As the proportion of Th17 cells induced by donor IBD A was particularly high, we hypothesized that the induction of Th17 cells by this microbiota was due to the presence of a specific strain that drives their induction and that perhaps the abundance of this strain was modulated following DMT, leading to the reduction in Th17 cells. Using 16 strains from the IBD A microbiota, we assembled eight subcommunities, each with four strains, using an orthogonal design (28). Each strain was present in two different subcommunities. We colonized groups of germ-free B6 mice with the eight subcommunities and assessed the impact of each on the proportion of colon Th17 cells (Fig. 3A). The eight subcommunities induced varied proportions of IL-17A⁺ Th17 cells (*P* = 0.0004, ANOVA; Fig. 3B). We quantified the association of each strain with the proportion of Th17 cells and that found a single strain, *E. coli* A6, positively associated with the proportion of colon RORγt⁺ and IL-17A⁺ Th17 cells (*P* = 0.0035, *t* test; Fig. 3C and SI Appendix, Fig. S2A). Of note, a second strain of *E. coli*,

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**Fig. 1.** Defined microbiota transplant enables donor strain engraftment. (A) Germ-free mice were first colonized with one of three defined IBD donor microbiotas before microbiota transplant with one of five defined HD microbiotas 3 wk later. (B) The average contribution of IBD donor- and HD-derived strains to the microbiota 3 wk after transplant as a proportion of the total community. (C) The average number of IBD donor- and HD-derived strains that comprise the microbiota 3 wk after transplant. (D) The average proportion of strains from each HD that engraft when given as a microbiota transplant to each IBD microbiota-colonized recipient. (E) The average proportion of strains from each IBD donor that persist following each microbiota transplant. (F) The average total number of strains comprising the microbiota of mice colonized with IBD donor-derived microbiota before and after transplant and the change in the total number of strains following transplant. Plots in D–F show the mean of each group of mice ± SE. *P* < 0.05, *** *P* < 0.001, ns: not significant by ANOVA with Tukey correction.
isolated from IBD A (E2) was not associated with Th17 cells in this screen ($P = 0.94$, t test; SI Appendix, Fig. S2A). The effect appeared to be specific to RORγt+ effector T cells, as the proportion of RORγt+ FoxP3+ Treg cells was not associated with E. coli A6 (SI Appendix, Fig. S2B). To confirm the specific ability of E. coli A6 to induce Th17 cells, we generated a consortium of the strains isolated from IBD donor A excluding only E. coli A6. Mice colonized with this consortium lacking only E. coli A6 (ΔA6) had a significantly reduced proportion of colon IL-17A+ Th17 cells compared to mice colonized with the complete community from donor IBD A (Fig. 3D). These data suggested that E. coli A6 is required for Th17 cell induction in the context of microbiota IBD A. We next considered if E. coli A6 could have a similar immunomodulatory effect in a different setting. We constructed an artificial community of seven common commensal species (see Methods for details) and colonized groups of germ-free mice with this consortia with and without the addition of E. coli A6. In the context of this nonnative community, E. coli A6 significantly increased the proportion of lamina propria Th17 cells, but to a lesser extent than was observed for the original IBD-A community (SI Appendix, Fig. S2C). Strains of AIEC that induce Th17 cells in mice have been described (18). E. coli A6 was poorly invasive and adherent in a Caco-2 cell assay, did not survive in a macrophage cell line as compared to known strains of AIEC, and thus did not satisfy the phenotypic criteria for AIEC (SI Appendix, Fig. S2D). It was also negative for the pduC virulence factor previously associated with Th17 induction (18). These results demonstrate that non-AIEC E. coli strains can also induce Th17 cells.

CD4+ T cells from the mesenteric lymph nodes (mLN) of mice colonized with IBD A secreted IL-17A when restimulated ex vivo with dendritic cells loaded with a lysate of E. coli A6 (Fig. 3E) (29). This response was prevented by MHC-II blockade, suggesting a cognate interaction between the Th17 cells and DC-presenting antigen from E. coli A6 (Fig. 3E). Lamina propria Th17 cells induced by the model Th17-inducing bacterium SFB are oligoclonal and characterized by an expansion of Th17 cells utilizing the Vβ14 T cell receptor (29). Using a broad panel of anti-Vβ chain antibodies, we tested if a similar expansion occurred in mice colonized with E. coli A6. In contrast to mice colonized with SFB, we did not find an expansion of Vβ14+ CD4 T cells in the Th17 compartment of the colon, small intestine, or mLN and did not see significant enrichment of any particular Vβ chain in mice colonized with IBD A compared to SFB-containing microbiota (SI Appendix, Fig. S2E).

