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Fecal microbiota transplant treatment for recurrent *Clostridioides difficile* infection enhances adaptive immunity to TcdB

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Abbreviations:

CDI	*C. difficile* infection
FMT	Fecal microbiota transplant
TCR	T cell receptor
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
$TcdB_{CROPS}$	C-terminal combined repetitive oligopeptides domain of TcdB

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INTRODUCTION

Clostridioides difficile infection (CDI) is the leading cause of gastroenteritis-associated death in North America [1]. First line treatment is typically vancomycin, which kills vegetative forms and eliminates production of the two pathogenic toxins: TcdA and TcdB [2]. However, 25-35% of patients experience recurrent disease [1]. Fecal microbiota transplant (FMT) is an effective second-line therapy for recurrent CDI [3], but remains difficult to access, carries infectious risk if not done carefully, and multiple treatments may be needed.

Our study aim was to gain a better mechanistic understanding of why FMT is an effective therapy in recurrent CDI. As we previously identified a deficit of T helper type 17 (Th17) cells within circulating TcdB-specific $CD4^+$ T cells in recurrent CDI patients [4], we hypothesised FMT would alter the proportions of TcdB-specific Th subsets. Understanding how FMT affects immune memory responses will enable its fine-tuning and wider application.

METHODS

Identification of antigen-specific T cells. To detect antigen-specific $CD4^+$ T cells, the whole blood activation-induced CD25 and OX40 (CD134) marker assay was performed as described [4].

ELISA. ELISA was used to quantify anti-TcdA, TcdB or $TcdB_{CROPS}$ IgG and IgA in plasma as described [4].

Additional methods described in supplemental data.

RESULTS

Fecal microbiota transplant increases TcdB-specific Th17 cells and antibodies

To assess effects of FMT on adaptive immunity to *C. difficile*, we recruited n=22 recurrent CDI patients. All were on vancomycin suppression before FMT, 16/22 (68%) were female, with median age 61 (range 18-95 years). There was no difference in the age range of patients with mild versus severe disease (assessed by requirement for hospitalisation and IV fluids). FMT was curative for 20/22 patients, defined by absence of CDI recurrence without vancomycin prophylaxis for 3 months after FMT (n=1 relapse and n=1 lost to follow up). Blood was collected prior to, and 8-12 weeks following, FMT. $CD4^+$ T cells specific for TcdA or TcdB, or Pediacel (childhood vaccine-induced T cell memory), or polyclonally-stimulated by staphylococcus enterotoxin B (SEB; assay positive control), were quantified by incubation with antigen for 44h and flow cytometric detection of induced co-expression of CD25 and OX40 (OX40 assay, **Supplemental Figure 1**) [4]

Although the total frequencies TcdA- and TcdB-specific $CD4^+$ T cells were not altered by FMT, there was a significant increase in the proportions of TcdB-specific Th17 cells and decrease in TcdB-specific Th2 cells (**Figure 1A and Supplemental Figure 1**; too few TcdA-specific $CD4^+$ T cells to phenotype). TcdB-specific cells sorted and restimulated from 2 patients showed increased secretion of Th17 cell cytokines IL-17A and IL-22 post-FMT compared to pre-FMT (**Supplemental Figure 1D**). These data support our surface marker analysis (**Figure 1A**) and suggest the expanded TcdB-specific Th17 cells are functional. There was also a significant reduction in the frequency of Pediacel-specific $CD4^+$ T cells post-FMT, but no changes in the Th cell subset proportions (**Figure 1B**) or association with age and magnitude of T cell responses (data not shown). There were no changes in frequency or phenotype of SEB-

stimulated $CD4^+$ T cells (**Figure 1C**), so increased Th17 cells post-FMT were specifically a feature of responses to TcdB.

Interestingly, the levels of anti-TcdA, TcdB and $TcdB_{CROPS}$ IgA and IgG were significantly increased post-FMT, indicating FMT affects both cellular and humoral immunity; these antibodies may mediate protective immunity (**Figure 1D**). Importantly, anti-Pediacel IgG and IgA levels did not change post-FMT (**Figure 1E**), indicating that FMT-associated changes in vaccine-generated immunity is restricted to circulating $CD4^+$ T cells.

