Host immunity modulates the efficacy of microbiota transplantation for treatment of *Clostridioides difficile* infection

Eric R. Littmann¹, Jung-Jin Lee², Joshua E. Denny³, Zahidul Alam³, Jeffrey R. Maslanka³, Isma Zarin³, Rina Matsuda⁴, Rebecca A. Carter⁵, Bože Susac⁵, Miriam S. Saffern⁵, Bryton Fett², Lisa M. Mattei², Kyle Bittinger² & Michael C. Abt³

Fecal microbiota transplantation (FMT) is a successful therapeutic strategy for treating recurrent *Clostridioides difficile* infection. Despite remarkable efficacy, implementation of FMT therapy is limited and the mechanism of action remains poorly understood. Here, we demonstrate a critical role for the immune system in supporting FMT using a murine *C. difficile* infection system. Following FMT, *Rag1* heterozygote mice resolve *C. difficile* while littermate *Rag1−/−* mice fail to clear the infection. Targeted ablation of adaptive immune cell subsets reveal a necessary role for CD4⁺ Foxp3⁺ T-regulatory cells, but not B cells or CD8⁺ T cells, in FMT-mediated resolution of *C. difficile* infection. FMT non-responsive mice exhibit exacerbated inflammation, impaired engraftment of the FMT bacterial community and failed restoration of commensal bacteria-derived secondary bile acid metabolites in the large intestine. These data demonstrate that the host’s inflammatory immune status can limit the efficacy of microbiota-based therapeutics to treat *C. difficile* infection.
The microbiota, consisting of the community of bacterial, viral, fungal, and protozoa organisms that inhabit a mammalian host, can impact susceptibility to a range of diseases including cancer, diabetes, allergy, obesity, and infection. An altered or dysbiotic intestinal microbiota is observed in all of these disease states suggesting that resetting the intestinal microbiota with a composition of bacteria from healthy individuals could treat disease. This theory is supported by animal studies demonstrating alleviation of disease through transplantation of bacteria into the diseased host’s intestinal tract. Significant advancements have been made in defining the community of microbial species that are important in supporting human health. Translating these findings into viable microbiota-based therapies has had limited success in the clinic, however, and is not yet considered a treatment option for most diseases impacted by microbial dysbiosis. One notable exception is the use of fecal microbiota transplantation (FMT) to treat recurrent Clostridium difficile infection. FMT treatment of C. difficile infection is the first microbiota-based therapy clinically proven to ameliorate disease. Before such treatment strategies can be broadly implemented, the underlying principles determining success for microbiota-based therapy need to be elucidated.

C. difficile, a gram positive, spore-forming, obligate anaerobe is currently the most common nosocomial infection encountered by hospitalized patients, with nearly a half million patients infected and an estimated 13,000–30,000 deaths annually in the USA alone. This opportunistic pathogen infects the large intestine following perturbation of the intestinal microbiota. Vancomycin or fidaxomicin are first line treatment options that effectively target the vegetative form of C. difficile, but fail to resolve the underlying condition that promotes infection, a dysbiotic microbiota. Recurrence of C. difficile infection due to repeated failed antibiotic treatments is estimated as high as 25–35% and can cause potentially lethal fulminant colitis. This high recurrence rate following standard antibiotic treatment has spurred the development of new treatment modalities such as FMT. In 2013, the first controlled, double-blinded study reported the superior efficacy of FMT compared to antibiotics in treatment of recurrent C. difficile infection. Subsequent studies demonstrate FMT has an 80–90% cure rate in individuals and this treatment option is now incorporated as standard care for recurrent disease in both Europe and the USA.

Intestinal dysbiosis in C. difficile-infected patients is characterized by a loss of bacterial diversity and altered production of microbiota-derived intestinal metabolites. Successful FMT restores homeostasis both in the intestinal microbial community and metabolism. For example, following FMT, commensal bacteria that convert host-derived primary (1°) bile acids into secondary (2°) bile acids repopulate the large intestine and 2° bile acid pools are restored to levels observed in healthy individuals. The presence of 2° bile acid converting bacteria promotes colonization resistance against C. difficile and restoration of the 2° bile acid pools is associated with successful FMT in recurrent C. difficile-infected patients. These reports provide evidence that efficacy of the FMT is dependent on the bacterial consortium successfully engraving in the intestine of the infected host to restore the intestinal microenvironment to pre-infection conditions and create an inhospitable environment for C. difficile. The role of host immune factors in supporting FMT engrainment and subsequent clearance of C. difficile has not been explored and is the focus of this report.

In this study, we demonstrate that multiple strains of immunodeficient mice, all of which lack CD4+ Foxp3+ T-regulatory (Treg) cells, exhibit increased intestinal inflammation compared to immunocompetent mice when persistently infected with C. difficile and fail to resolve infection following FMT. The transplanted bacteria from the FMT inoculum do not completely engraft in the large intestine of FMT non-responsive mice and the intestinal metabolite profile is not restored to pre-infection levels. These data reveal an important role for the host immune system in supporting bacterial engrainment and subsequent resolution of C. difficile infection.