We analyzed the relative abundance of the IBD-donor–derived strains in mice both before and following DMT with each of the five HD microbiotas. The abundance of the Th17-inducing E. coli A6 strain was significantly reduced in feces of mice following four of the five DMTs, relative to mice colonized with IBD A alone (Fig. 4A and SI Appendix, Fig. S3A). The relative and absolute abundance of E. coli A6 in the fecal microbiota before and after DMT was associated with the proportion of colon RORγt+ Th17 cells (Fig. 4B and SI Appendix, Fig. S3B). This suggests a mechanism whereby, in the context of the IBD A microbiota, DMT with HD microbiotas reduces the relative abundance of a specific strain, resulting in a reduction in lamina propria Th17 cells.

### Induction of Regulatory T Cells following DMT

The RORγt+ subset of intestinal regulatory T cells enforces tolerance to microbiota (30). RORγt+ Treg cells are dynamically and variably induced by different microbiotas (31) and are specifically deficient in mice colonized with microbiotas from donors with IBD (15). We hypothesize that one rational aim of microbiota-targeted therapy for IBD would be to increase the in situ differentiation and stability of RORγt+ Treg cells. When colonized alone, each of the three IBD recipient microbiotas (IBD A–C) induced a relatively low proportion of RORγt+ Treg cells in both colon and ileum of mice (Fig. 5A and B). Three weeks following microbiota transplant, the proportion of RORγt+ Treg cells in the colon of recipient mice in all cases significantly increased relative to mice colonized with each IBD microbiota alone (Fig. 5A). We observed no “super donor” microbiota that consistently induced a higher proportion of RORγt+ Treg cells across all recipient microbiotas, and mice colonized with each of the three IBD donor microbiotas were similarly amenable to immune modulation by DMT in the colon.

Modulation of RORγt+ Treg cells was less pronounced in the ileum where significant increases were limited to three of the DMTs only in mice colonized with IBD A (Fig. 5B). In addition to the RORγt+ subset of Treg cells, we also observed modulation of the total gut FoxP3+ Treg population (SI Appendix, Fig. S4A and B). Microbiotas that induce high proportions of RORγt+ Treg cells are also associated with reduced activation of colon dendritic cells (15, 30). Consistent with these observations, the expression of CD86 was lower on both total and CD11b+CD103+ (double positive) colonic dendritic cells from mice colonized with HD 1 than IBD A and are reduced post DMT (SI Appendix, Fig. S4C).

The proportion of total FoxP3+ Treg in colon and ileum was less consistently altered following DMT (SI Appendix, Fig. S4A and B). Interestingly, the proportion of colon FoxP3+ Treg cells in each recipient donor microbiota combination was predictable from the baseline induction by the donor microbiota alone ($R^2 = 0.47$, $P = 0.001$, t-test) (SI Appendix, Fig. S4D). However, the proportion of RORγt+ Treg cells was not predictable from the baseline induction of these cells by the donor and recipient...
from mLN CD4+ T cells isolated from mice colonized with IBD A and restimulated ex vivo with anti-CD3 and anti-CD28 or dendritic cells loaded with donors alone, suggested that RORγt proportion was unrelated to the proportion induced by the induction following every tested DMT, and that the posttrans- 
test; Fig. 6 and C. The proportion of IL-17A+ CD4+ T cells and RORγt+ Th17 cells in the colon lamina propria of mice colonized with the complete IBD A microbiota or a modified version of IBD A lacking E. coli strain A6 (ΔA6). (E) IL-17A secretion from mLN CD4+ T cells isolated from mice colonized with IBD A and restimulated ex vivo with anti-CD3 and anti-CD28 or dendritic cells loaded with E. coli strain A6, with or without an MHC-II blocking antibody. Plots show the mean and SE of each group of mice, and P values were calculated by t test (C and D), ANOVA (B), and ANOVA with Tukey correction (E). **P < 0.01, ***P < 0.001, ****P < 0.0001.

