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Mucosa-Associated Invariant T Cells Undergo Phenotypic Changes Following Fecal Microbiota Transplantation

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Abstract

Fecal microbiota transplantation (FMT) has emerged as the most effective therapy for recurrent *Clostridioides difficile* infections (rCDI); yet the role of immune cells in the response to FMT remains poorly understood. Mucosa-associated invariant T (MAIT) cells are bacterial metabolite-reactive T cells found in the peripheral blood and mucosal tissues. We posited that MAIT cells are in the perfect position to respond to the influx of microbial metabolites associated with FMT. We collected peripheral blood from rCDI patients prior to FMT, 1-week post-FMT, and 1-month post-FMT, to explore if MAIT cell phenotypic characteristics or functions change following treatment. Following FMT, MAIT cells moved away from a state of exhaustion and displayed increased cytokine production. While pre-FMT MAIT cells were predominantly MAIT-17, post-FMT MAIT cells in some patients shifted to become neither MAIT-17, MAIT-1, nor MAIT-2. The data indicates that FMT may reverse MAIT cell exhaustion after a long battle with rCDI.

Keywords

Clostridioides difficile, fecal microbiota transplantation, mucosa-associated invariant T cell, innate immunity, mucosal immunology, riboflavin derivatives, MHC-related protein 1

Summary for Lay Audience

Clostridioides difficile is a bacterium that can cause infection resulting in diarrhea, fluid loss, or even death, when it gets into the intestinal tract. Since *C. difficile* has multiple ways to avoid destruction by antibiotics, a new effective treatment called fecal microbiota transplantation (FMT) is being widely adopted for those with the chronic form of the disease. FMT involves taking healthy gut bacteria from one person and transferring them into the intestinal tract of a person with *C. difficile* infection. It is thought that the healthy bacteria take up space and nutrients, eventually forcing *C. difficile* out. While FMT is successful in getting rid of *C. difficile* in most cases, the way the immune system responds to FMT is still not well understood. One immune cell type, mucosa-associated invariant T (MAIT) cells, is found in mucosal tissues and the blood. MAIT cells also respond specifically to bacterial by-products, such as those from the FMT bacteria. We therefore theorized that MAIT cells are in the perfect position to interact with FMT. With this in mind, we drew blood from people with *C. difficile* infections prior to FMT, then again one week and one month post-FMT. The activation level of blood MAIT cells, as well as what types of anti-bacterial molecules they produced, were then examined. We found MAIT cells to express high levels of markers associated with exhaustion — a state where MAIT cells do not function properly— prior to FMT. Following FMT, however, MAIT cells moved away from a state of exhaustion and were able to produce higher levels of anti-bacterial molecules. My findings suggest that FMT may help to restore MAIT cells' antibacterial functions following their battle with *C. difficile*. FMT can be costly and is not widely accessible at present. With improvements in our understanding of the immune response to FMT, new treatment strategies and tools to optimize or even replace FMT may emerge.

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5-A-RU	5-amino-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formyl pterin
APC	Antigen-presenting cell
BFA	Brefeldin A
BMI	Body mass index
BTLA	B and T lymphocyte attenuator
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDI	*C. difficile* infection
CDT	*C. difficile* transferase
cRPMI	Complete Roswell Park Memorial Institute medium
CTLA4	Cytotoxic T-lymphocyte antigen-4
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FSC	Forward scatter
FVD	Fixable Viability Dye eFluor780

GATA3	GATA binding protein 3
GI	Gastrointestinal
gMFI	Geometric mean fluorescence intensity
GTD	Glucosyltransferase domain
HCV	Hepatitis C virus
IAV	Influenza A virus
IBS	Irritable bowel syndrome
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
ILCs	Innate lymphoid cells
LAG3	Lymphocyte activation gene 3
MAIT	Mucosa-associated invariant T (cells)
MHC	Major histocompatibility complex
MR1	MHC class I related protein
NFAT	Nuclear factor of activated T cells
NK	Natural Killer (cells)
NOD	Nucleotide-binding oligomerization domain 1
PBMCs	Peripheral blood mononuclear cells
PC	Principal component

PCA	Principal component analysis
PD-1	Programmed cell death protein 1
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PSA	Polysaccharide A
PSI	Pounds per square inch
rCDI	Recurrent CDI
REB	Research ethics board
RORγT	Retinoid-related orphan receptor gamma T
SLP	Surface layer proteins
SSC	Side scatter
T-bet	T-Box transcription factor
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
TCR	T cell receptor
T_{FH}	T follicular helper (cells)
TGF	Transforming growth factor
$T_{H()}$	T helper type () (cells)
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domain

TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{reg}	Regulatory T (cells)
VISTA	V-type immunoglobulin domain-containing suppressor of T cell activation
WT	Wild type

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Chapter 1

1 Introduction

1.1 *Clostridioides difficile*

1.1.1 Rise of *C. difficile* in Healthcare Settings

Antibiotics are one of our most important medical discoveries that have saved countless lives. Unfortunately, the use of potent, broad-spectrum antibiotics can also kill the healthy intestinal microbiota and leave patients susceptible to infection by pathogenic bacteria[1–3]. Normally, the intestinal microbiota serves to take up physical adhesion sites and nutrients, while producing antimicrobial peptides that impede colonization by pathogens[4–7]. Antibiotic-mediated perturbation of the intestinal microbiota therefore removes barriers to infection by enteric pathogens. In fact, antibiotic use is recognized as a major risk factor for infection by the enteric pathogen *Clostridioides difficile*[1,2]. *C. difficile* infection (CDI) can lead to severe intestinal inflammation, and CDI is one of the main causative agents of gastroenteritis-associated deaths[8]. The steady rise in *C. difficile*-associated fatalities, coupled with the fact that it causes nearly 500,000 infections each year in the United States of America alone, makes finding effective treatments a priority[8,9].

The overuse of antibiotics increases the risk of CDI and can also be ineffective at treating the resulting infections. Emerging strains such as the toxin-producing ribotype 027, which possess resistance to both vancomycin and metronidazole, have quickly spread around the world[10,11]. While some CDIs can be treated with moderate efficacy using vancomycin or metronidazole, up to 35% of patients still experience recurrence of infection and symptoms[12]. As patients experience more rounds of recurrence, their risk of further recurrent episodes increases[13]. These recurrence rates are particularly notable with the more hypervirulent strains, and as *C. difficile* progressively acquires broad antibiotic resistance, treatment will become more difficult[13,14].

1.1.2 Biology and Transmission

Clostridioides difficile is an anaerobic, spore-forming, Gram-positive bacterium[15]. These endospores (spores) are the main transmissible form of the bacteria and are highly resistant to destruction even in an aerobic environment[15]. The durability of the spores allows *C. difficile* to transmit between individuals via the fecal-oral route, since it can survive the harsh acidity found in the stomach as it makes its way to the intestinal tract[16]. Furthermore, when spores are shed from an infected person, they can resist desiccation and survive on surfaces for months on end[17]. Even when these surfaces are treated with quaternary ammonium-based detergents, or an alcohol-based hand sanitizer for skin, the spores are not killed[15,17]. This necessitates the use of sodium hypochlorite (bleach) or other chlorine-based disinfectants to clean surfaces[17]. Additionally, the use of thorough handwashing or gloves when working with CDI patients can reduce personal infection and the spread of the spores to other people[17].

Once spores are ingested, they make their way through the stomach and into the intestinal tract. In the small intestine, the spores encounter primary bile salts released by the gall bladder[18]. This bile is largely comprised of chenodeoxycholate and cholate conjugated to glycine or taurine[18]. Taurocholate along with glycine sends a germinant signal to *C. difficile* spores, triggering their germination into vegetative cells that are no longer metabolically dormant[18]. In contrast, normally the healthy microbiota would metabolize cholate derivatives into deoxycholate, and this secondary bile salt can inhibit *C. difficile* growth. When the microbiota is perturbed, *C. difficile* travels through the intestinal tract and begins to germinate until it arrives at its destination, namely the large intestine.

Once in the large intestine, many pathogenic strains of *C. difficile* can produce two different glucosylating cytotoxins: toxin A (TcdA) and toxin B (TcdB)[19,20]. TcdA and TcdB are large toxins, with molecular weights of 308 kDa and 270 kDa, respectively[20]. These toxins bind to receptors on the surface of colonic epithelial cells to facilitate their internalization[21–23]. The toxins then use a pore-forming domain to allow the release of their glucosyltransferase domain (GTD) into the cytosol[24]. In order for just the GTD to be released, it is cleaved from the rest of the toxin in an autocatalytic manner[25,26]. In the cytoplasm, the GTDs of TcdA and TcdB move to glucosylate members of the Rho