Results
FMT mediated clearance of C. difficile infection resolves intestinal inflammation. Fecal microbiota transplantation is a proven treatment for recurrent C. difficile infection, however, whether the host’s immune system contributes to FMT-mediated resolution of C. difficile is unknown. To address this question, we first established a murine model of C. difficile infection followed by FMT treatment (Fig. 1a). Similar to previous reports in both mice and humans, FMT reduced C. difficile burden and toxin levels in the large intestine of persistently infected C57BL/6 mice to below the limit of detection while sham PBS treatment did not impact C. difficile burden (Supplementary Fig. 1A,B). By day 10 post-FMT, recipient mice had resolved C. difficile infection-driven intestinal inflammation characterized by immune cell infiltration, submucosa edema (Supplementary Fig. 1C), large intestine crypt elongation (Supplementary Fig. 1D), and elevated mRNA expression of proinflammatory genes in the proximal colon (Supplementary Fig. 1E). These data establish that FMT can indirectly shape intestinal immune homeostasis via resolution C. difficile infection and provoke further investigation into the role of the immune system in supporting FMT-mediated C. difficile resolution.

Immunodeficient Rag1−/− mice exhibit impaired resolution of C. difficile infection following FMT. If FMT resolves C. difficile infection independently of the host immune system, FMT therapy should be equally successful in immunodeficient hosts. To test this null hypothesis and to begin to dissect the role of the immune system in supporting a FMT, T and B cell deficient Rag1−/− mice and littermate control, Rag1 heterozygous mice (Rag1HET) were compared using our C. difficile infection followed by FMT experimental system (Fig. 1a). In agreement with previous reports, Rag1HET and cohoused Rag1−/− mice exhibited similar weight recovery from acute C. difficile disease (Fig. 1b) and both groups established a comparable persistent C. difficile infection in the large intestine (Supplementary Fig. 2A). Following establishment of persistent infection, Rag1HET and Rag1−/− mice were administered a FMT, separated into individually housed cages after FMT to prevent microbial cross contamination, and C. difficile was monitored in the feces. Rag1HET mice resolved C. difficile infection following FMT and had no detectable C. difficile toxin present in its cecal content (Fig. 1c, d). In contrast, C. difficile burden and toxin titers persisted in the large intestine of FMT-treated Rag1−/− mice thus refuting our null hypothesis (Fig. 1c, d). Histological examination of the cecum of C. difficile-infected Rag1HET and Rag1−/− mice demonstrated pronounced submucosa edema, cellular infiltration, and crypt elongation in Rag1−/− mice compared to Rag1HET mice (Fig. 1e). Following FMT, intestinal inflammation was resolved in Rag1HET mice but not in FMT-treated Rag1−/− mice (Fig. 1e, f). These observations suggest that the immune status of the FMT recipient is an important determinant in the ultimate success of FMT therapy.
C. difficile-infected Rag1−/− mice exhibit exacerbated intestinal inflammation compared to Rag1HET mice despite similar microbiota composition. We next analyzed the intestinal microbiota composition of Rag1HET and Rag1−/− mice by bacterial 16S rRNA marker gene profiling on fecal samples from Rag1HET and Rag1−/− mice prior to antibiotic treatment, at the day of infection, and following establishment of persistent infection (day 36 post-infection [p.i.]). The null hypothesis that there was no difference in the microbial community of Rag1HET and Rag1−/− mice was tested to determine if intrinsic differences in the microbiota could explain the differential outcome following FMT. Analysis of unweighted (Fig. 2a), weighted (Supplementary Fig. 3A) UniFrac or Bray-Curtis (Supplementary Fig. 3B) distances between samples did not delineate beta diversity differences in bacterial composition between Rag1HET and Rag1−/− at day 36 p.i., despite significant shifts in microbiota composition of both groups following antibiotic treatment and subsequent C. difficile infection. Comparison of 16S rRNA bacterial community profiles between Rag1HET and Rag1−/− mice by relative bacterial genera abundance (Fig. 2b and Supplementary Fig. 3c, d), UniFrac distances (Fig. 2c and Supplementary Fig. 3E–G) and unsupervised hierarchical clustering (Fig. 2d and Supplementary Fig. 3H) throughout the course of the experiment using both the CD196 and highly pathogenic VP110463 C. difficile strains42. Despite cohabitation and continuous microbial exposure from FMT-treated C57BL/6 mice, FMT-treated Rag1−/− mice exhibited significantly higher C. difficile burden (Supplementary Fig. 2B, D) and toxin titers in the feces (Supplementary Fig. 2C, E) compared to naïve, C. difficile-infected PBS treated, or C. difficile-infected FMT-treated Rag1HET and Rag1−/− mice. n = 3 naïve Rag1HET and Rag1−/− mice; n = 3 C. difficile-infected PBS treated Rag1HET mice; n = 4 C. difficile-infected PBS treated Rag1−/− mice; n = 6 C. difficile-infected FMT-treated Rag1HET mice; n = 4 C. difficile-infected FMT-treated Rag1−/− mice. Scale bar = 100 μm. Data is representative of two independent experiments. Statistical significance was calculated by a two-sided unpaired Mann-Whitney test; *p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as mean values ± SEM. b.d. below detection.
Fig. 3H) do not lead us to reject the null hypothesis that there was no microbial community level differences between groups. A PERMANOVA test of UniFrac distance indicated a statistically significant difference between the microbiota of Rag1\(^{-/-}\) and Rag\(1^HET\) mice prior to antibiotics as has been previously reported\(^43\), but community differences were not statistically significant at later timepoints (Supplementary Table 1). Analysis of the 16S rRNA bacterial gene profile from a second validation cohort of C. difficile infected Rag1\(^{-/-}\) and Rag\(1^HET\) mice at time of FMT (day 32 p.i.) also failed to reject the null hypothesis as determined by unweighted UniFrac distances (Supplementary Fig. 4A, weighted UniFrac distances (Supplementary Fig. 4B), the magnitude of distances between groups (Supplementary Fig. 4C, E), unsupervised hierarchical clustering, and PERMANOVA tests of UniFrac distance between groups (Supplementary Fig. 4D, F and Supplementary Table 2).