The IBD microbiota has reduced alpha diversity (32, 33) and lower density than the microbiota from HDs (1, 23, 24, 34). The proportion of intestinal Treg cells can be influenced by microbiota composition (14–16, 21, 31), but can also be regulated independently of composition in response to changes in microbiota density (1). Following FMT for rCDI in humans, both the alpha diversity and the density of the gut microbiota increased (1, 35–38). We therefore examined if the increase in RORγt+Treg cells that we observe following DMT in our model could be attributed to changes in either alpha diversity or microbiota density (Fig. 6). We calculated microbiota density as the amount of DNA extracted from a fecal sample divided by the original sample mass (1, 39). Alpha diversity increased following some of the DMTs, particularly those involving IBD A and C, but this was not consistent for all tested pairs of IBD and HD microbiotas (P = 0.07, paired t test; Fig. 6 A and B). However, microbiota density significantly increased after DMT (P = 0.004, paired t test; Fig. 6 C and D).

Increased fecal microbiota density has been reported in individuals receiving FMT to treat rCDI (1). To assess the relevance of microbiota density modulation in FMT used in the context of IBD, we analyzed data from a recent placebo-controlled clinical trial of FMT in individuals with ulcerative colitis (9). This study reported endoscopic remission in ~20% of individuals receiving an intensive multidonor FMT regime. Strikingly, microbiota density was higher following 8 wk of intensive FMT therapy in individuals in which endoscopic remission was achieved than in those where FMT did not lead to endoscopic remission (P = 0.024; Welch two sample t test, Fig. 6E).

In the gnotobiotic mouse model, microbiota density but not alpha diversity was significantly associated with the proportion of colonic RORγt+ Treg and total FoxP3+ Treg cells (R² = 0.23, P = 0.01 and R² = 0.14, P = 0.01, respectively, f-test; Fig. 6F and SI Appendix, Fig. S5 A–C). We find no association between microbiota density and the proportion of lamina propria Th17 cells that we observe following DMT in our model could be attributed to changes in either alpha diversity or microbiota density (Fig. 6). We calculated microbiota density as the amount of DNA extracted from a fecal sample divided by the original sample mass (1, 39). Alpha diversity increased following some of the DMTs, particularly those involving IBD A and C, but this was not consistent for all tested pairs of IBD and HD microbiotas (P = 0.07, paired t test; Fig. 6 A and B). However, microbiota density significantly increased after DMT (P = 0.004, paired t test; Fig. 6 C and D).

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We have previously shown that induction of RORγ Treg cells can protect mice from intestinal inflammation in a microbiota-dependent model of colitis. To test this, we colonized groups of Rag-deficient germ-free mice with microbiota IBD A or IBD C followed after 2 wk with a DMT of either microbiota HD-1 or HD-3. Colitis was induced after a further 2 wk by the transfer of naive (CD45RB<sup>HI</sup> CD25<sup>−</sup>) CD4<sup>+</sup> T cells, and colitis was monitored by changes in body mass. As expected (15), mice colonized with only an IBD microbiota developed more severe disease than those colonized with only a HD microbiota (Fig. 7B and C). Mice that received DMT before colitis induction lost significantly less weight than those that did not receive DMT (Fig. 7B and C), demonstrating that HD-derived DMT can protect from intestinal inflammation in this model.

**Discussion**

Compositional changes to the gut microbiota are observed in many human diseases. Fecal microbiota transplantation is predicated on the assumption that an altered microbiota contributes to disease and restoring the composition of the microbiota to a "normal" state will provide therapeutic benefit. Despite a steady increase in FMT trials for different indications, we have little data to make predictions about how a treatment recipient will respond to a microbiota transplant and what are the desirable traits of a good donor and the molecular markers to indicate success beyond standard clinical end points. Manipulation of microbiota community characteristics, including colonization resistance, microbiota fitness, and microbiota density are likely key to the success of FMT in the context of recurrent *C. difficile* infection. However, the application of FMT in the context of autoimmune or inflammatory diseases likely requires achieving an element of specific immune modulation either in the gut, at a distal tissue site, or systemically. Immune modulation directed by microbiota manipulation could be achieved through a combination of suppression or elimination of proinflammatory effector strains and expansion or addition of tolerance-promoting strains or community characteristics. To advance microbiota manipulation as a potential therapeutic for immune-mediated disease, and to provide a solid scientific basis for the expanded use of such therapies beyond rCDI, we must better understand the potential of microbiota manipulation to alter specific immune populations and ultimately track these immune populations in FMT clinical trials (43–45).