FMT does not alter the polyclonal TcdB-specific TCR repertoire

We analysed the TCR repertoire of TcdB-specific $CD4^+$ T cells by sequencing the β chain (amino acid sequences analysed) for n = 5 recurrent CDI patients, n=3 of which had paired pre- and post-FMT samples. TcdB-specific $CD4^+$ T cells were polyclonal: > 75% of productively rearranged templates were a unique clonotype and Simpsons' clonality indexes < 0.03, indicated a low frequency of identical clonotypes (**Supplemental Figure 2A**). There was little overlap of patient TCR repertoires, with low Jaccard similarity indexes (100% overlap = index of 1, **Supplemental Figure 2B**). However, we found several clonotypes present in at least two individuals, possibly representing public clonotypes (**Supplemental Figure 2B**). There was no bias in TCR beta chain variable region (TRBV) usage (**Supplemental Figure 2C**). Comparing n=3 patients pre- and post-FMT, we saw no alterations in TCR repertoire clonality or TRBV usage; and even within patients the repertoire overlap remained low (Jaccard similarity indexes < 0.1; **Supplemental Figure 2D-F**).

DISCUSSION

We show here that successful FMT therapy is associated with increased proportions of TcdB-specific Th17 cells as well as IgG and IgA antibodies specific for TcdA and TcdB. These data suggest that a mechanism of action of FMT could be enhanced pathogen-specific immunity. We previously found that T cell, but not antibody, responses to TcdB distinguished CDI patients from healthy controls and positively correlated with disease severity [4]. Moreover, TcdB-specific $CD4^+$ T cells were predominantly Th17 cells, which were significantly reduced in recurrent CDI patients compared to healthy controls. Evidence shown here that successful FMT increases TcdB-specific Th17 cells raises the hypothesis that these specialised cells, known to be crucial for gut homeostasis [5], are important for protective immunity to *C. difficile*.

A diverse TCR repertoire is important for effective immunity [6], but chronic antigen exposure, for example through reinfections, can narrow the repertoire [7]. We observed a polyclonal TCR repertoire of TcdB-specific $CD4^+$ T cells, similar to the Th17-biased response to *Candida albicans* [8]. Evidence that FMT did not affect the TCR repertoire suggests that alterations in Th cell proportions within TcdB-specific T cell memory is not due to large clonal expansions.

Collectively, these data indicate that positive effects of FMT in recurrent CDI patients may not only be due to changes in the microbiome, but also through improving $CD4^+$ T cell and antibody mediated immunity to *C. difficile* toxins. These results are important for the design of disease monitoring strategies and highlight that future study of how FMT influences pathogen-specific immunity is warranted: specifically, determining if effectively restoring the TcdB-specific cellular repertoire to healthy control proportions contributes to treatment success of FMT.

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FIGURE LEGEND

Figure 1. Proportions of Th17 TcdB-specific $CD4^+$ T cell are increased post-FMT. Blood was collected from n=22 recurring CDI patients immediately prior to FMT and 8-12 weeks post-FMT. OX40 assays were performed and the magnitude and phenotype of responses are shown for **(A)** TcdB (phenotype data from n=7); **(B)** Pediacel (phenotype data from for n=8) and **(C)** SEB (phenotype data from for n=16). Gating for Th cell subsets is shown in Supplemental Figure 1; cut-off for analysis was >30 events in parent gate; samples with < 30 $CD25^+OX40^+$ cells were not analysed for Th subset proportions. **(D)** Anti-TcdA, TcdB and $TcdB_{CROPS}$; and **(E)** anti-Pediacel IgG and IgA levels were measured by ELISA for n=19 recurring CDI patients pre- and post-FMT. Wilcoxon signed rank tests were performed. Healthy control (HC) median (solid line) and interquartile range (dotted lines) are shown from previously published analysis [4].