16S rRNA marker gene profiling does not yield consistent species-level resolution of microbial communities\(^44\), and therefore...
may not reveal microbial differences that drive differential outcomes following FMT in C. difficile-infected Rag1<sup>HET</sup> and Rag1<sup>−/−</sup> mice. To address whether species-level differences at the time of FMT could drive FMT failure in C. difficile infected mice independent of the host’s immune status, the cecal content of C. difficile-infected C57BL/6 or Rag1<sup>−/−</sup> mice was transferred into antibiotic-treated Rag1<sup>−/−</sup> or Rag1<sup>HET</sup> recipient mice, respectively (Supplementary Fig. 5A). Recipient mice established a C. difficile infection derived from the donor transplant, recovered from initial disease morbidity and then received a FMT from naïve C57BL/6 mice donors (Supplementary Fig. 5A). Rag1<sup>−/−</sup> mice receiving the microbiota from a C. difficile infected Rag1<sup>−/−</sup> mouse were still capable of resolving C. difficile infection following FMT (Supplementary Fig. 5B–D). In contrast Rag1<sup>−/−</sup> mice receiving the microbiota from a C. difficile infected C57BL/6 mouse failed to clear C. difficile following FMT (Supplementary Fig. 5B–D). Next, germ-free (GF) C57BL/6 mice were cohoused with Rag1<sup>−/−</sup> or Rag1<sup>HET</sup> mice and subsequently treated with antibiotics, infected with C. difficile and then administered a FMT (Supplementary Fig. 5E). C. difficile infected GF mice cohoused with either Rag1<sup>−/−</sup> or Rag1<sup>HET</sup> mice prior to infection were equally capable of resolving C. difficile infection (Supplementary Fig. 5F). This experimental result demonstrates that the microbiota from naïve Rag1<sup>−/−</sup> mice does not inherently imprint FMT failure in immuneocompetent mice. Combined, these data provide evidence that microbiota composition is not intrinsically sufficient to prevent Rag1<sup>−/−</sup> mice from resolving C. difficile infection following FMT.

Next, the intestinal inflammatory milieu of Rag1<sup>HET</sup> and Rag1<sup>−/−</sup> mice was assessed at time of FMT. C. difficile-infected Rag1<sup>−/−</sup> mice exhibited elevated expression of proinflammatory immune response genes in the colon (Il22, Cxcl1, Cxcl2, Tnfα, Nos2, Reg3γ, and Reg3β) compared to C. difficile-infected Rag1<sup>HET</sup> mice (Fig. 2e). Lack of T and B cells in Rag1<sup>−/−</sup> mice can confute interpretation of mRNA transcripts quantification from whole colon tissue. Therefore, total protein levels were measured to assess the in vivo concentration of these proinflammatory cytokines and chemokines regardless of contributing cellular composition. In agreement with gene transcriptional expression, C. difficile-infected Rag1<sup>−/−</sup> mice had increased protein levels of proinflammatory cytokines and chemokines in the large intestine at time of FMT compared to C. difficile-infected Rag1<sup>HET</sup> mice (Fig. 2f). Gastrointestinal infection-induced inflammation is associated with bystander γ-Proteobacteria bloom in the intestine<sup>45,46</sup>. Indeed 16S RNA marker gene profiling revealed a trend, though not statistically significant, toward more γ-Proteobacteria in C. difficile infected Rag1<sup>−/−</sup> mice (Fig. 2g). These data support the hypothesis that FMT failure in Rag1<sup>−/−</sup> mice is driven by host immunity shaping intestinal environment at the time of FMT engraftment.

**CD4<sup>+</sup> T cells support FMT-mediated resolution of C. difficile infection.** The inability of Rag1<sup>−/−</sup> mice to resolve C. difficile infection following FMT suggests adaptive immunity is important for FMT-mediated resolution of C. difficile infection. To determine the cellular components of the adaptive immune system that supports FMT, mice deficient in B cells (μMT<sup>−/−</sup> mice), CD8<sup>+</sup> T cells (β<sub>2</sub>M<sup>−/−</sup> mice) or CD4<sup>+</sup> T cells (MHC Class II<sup>−/−</sup> mice) were screened for FMT responsiveness following C. difficile infection in comparison to cohoused C57BL/6 mice. All four groups of mice exhibited similar morbidity and mortality following acute C. difficile infection and exhibited persistent C. difficile colonization (Supplementary Fig. 6A–C<sup>47</sup>). Following FMT, C57BL/6, μMT<sup>−/−</sup>, β<sub>2</sub>M<sup>−/−</sup> mice resolved C. difficile infection (Fig. 3a) while MHC Class II<sup>−/−</sup> (C-II<sup>−/−</sup>) mice failed to clear C. difficile (Fig. 3b) and maintained high toxin levels in the cecum (Fig. 3c). These data indicate that CD4<sup>+</sup> T cells not B cells or CD8<sup>+</sup> T cells are necessary for supporting FMT-mediated resolution of C. difficile infection.