We report a set of gnotobiotic mouse experiments that support the preclinical assessment of LBPs and microbiota transplants in the context of human inflammatory disease-associated microbiota. In this study, we used mice colonized with defined IBD donor microbiotas and assessed how the immunophenotype of these hosts responds to DMT with HD microbiotas with known immunomodulatory properties across a total of 14 different microbiota transplants. The gut immune landscape of the mice is consistently reshaped following DMT. We demonstrate that introduction of HD microbiotas to mice colonized with IBD microbiotas can lead to an increase in the proportion of Treg cells, including the potently immunoregulatory RORγ Treg subset. We find that the increase in RORγ Treg cells occurs with a restoration of microbiota density following DMT. Microbiota density is decreased in humans with IBD and is correlated with the proportion of gut Treg cells in specific pathogen-free mice (1, 23). We found increased microbiota density in individuals with ulcerative colitis reaching endoscopic remission following intensive FMT in a recent placebo-controlled clinical trial (9), relative to individuals in whom endoscopic remission did not occur. We therefore hypothesize that the DMTs in mice restore the microbiota to a compositional or structural state that supports a greater microbiota density, which leads to induction of RORγ Treg
cells. In humans receiving FMT for ulcerative colitis, a higher microbiota density following treatment associates with better endoscopic outcomes, suggesting a shift toward a more tolerance-promoting mucosal environment. Interestingly, the increase in RORγt+ Treg cells was not correlated with increases in alpha diversity, which is frequently associated with health and is one of the most commonly used metrics of dysbiosis. This suggests that microbiota density may be a metric of microbiota health as it relates to the tolerogenic Treg compartment and could be a useful biomarker for noninvasive monitoring of fecal microbiota transplant (1).

Microbiotas from donors with IBD induce a greater proportion of Th17 cells in the gut than do HD microbiotas (15, 18). Focusing on mice colonized with a microbiota from a donor with Crohn’s disease, we found that DMT using HD microbiotas suppressed the proportion of gut Th17 cells. This reduction in Th17 cells following DMT was not consistently associated with changes in microbiota density or alpha diversity. Instead, we found that a specific strain of E. coli was a primary driver of Th17 cell enrichment in mice colonized with this donor consortia and that decreased Th17 cells following DMT was correlated with a significant decrease in the abundance of this strain. Whereas RORγt+ Treg cell proportions were modulated by DMT following broad changes to the microbiota density, the reduction of Th17 cells following microbiota transplantation in mice colonized with this CD donor microbiota may be attributed to depletion of one specific strain. This more broadly reflects a different rationale being used to design microbiota-based therapeutics. Some approaches, such as FMT, aim to reverse dysbiosis and restore a broad structural and compositional state that is associated with health. Other approaches, such as the use of phage therapy or CRISPR/Cas-based strategies (4, 13), aim to...
target specific strains implicated in the particular disease. If microbiota-targeted therapies are to be successfully used in the context of complex diseases beyond rCDI, it is likely that a combination of these strategies will be required.

Methods

Human Microbiota Consortia. Stool samples from IBD donor A (Crohn’s disease) and donors B and C (UC) were collected from de-identified donors in the context of complex diseases beyond rCDI, it is likely that a combination of these strategies will be required.

Mice and Gnotobiotic Methods. Germ-free C57BL/6J mice were bred in isolators at the Mount Sinai Precision Immunology Institute Gnotobiotic Facility. SPF C57BL/6 mice were purchased from Jackson Laboratories. Mice were colonized at approximately 6 wk old by a single gavage of a defined microbiota consortia. Following colonization, mice were housed in autoclaved filter-top cages, fed an autoclaved diet (SK67, LabDiet) and water, and handled under aseptic conditions. Some mice received a defined microbiota transplant, administered as a single gavage 3 wk after initial primary colonization. At the time of DMT, mice were transferred to a new autoclaved cage to minimize the carryover of the preexisting microbiota from the cage environment. Where indicated, mice were provided antibiotics at the following concentrations: ampicillin 1 mg/mL, ciprofloxacin 0.1 mg/mL, clindamycin 0.267 mg/mL, polymyxin B 0.1 mg/mL, or vancomycin 0.5 mg/mL in drinking water with 2% sucrose, prepared as previously described (1). FoxP3YFP–/– (B6.129(Cg)-Foxp3tm4(YFP/icre)Ayr) and Rorcfl/fl (B6.Cg-Rorcfl/flJ) were purchased from Jackson Laboratories and crossed in-house under SPF conditions.