Intestinal immune homeostasis is regulated, in part, by the balance of CD4<sup>+</sup> T regulatory cells (T<sub>reg</sub>) and T helper 17 (T<sub>17</sub>) cells responding and interacting with cues from the intestinal microenvironment<sup>48,49</sup>. C. difficile infection elicited a significant expansion in frequency (Fig. 3d) and total number of IL-17α<sup>+</sup> competent (Fig. 3e) and IL-22<sup>+</sup> competent (Fig. 3f) CD4<sup>+</sup> T cells in the large intestine lamina propria. Further, ten days following FMT the T<sub>17</sub> cell population remained significantly elevated relative to naïve mice despite resolution of C. difficile infection at this timepoint (Fig. 3d–f). To test the potential role of T<sub>17</sub> cells in supporting FMT we genetically ablated the signature effector molecules for this T cell subset, IL-17α or IL-22, and assessed FMT-mediated resolution following C. difficile infection. Both Il17a<sup>−/−</sup> (Supplementary Fig. 7A) and Il22<sup>−/−</sup> mice (Supplementary Fig. 7B) exhibited equivalent capacity to reduce C. difficile burden following FMT compared to littermate or cohoused wild-type mice. T<sub>17</sub>-deficient (Tbx21<sup>−/−</sup>) mice that lack the capacity for T<sub>17</sub> cell differentiation were also capable of resolving C. difficile infection following FMT (Supplementary Fig. 7C). These data suggest FMT-mediated resolution of C. difficile infection can occur independent of the T<sub>17</sub> and T<sub>17</sub> CD4<sup>+</sup> T cell lineage.

**Depletion of CD4<sup>+</sup> T-regulatory cells impairs FMT-mediated resolution of C. difficile infection.** In parallel with T<sub>17</sub> cell expansion, the Foxp3<sup>+</sup> and Foxp3<sup>+</sup> Rorgt<sup>+</sup> T<sub>reg</sub> cells significantly increased in the large intestine lamina propria following C. difficile infection and remained elevated following FMT compared to naïve mice (Fig. 3g–i). To directly test the role of T<sub>reg</sub> cells in FMT-mediated resolution of C. difficile, the Foxp3<sup>DT</sup> knockin mice system<sup>50</sup> was used to selectively deplete T<sub>reg</sub> cells in the C. difficile infected mice prior to FMT (Fig. 4a). Continued administration of diphertheria toxin (DT) drives systemic autoimmunity and mortality in these mice<sup>50</sup>, therefore DT or PBS was administered only at day 8 and 9 post-infection, during the recovery from the acute phase of infection, to temporarily delete T<sub>reg</sub> cells. Diphertheria toxin administration resulted in a temporary 10–15% loss in weight in C. difficile infected mice (Fig. 4b), but minimal weight loss in antibiotic treated, uninfected mice (Supplementary Fig. 7D), suggesting the loss of T<sub>reg</sub> cells in combination with persistent C. difficile infection reactivated intestinal inflammation. DT treatment in Foxp3<sup>DT</sup> mice did not alter C. difficile burden prior to FMT compared to PBS-treated Foxp3<sup>DT</sup> mice (Fig. 4c). Following FMT, however, DT-treated Foxp3<sup>DT</sup> mice exhibited a significantly delayed resolution of C. difficile infection compared to PBS-treated Foxp3<sup>DT</sup> mice (Fig. 4c). Transient ablation of the large intestinal T<sub>reg</sub> cells by DT administration was confirmed at day 12 p.i. (Fig. 4d–f), and, in agreement with a previous report<sup>51</sup>, the T<sub>reg</sub> cell population began to return by day 21 p.i. (day of FMT; Fig. 4d, g). Despite comparable total numbers, the relative proportion of T<sub>reg</sub> cells compared to T<sub>17</sub> and T<sub>17</sub> CD4<sup>+</sup> T cell subsets remained diminished in DT-treated Foxp3<sup>DT</sup> mice (Fig. 4h). The repopulated T<sub>reg</sub> cell compartment was not sufficient to limit intestinal inflammation as absolute numbers of T<sub>17</sub>, T<sub>17</sub>1 CD4<sup>+</sup> T cells, infiltrating inflammatory monocytes and neutrophils was increased in C. difficile-infected DT-treated Foxp3<sup>DT</sup> mice compared to C. difficile-infected PBS-treated Foxp3<sup>DT</sup> mice (Fig. 4i). Further, C. difficile-infected DT-treated Foxp3<sup>DT</sup> mice had significantly elevated expression of proinflammatory immune defense genes in the colon at day 21 p.i. compared to C. difficile-infected PBS-treated Foxp3<sup>DT</sup> mice (Fig. 4j), similar to the enhanced proinflammatory signature
observed in C. difficile infected Rag1−/− mice prior to FMT (Fig. 2e). These results demonstrate that depletion of the Treg cells enhances expression of proinflammatory immune defense genes in the context of C. difficile infection and is sufficient to impair FMT-mediated resolution.

Continued administration of DT administration up through FMT also impaired resolution of C. difficile infection (Supplementary Fig. 7E), while DT treatment initiated immediately prior to FMT did not alter C. difficile resolution (Suppl. Fig 7F). These results indicate immune activation downstream of Treg cell depletion is needed to re-shape the intestinal environment into a state that is refractory to FMT. Combined, the data presented support a role for CD4+ Treg cells in promoting the success of FMT therapy.

**FMT fails to engraft in C. difficile infected non-responsive mice.** To better understand the etiology of FMT failure, intestinal microbial community profiling was conducted following FMT in C. difficile infected Rag1−/− and C-II−/− mice and compared to Rag1HET and C57BL6 mice respectively. The microbiota of C. difficile infected FMT-treated Rag1HET (Fig. 5a–d) or C. difficile infected FMT-treated C57BL6 mice (Fig. 5e–h) shifted to
phenotypically resemble the composition of the FMT inoculum, thereby demonstrating successful FMT engraftment. In contrast, the microbiota from *C. difficile* infected FMT-treated Rag1$^{-/-}$ (Fig. 5a–d) or *C. difficile* infected FMT-treated C-II$^{-/-}$ mice (Fig. 5e–h) remained distinct from the FMT inoculum. Analyses of weighted UniFrac (Supplementary Fig. 8a, e), or Bray-Curtis (Supplementary Fig. 8B, F) distances confirmed FMT engraftment in Rag1$^{HET}$ and C57BL/6 mice. In contrast, *C. difficile* infected FMT-treated Rag1$^{-/-}$ and C-II$^{-/-}$ mice failed to engraft the FMT inoculum. Analyses of the microbial community profile by relative bacterial genera abundance (Fig. 5b, f), unweighted or weighted UniFrac distance relative to the FMT (Fig. 5c, g and Supplementary Fig. 8C, G), unsupervised hierarchical clustering (Fig. 5D, H and Supplementary Fig. 8d, h) and PERMANOVA test (Supplementary Table 3) all demonstrated the microbiota of FMT-responsive mice more closely resembled the FMT inoculum than the microbiota of FMT non-responsive mice. *C. difficile* infected Rag1$^{HET}$ or Rag1$^{-/-}$ mice receiving PBS instead of FMT did not exhibit significant microbiota composition changes (Supplementary Fig. 9A, B) and remained colonized with *C. difficile* (Supplementary Fig. 9C).

To identify the microbial species that differential engraft in FMT responsive vs. non-responsive mice, the 16S rRNA sequence dataset from *C. difficile* infected Rag1$^{HET}$ or Rag1$^{-/-}$ mice before and after FMT was analyzed for amplicon sequence variants (ASVs) that met the following criteria: (1) present in the FMT inoculum, (2) fold change of 1.5 or higher, (3) fold change of 2.5 or higher, and (4) fold change of 5 or higher.

**Figures and Tables:**
- **Fig. 5a:** Phenotypic similarity of FMT engraftment.
- **Fig. 5b:** Weighted UniFrac analysis of microbial communities.
- **Fig. 5c:** Bray-Curtis distances between treatments.
- **Fig. 5d:** Percent of initial weight over time.
- **Fig. 5e:** CD4+ T cell subsets over time.
- **Fig. 5f:** Fold induction of selected genes.

**Tables:**
- **Supplementary Table 3:** Summary of PERMANOVA test results.
- **Supplementary Fig. 8:** Additional UniFrac and Bray-Curtis analyses.

**Legend:**
- **PBS:** Placebo
- **DT:** Donor transfer
- **FMT:** Fecal microbiota transplantation
- **NS:** Not significant
- ***p**:** Significance level (p-value)
inoculum, (2) absent in C. difficile infected Rag1<sup>HET</sup> or C. difficile infected Rag1<sup>+/−</sup> mice prior to FMT, (3) absent in C. difficile infected PBS-treated Rag1<sup>HET</sup> or Rag1<sup>−/+</sup> mice, (4) absent in C. difficile infected FMT-treated Rag1<sup>−/+</sup> mice, and (5) present in C. difficile infected FMT-treated Rag1<sup>HET</sup> mice. Despite these criteria, 279 ASVs met all five conditions with the majority of ASVs identified not distinguishable at the species levels (Supplementary Fig. 10).

FMT rejection required C. difficile-driven inflammation as ABX-treated, uninfected Rag1<sup>−/+</sup> mice (Supplementary Fig. 11A) successfully engrafted the FMT inoculum as determined by relative bacterial genera abundance (Supplementary Fig. 11B) unweighted (Fig. 11c), weighted (Supplementary Fig. 11D) UniFrac distances, beta diversity dissimilarity relative to the FMT (Supplementary Fig. 11E, G), and unsupervised hierarchical clustering (Supplementary Fig. 11F, H). Combined, these data demonstrate that the host’s immune activation status can determine which transplanted bacteria successfully engraft in a C. difficile-infected host.

FMT fails to restore intestinal metabolites in C. difficile-infected, FMT non-responsive mice. Successful FMT treatment in recurrent C. difficile infected patients correlates with restoration of the intestinal metabolites to pre-infection concentrations<sup>34,35</sup>. Therefore, targeted metabolite profiling was conducted on caecal content from naïve, C. difficile-infected, and C. difficile-infected FMT-treated Rag1<sup>HET</sup> and Rag1<sup>−/+</sup> mice to assess whether FMT bacterial engraftment failure functionally impaired restoration of the intestinal metabolites. Amino acid<sup>32,33</sup>, short chain fatty acid (SCFA)<sup>34,35</sup>, primary and secondary bile acid<sup>13,56–60</sup> metabolites were selected based on reports demonstrating C. difficile colonization is influenced by the availability of these metabolites in the intestine. The metabolite composition in the cecum distinctly shifted between naïve and C. difficile infected mice (Fig. 6a, b), however, no significant difference was observed between Rag1<sup>HET</sup> and Rag1<sup>−/+</sup> mice prior to FMT (Fig. 6b and Supplementary Fig. 12A, B). Following FMT, the amino acids, SCFAs, 1<sup>st</sup> and 2<sup>nd</sup> bile acids profile of Rag1<sup>HET</sup> mice but not Rag1<sup>−/+</sup> mice was restored to resemble the composition of naïve mice (Fig. 6a, b). These data demonstrate that failed engraftment of FMT bacterial species led to functionally impairment at the metabolite level. Following FMT, 2<sup>nd</sup> bile acid derivatives were the most significantly enriched in C. difficile infected FMT-treated Rag1<sup>HET</sup> mice compared to C. difficile infected FMT-treated Rag1<sup>−/+</sup> mice (Fig. 6c and Supplementary Fig. 12C). Specifically, deoxycholic acid, lithocholic acid, taurodeoxycholic acid, and omega-muricholic acid concentrations in the cecum of C. difficile infected FMT-treated Rag1<sup>HET</sup> mice were all restored to resemble the profile of naïve mice (Fig. 6d and Supplementary Fig. 8C). In contrast the 2<sup>nd</sup> bile acid pool remained nearly undetectable in C. difficile infected FMT-treated Rag1<sup>−/+</sup> mice (Fig. 6d and Supplementary Fig. 12C) while the 1<sup>st</sup> bile acid pool remained elevated compared to naïve mice (Supplementary Fig. 12D, E). These data are in agreement with the observation that a canonical 2<sup>nd</sup> bile acid converter bacterial species, Clostridium scindens, engrafted in C. difficile infected FMT-treated Rag1<sup>HET</sup> mice but not C. difficile infected FMT-treated Rag1<sup>−/+</sup> mice (Supplementary Figs. 10 and 12F). Similarly, C. difficile infected FMT-treated CII<sup>−−</sup> mice exhibited significantly reduced cecal levels of deoxycholic acid and lithocholic acid compared to FMT-treated C57BL/6 mice (Supplementary Fig. 12G). These data identify intestinal metabolites composition, specifically 2<sup>nd</sup> bile acids, as biomarkers for FMT engraftment failure in mice that exhibit elevated expression of immune defense genes. Combined, this report demonstrates that the immune status of the host is a critical factor in determining FMT therapy success in the context of C. difficile infection.