Lymphocyte Isolation. Gut tissues were prepared as previously described (15). Briefly, cleaned gut tissues were deepithelialized in 5 mM ethylenediaminetetraacetic acid (EDTA), 15 mM Hepes, and 5% fetal bovine serum (FBS) in Hank’s Balanced Salt Solution (HBSS) before digestion with 0.5 mg/mL Collagenase Type IV (Sigma Aldrich) and 0.25 mg/mL DNase I in HBSS with 2% FBS. Lymphocytes enriched by passing the cell suspension sequentially through 100- and 40-μm strainers. For enzyme-linked immune adsorbent spot (EISPOT) experiments, CD4+ T cells were isolated from mLNs using magnetic isolation (CD4 microbeads, Miltenyi Biotec) and dendritic cells were isolated from the spleens of naive SPF C57BL/6 mice (Jackson Laboratories) by magnetic isolation (CD11c microbeads, Miltenyi Biotec) using an AutoMACS instrument (Miltenyi Biotec) following the manufacturer’s instructions.

T Cell Transfer Colitis. Colitis experiments were performed as previously described (15). Briefly, CD4+ T cells were magnetically enriched (Magsort CD4 Enrichment Kit; Thermo Fisher) from spleens of naive SPF C57BL/6 (Jackson Labs), FoxP3YFP–/– x Rorcfl/fl; mice, or FoxP3YFP–/– x Rorcfl/fl; cells before fluorescence-activated cell sorting purification of CD4+ CD25+FoxP3+CD127–/– cells. Rag1−/− C57BL/6 mice were administered 1 × 10^6 naive CD4 T cells by intraperitoneal injection and monitored for changes in body mass weekly.

Flow Cytometry. Flow cytometry of CD4+ T cells was performed as previously described (15). The following antibodies were used for T cell phenotyping: CD45 APC-Cy7 (Biolegend), CD4 APC (Biolegend), FoxP3 PE (Thermo Fisher/ebioscience), RORγt PerCp-Cy5.5 (BD Bioscience), and IL-17A-PE (BD Bioscience), and dead cells were excluded using Zombie Aqua (Biolegend). To detect intracellular IL-17A, isolated lymphocytes were first restimulated with 5 ng/mL phorbol 12-myristate 13-acetate and 50 ng/mL ionomycin in the presence of monensin (Biolegend) for 3.5 h. FoxP3 and RORγt expression was analyzed in unstimulated cells. For analysis of dendritic cells, the following antibodies were used: CD45 BrilliantViolet 750 (BD Bioscience), MHC-II PerCp-Cy5.5 (Biolegend), CD86 APC (Biolegend), and CD64 BrilliantViolet 786 (BD Bioscience), and CD86 BrilliantViolet 605 (Biolegend), and dead cells were excluded with Fixable Viability Dye eFluor780 (ThermoFisher/ebioscience). The Vβ1 chain repertoire analysis used the following antibodies: Vβ2 AF647 (Biolegend), Vβ3 BrilliantViolet 510 (BD), Vβ5.1/5.2 PE-Cy7 (Biolegend), Vβ6 BrilliantViolet 650 (BD), Vβ7 FITC (Biolegend), Vβ8.2 APC-Viso770 (Miltenyi), Vβ10 BrilliantViolet 711 (BD), Vβ11 BrilliantViolet 421 (BD), Vβ12 BrilliantViolet 480 (BD), Vβ13 PerCp-eFluor710 (ThermoFisher/ebioscience), Vβ14 biotin (ThermoFisher/ebioscience) with streptavidin Qdot800 (ThermoFisher), Vβ17a BrilliantViolet 560 (BD), CD45 BrilliantViolet 750 (BD), FoxP3 PE (ThermoFisher/ebioscience) RORγt APC (BD) and CD45 BrilliantViolet 570 (Bioscience). T cell phenotyping data were generated using an LSRII instrument (BD Biosciences), Vβ chain and dendritic cell phenotype data were acquired using an Aurora spectral cytometer (Cytek). Baseline immune profiles for the three recipient communities and four of the five donor communities (except donor 5) were previously published (15) and are included here as baseline reference points to compare with the results of the DMT.

EISPOT Assay. EISPOT experiments for the detection of microbe-specific T cell responses were performed as previously described with some minor modifications (29). CD4+ T cells were harvested from the mLNs of anotobes that were isolated from the spleens of naïve SPF C57Bl/6 mice (Jackson Laboratories) by magnetic isolation (CD11c microbeads, Miltenyi Biotec)
using an AutoMACS instrument (Miltenyi Biotec) following the manufacturer’s instructions. Bacterial strains were grown to stationary phase in Luria-Bertani (LB) media and bacterial antigen by autoclaving the washed bacteria in phosphate-buffered saline (PBS). Dendritic cells were plated at a density of 50,000 cells/well and pulsed with antigen (1:100 dilution of the stationary phase culture) overnight. Isolated CD4+ T cells were washed three times with PBS at 3 h post-infection, serially diluted, and plated on LB agar. For invasion assays, cells were washed three times with PBS, and extracellular bacteria were killed with 100 μg/mL gentamicin for 1 h. Intracellular bacteria were enumerated as described above. The adhesion and invasion levels were expressed as the total number of colony-forming units per milliliter recovered per well. J774 macrophages were seeded in 24-well plates at a density of 2 × 10^6 cells/mL. The next day they were infected with E. coli at a MOI of 10 for 1 h. After the infection period, cells were washed three times with PBS, and extracellular bacteria were killed with 100 μg/mL gentamicin before gentamicin concentration was dropped to 20 μg/mL for longer infection periods. Survival after 24 h was expressed as the mean percentage of bacteria recovered after 1 h post-infection, defined as 100%. For cell culture assays, a noninvasive E. coli strain (DH5α) and an AIEC strain CU 541–15 or LF82 were used as negative and positive controls, respectively (49). To determine the motility of the E. coli strains, E. coli was grown overnight at 37 °C in LB broth. Two microliters of overnight culture was inoculated onto soft agar (1% tryptone, 0.5% NaCl, 0.25% agar), and plates were incubated at 30 °C overnight. Motility was determined by measuring the diameter of the ring formed by each strain.

**Data Availability.** Anonymized next-generation sequencing (NGS) data have been deposited in the National Center for Biotechnology Information under accession nos. PRJNA518912 and PRJNAS89044.

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**Genome and Metagenome Sequencing.** DNA was extracted from isolated bacterial strains by mechanical dissociation by bead beating in 282 μL of DNA buffer A (20 mM Tris, pH 8.0, 2 mM EDTA, and 200 mM NaCl), 200 μL of 20% sodium dodecyl sulfate (SDS) (w/v), 550 μL of Phenol:Chloroform:IAA (25:24:1), 268 μL of Buffer PM (Qiagen), and 400 μL of 0.1-mm-diameter zirconia/silica beads (20). DNA was extracted from mouse feces by bead beating in NDSa Inactivation Buffer (0.5% SDS, 0.5 mM EDTA, 20 mM Tris, pH 8.0, with 200 μL of 0.1-mm-diameter zirconia/silica beads (1)). DNA from bacterial isolates and feces was isolated using QiaQuick (Qiagen) columns and quantified by Qubit assay (Life Technologies). Sequencing libraries were generated from sonicated DNA with the NEBNext Ultra II DNA Library Prep kit (New England BioLabs). Ligation products were purified with SPRIselect beads (Beckman Coulter), and enrichment PCR was performed with NEBNext Ultra Q5 Master Mix (New England BioLabs). Samples were pooled in equal proportions and size-selected using 0.6× followed by 0.2× of AMPure XP beads (Beckman Coulter) before sequencing with an Illumina HiSeq Ultra 3000 instrument of the stationary phase culture overnight. Isolated CD4+ T cells were grown in 24-well plates for 2 d, and confluent monolayers were incubated with 0.5 μL of 20% L of 20% geno-globin sample. Genomes for strains in HD-5 were downloaded from NCBI; accession numbers can be found in Dataset S1 (14). Abundances were scaled to the size of each specific genome as previously described (48). Microbiota density was calculated as the mass of DNA extracted from a fecal sample, divided by the mass of the sample (the manufacturer’s instructions (R&D Systems)).

E. coli Adhesion, Invasion, and Motility Assays. Colonial epithelial cell line Caco-2 (ATCC HTB-37) and murine macrophage cell line J774 (ATCC TIB67) were obtained from ATCC and grown according to ATCC protocols. Caco-2 cells

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