Discussion

The clinical success of fecal transplants to treat recurrent C. difficile is an encouraging development for the treatment of this disease as well as other diseases associated with microbiota dysbiosis. The transplant’s mechanism of action in C. difficile infection is postulated to be transplanted bacteria directly altering the intestinal environment to render it inhospitable for C. difficile. In this report, we find that successful engraftment of transplanted bacteria is dependent on the immune status of the recipient, specifically, immunodeficient hosts that lack CD4<sup>+</sup> Foxp3<sup>+</sup> T<sub>reg</sub> cells fail to resolve C. difficile infection following FMT. Persistent C. difficile infection in Rag1<sup>−/+</sup> mice or Foxp3<sup>−</sup> T<sub>reg</sub> cell-depleted mice led to exacerbated induction of inflammatory mediators in the large intestine compared to immunocompetent littermates. These FMT non-responsive mice exhibited impaired engraftment of the donor bacterial populations. Engraftment failure resulted in an inability to restore intestinal metabolite to pre-infection concentrations and ultimately failure to resolve C. difficile infection.
Foxp3$^+$ T$_{reg}$ cells serve an important balance in limiting host inflammation in the intestine. Unchecked, inflammatory mediators produced by the host may inhibit specific transplanted bacteria critical for the resolution of *C. difficile* infection. Host-derived inflammatory byproducts can be used by pathogens and bystander commensal species alike to promote their growth. For example, Baumler and colleagues observed that host production of reactive oxygen and reactive nitrogen species induced by *Salmonella* infection could support expansion of both the pathogen and commensal *Enterococcus* species while limiting the growth of commensal *Clostridia* species. Further studies have demonstrated that ethanolamine, nitrites, formate, and lactate metabolites induced by inflammation can support selective bacterial growth in the intestine. Thus, a potential positive feedback loop exist by which the inflammatory environment induced by infection concurrently promotes the survival of select inflammation-tolerant bacterial species and inhibits engraftment of transplanted inflammation-sensitive bacteria. This feedback loop may be potentiated in the absence of functional immunoregulatory mechanisms, such as T$_{reg}$ cells that normally act as checkpoints that limit expansion of inflammation-tolerant bacteria. For example, butyrate drives PPAR-γ signaling in epithelial cells and augments the intestinal T$_{reg}$ cell population ultimately depriving Enterobacteriaceae species of an oxygen electron donor.
acceptor. The inflammatory byproducts produced in the intestine of FMT non-responsive hosts with defective immunoregulatory mechanisms may establish an environment that prevents the engraftment of inflammation-sensitive bacterial species. Indeed, in this report we observed 279 unique bacterial ASVs that successfully engrafted in Rag1\textsuperscript{HET} mice but could not engraft in Rag1\textsuperscript{−/−} mice, supporting this concept.

This report conducted a limited intestinal metabolite screen of amino acids, SCFA, 1\textsuperscript{st} and 2\textsuperscript{nd} bile acids targeted to metabolites that have known roles in modulating \textit{C. difficile} colonization in the intestine. FMT non-responsive mice failed to restore 2\textsuperscript{nd} bile acid pools in the cecum. Primary and secondary bile acid levels are closely linked with \textit{C. difficile} susceptibility. Host-derived primary bile acids are germinants for \textit{C. difficile} spores while select commensal bacteria species capable of converting 1\textsuperscript{st} bile acids to 2\textsuperscript{nd} bile acids can inhibit \textit{C. difficile} growth. Our results indicate 2\textsuperscript{nd} bile acids may serve as a useful biomarker for successful FMT engraftment and support a role for 2\textsuperscript{nd} bile acid restoration as one of the possible mechanisms that contribute to resolution of \textit{C. difficile}. The metabolite screen performed in this study was limited in scope. A more extensive characterization of intestinal metabolome is needed to understand the relative contribution of bacterial-derived metabolites in FMT success and how they are shaped by the host’s immune system.

Three recent reports observed that secondary bile acid derivatives can promote T\textsubscript{reg} cell development in the colon by directly signaling on T\textsubscript{reg} cells or indirectly signaling on dendritic cells that in turn promote T\textsubscript{reg} cell development. Our data demonstrate low to absent 2\textsuperscript{nd} bile acid pools in \textit{C. difficile} infected mice prior to FMT indicating that 2\textsuperscript{nd} bile acids are likely not promoting peripheral T\textsubscript{reg} cell development at the time FMT. Following FMT, however, restoration of 2\textsuperscript{nd} bile acids could promote T\textsubscript{reg} cell expansion and drive a feed forward loop that further reduces intestinal inflammation and enables repopulation of inflammation-sensitive commensal bacteria. FMT has previously been shown to promote the induction of anti-inflammatory properties via boosting IL-10 production in the context of a DSS-colitis mouse model. The timing and context of this potential 2\textsuperscript{nd} bile acid - T\textsubscript{reg} cells feed forward mechanism in promoting bacterial engraftment will require further study.

This report assessed the ability of bacterial components of a FMT to engraft and resolve \textit{C. difficile} infection in hosts with different immune statuses. The relative contribution of bacteriophages transplanted in the FMT in resolving \textit{C. difficile} infection was not assessed. A recent study found donor-derived bacteriophage populations to be associated with FMT success. Bacteriophages may directly act on \textit{C. difficile} itself through lytic processes or stimulate immune cell activation in the intestine. The relative contribution of bacteria and the bacteriophages in interacting with the immune system and supporting \textit{C. difficile} resolution following FMT will require future exploration.

Use of FMT therapy in immunocompromised patient cohorts remains limited due to concerns about adverse effects. Encouragingly, several case reports have described resolution of recurrent \textit{C. difficile} in HIV\textsuperscript{+}, transplant-reipient, and cancer patients. A multicenter, retrospective study found the FMT success rate in treating recurrent \textit{C. difficile} in IBD patients on immunosuppressive therapy was comparable to the FMT success rates reported in IBD-free \textit{C. difficile} patients though sustained engraftment of the transplanted bacteria was not assessed. A subsequent study found the microbiota of immunocompromised hematopoietic stem cell recipients drifted away from the FMT inoculum over time while recipients without overt immune defects exhibit long-term engraftment following FMT. Combined, these reports suggest microbiota-based therapeutics for immunocompromised patients is possible, however, which immune pathways are dispensable will require further investigation and may vary depending on the disease being treated. For example, this report found that the immune deficiencies in the B-cell, CD8\textsuperscript{+} T cell, T\textsubscript{H}17 and T\textsubscript{H}1 cell compartments did not impact FMT success while a defect in the T\textsubscript{reg} cell compartment impaired FMT-mediated resolution of \textit{C. difficile} infection. Similar to immunosuppressive intervention strategies that reduce the risk of organ transplant rejection, identifying and targeting specific immunological pathways that dictate microbiota transplantation success or failure will be important for the development of more informed clinical approaches that can harness the microbiota to shape host physiology and alleviate disease.

**Methods**

**Mice.** C57BL/6, Rag1\textsuperscript{−/−}, \textit{μMT}\textsuperscript{−/−}, B6.M\textsuperscript{−/−}, C-II\textsuperscript{−/−} (H2dlAb1-Ea), II12\textsuperscript{−/−}, Tbx21\textsuperscript{−/−}, and Foxp3\textsuperscript{TRT} mice were purchased from the Jackson Laboratory. II12\textsuperscript{−/−} mice were provided by R. Flavell (Yale University). All mouse strains were derived on a C57BL/6 background. All mice were bred and maintained in sterile autoclaved cages under specific pathogen-free conditions and kept on a grain-based diet (Labdiet 5053) at the Memorial Sloan Kettering Research Animal Resource Center or the University of Pennsylvania. Mice were provided autoclaved water ad libitum from water bottles. Sex and age-matched controls were used in all
experiments according to institutional guidelines for animal care. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan Kettering Cancer Center and University of Pennsylvania.

Antibiotic pretreatment, C. difficile infection, and Treg cell depletion. Two to four month old mice were administered drinking water supplemented with neomycin (Sigma; 0.25 g/L), metronidazole (0.25 g/L), and vancomycin (Novaplus) (0.25 g/L) for 72 h, and then replaced with normal water for the duration of the experiment. For experiments without littermates, mice were cohoused for three weeks prior to antibiotic treatment to allow for equilibration of the microbiota. Fortytwo hours following cessation of antibiotic water, mice received 200 μg of clindamycin (Sigma) by intraperitoneal injection. Twenty-four hours later, mice received ~1000 C. difficile spores (CD196, ribotype 027 strain87, or VPI10463, ribotype 087 strain ATCC #43255) via oral gavage. After infection, mice were monitored for C. difficile burden in the feces and weight loss.

**Fig. 6** FMT non-responsive Rag1⁻/⁻ mice fail to restore their intestinal metabolite profile to pre-infection conditions. C. difficile-infected Rag1⁻/⁻ and Rag⁻/⁻ mice were administered FMT or PBS, sacrificed 21 days later along with naïve mice and amino acid, short chain fatty acid (SCFA), 1° and 2° bile acid pools were analyzed in cecal content. a Heatmap of relative concentration of amino acids, SCFA, 1° and 2° bile acids in cecal content of naïve (n = 5), C. difficile-infected (n = 6) or C. difficile-infected FMT-treated (n = 4 Rag⁻/⁻; n = 6 Rag⁻/⁻) mice. b Principal coordinate analysis plot comparing metabolite profile in the cecum of naïve, C. difficile-infected, and C. difficile-infected FMT-treated Rag⁻/⁻ and Rag⁻/⁻ mice. Ellipses represent 95% confidence intervals. c Volcano plot of metabolites in the cecum of C. difficile-infected FMT-treated Rag⁻/⁻ and Rag⁻/⁻ mice. Blue circles indicate metabolites enriched in Rag⁻/⁻ mice. Red circles indicate metabolites enriched in Rag⁻/⁻ mice. Significance threshold criteria set at a two-fold change in concentration and an adjusted p-value of 0.05 using an unpaired t-test and adjusted for false discovery rate. d Concentration of individual 2° bile acids. n = 5 naïve Rag⁻/⁻ and Rag⁻/⁻ mice; n = 6 C. difficile-infected FMT-treated Rag⁻/⁻ mice; n = 6 C. difficile-infected FMT-treated Rag⁻/⁻ mice examined over two independent experiments. Statistical significance was calculated by a one-way ANOVA using Dunnett method for multiple comparison adjustments. DCA ***p < 0.0001, LCA *p = 0.043, TDCA *p = 0.014, ωMCA ***p = 0.002. Boxes represent median, first and third quartile. Whiskers extend to the highest and lowest data point. *p < 0.05, **p < 0.01, ***p < 0.001. TCDCA taurochenodeoxycholic acid, ωMCA alphamuricholic acid, BMCA betamuricholic acid, YMCA gammamuricholic acid, CDCA chenodeoxycholic acid, TCA cholic acid, TLCA taurolithocholic acid, TDCA taurodeoxycholic acid, GDCA glycodeoxycholic acid, LCA lithocholic acid, ωMCA omegamuricholic acid, DCA deoxycholic acid. b.d. below detection.
administered diphtheria toxin (20 μg/kg of body weight) or PBS (l.p.) on consecutive days at time points indicated.

**Quantification of C. difficile burden and toxin.** Fecal pellets or cecal content were resuspended in deoxyoxegated PBS, and ten-fold serial dilutions were plated on BHI agar supplemented with yeast extract, taurocholate, c-xylose, d-cysteine, and cefoxitin at 37 °C in an anaerobic chamber (Coy Labs) overnight. The presence of *C. difficile* toxins was determined using a cell-based cytotoxicity assay as previously described. Briefly, human embryonic lung fibroblast WI-38 cells (ATCC® CCL-75) were grown on 96-well plate overnight at 37 °C. Ten-fold serial dilutions of supernatant from resuspended cecal content were added to WI-38 cells, incubated overnight at 37 °C and the presence of cell rounding was observed the next day. The presence of *C. difficile* toxin B was confirmed by neutralization with antitoxin antisera (Techlab, Blacksburg, VA). The data are expressed as the log_{10} reciprocal value of the last dilution where cell rounding was observed.

**FMT treatment.** The donor source for FMT was fecal pellets from naive, C57BL/6 mice purchased from Charles River Laboratory and housed under specific pathogen-free conditions. Fecal pellets were collected fresh for each FMT and resuspended at a concentration of 0.2 g/mL of deoxyoxegated PBS under anaerobic conditions to preserve obligate anaerobe bacteria. FMT inoculation was flash spun to settle food debris and administered to mice via oral gavage (200 μL) and intrarectal instillation (100 μL) into chronically infected mice. Twenty-fours hours following initial FMT, mice receive a second dose of freshly prepared FMT via oral gavage (200 μL). To normalize FMT from experiment to experiment, fecal pellets were collected from the same colony of C57BL/6 mice. Following FMT, unless specified, mice were separated into individually housed cages.

**Bacterial DNA extraction, amplification, and analysis.** DNA was extracted from fecal pellet using a QiaGen PowerSoil kit (Qiagen) according to manufacturer’s instructions and 16S rRNA genes were amplified by PCR. Amplicons of the V4-V5 16S rRNA region were amplified and sequenced using an Illumina MiSeq platform as described in Buffie et al. Raw 16S sequence reads from experiments prior to FMT and with C. Diff–ve mice (Figs. 2 and 5–h, and Supplementary Fig. 3 and 8E–H) were quality filtered and clustered into 97% ASVs using UPARSE. Unweighted UniFrac distances were calculated using the phyloseq (v. 1.32.0)92 R package using R version 3.6.1 and hclust from the stats package. For diversity metrics including UniFrac distances, a rooted phylogenetic tree was generated: (DAG) and ASV representatives were classified using BLAST against the NCBI refseq database as previously described91. Unweighted UniFrac distances were calculated using the phyloseq (v. 1.32.0)92 R package using R version 3.6.1 and hclust from the stats package93. 16S rRNA sequencing data from experiments post-FMT treatment were processed and analyzed using the QIIME2 pipeline95. DADA296, implemented in QIIME2 for high variable positions were masked to reduce noise in a resulting phylogenetic tree. Forward primers of 8 f, 1131 f, and 518 f were used. Taxonomic analysis was performed using BLAST for comparison against standard curves of at least five points run in triplicate. Standard curves were run at the beginning and end of each metabolomics run. Quality control checks (blanks and standards) were run every eight samples.

**Cytokine and chemokine quantification.** Cytokine and chemokine concentrations were assessed for cytokine & chemokine concentrations using a Luminex bead array manufactured by BD Bioscience. This assay was performed according to manufacturer’s instructions. Quality control checks (blanks and standards) were run every eight samples. Statistical analysis. Results represent mean ± SEM from distinct biological samples. For comparison of *C. difficile* burden values, statistical significance was determined using a Mann–Whitney test for small samples size (*p < 0.05) and with a non-normal distribution. Statistical analyses were performed using Prism GraphPad software (*p < 0.05; **p < 0.01; ***p < 0.001). For 16S sequencing data, in order to test the null hypothesis of no differences in the study group centroids, a PERMANOVA test was implemented by the function adonis() in the vegan package v 2.5-3.
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