GTPase family by removing one glucose from UDP-glucose and transferring it to Thr35 in Cdc42 and Rac, and Thr37 in RhoA[20,27,28]. This causes inactivation of the proteins and dysregulation of the actin cytoskeleton[27,28]. TcdA and low concentrations of TcdB therefore cause apoptotic cell death and the breakdown of intestinal membrane barrier integrity[29]. Interestingly, higher concentrations of TcdB can cause necrotic cell death in a glucosyltransferase independent manner, likely through the production of high levels of reactive oxygen species[29]. The release of TcdA and TcdB also leads to the production of proinflammatory cytokines, further exacerbating the inflammation associated with infection[30]. In addition to TcdA and TcdB, 17-23% of *C. difficile* strains can also produce a binary toxin called *C. difficile* transferase (CDT)[31]. CDT promotes protrusions of microtubules from the surface of targeted colonic cells, making it easier for *C. difficile* to adhere to them[31].While toxin-producing strains of *C. difficile* often cause disease symptoms, non-toxigenic strains have been found to asymptomatically colonize up to 13% of people[32,33]. To a much lesser extent, some healthy people are asymptomatically colonized by toxigenic *C. difficile*[34].

Vegetative *C. difficile* cells can possess a host of antibiotic resistance mechanisms. Many of these are curtesy of mobile genetic elements acquired from other *C. difficile* strains or other species of bacteria altogether[14]. Nearly 11% of the genome of some *C. difficile* strains can be comprised of mobile genetic elements, providing resistance mechanisms to chloramphenicol, clindamycin, erythromycin, and tetracycline[14]. *Clostridioides difficile* can also produce penicillin-binding proteins and β-lactamases to further thwart cephalosporins and penicillin[14]. In addition to interfering with the function of the antibiotic itself, *C. difficile* may alter the targets of the antibiotics. While not completely characterized, it is predicted that resistance to metronidazole and vancomycin may be achieved in this way[14]. Given that *C. difficile* endospores are not fully metabolically active, they too can survive antibiotic treatment and potentially start another round of *C. difficile* infection[14].

Over the course of CDI, *C. difficile* continues to produce spores. The exact signals which lead to the formation of spores by *C. difficile* are unknown, though the trigger for other bacterial species' production of spores can include stressors such as nutrient

deficiencies[35]. When the signal is received, the vegetative cell asymmetrically divides to produce a mother cell and a forespore[35]. The mother cell then engulfs the forespore and assembles the components needed to become a mature spore around the forespore center[35]. Once the spore is completely assembled, the mother cell lyses to release the spore and continue the infection and transmission cycle[35].

1.1.3 Innate and Adaptive Immune Responses to *C. difficile*

The gut microbiota serves an important role in skewing the intestinal immune environment towards an anti-inflammatory phenotype. For instance, the commensal bacteria *B. fragilis* produces a molecule called polysaccharide A (PSA)[36]. It has been shown that *B. fragilis,* and even PSA alone, are capable of ameliorating colitis in animal models[36]. This protection is mediated in part by reducing the levels of interleukin (IL)-17 and IL-23, which are known to contribute to the induction and progression of colitis[36]. Treatment with PSA also resulted in increased production of IL-10 by $CD4^+$ T cells which was shown to be protective against colitis[36]. Similarly, colonization by commensal *Clostridium* spp., such as *C. scindens,* increases the amount of regulatory T cells (T_{regs}) in the colonic lamina propria[37,38]. Colonization by commensal *Clostridium* spp. also promotes production of transforming growth factor (TGF)-β by colonic epithelial cells, and the supernatants from these cell cultures can induce $CD4^+$ T cell differentiation into T_{regs}[37]. Overall, the presence of T_{regs} and anti-inflammatory cytokines in the intestinal environment favors a steady state of hyporesponsiveness towards microbial signaling[39]. The loss of these commensal signals that maintain the anti-inflammatory state, such as through antibiotic use, allows an easier transition to an inflammatory gut response.

In addition to cell death caused by TcdA and TcdB, *C. difficile* can also promote secretion of various cytokines and chemokines by intestinal epithelial cells. In the process of killing intestinal epithelial cells, TcdA promotes their secretion of the neutrophil chemotactic cytokine IL-8[40]. Despite being a Gram-positive bacterium, *C. difficile* produces a meso-diamino-pimelic acid-type peptidoglycan[41]. This molecule allows both toxigenic and non-toxigenic strains of *C. difficile* to activate the nucleotide-binding oligomerization domain 1 (NOD1) pattern recognition receptor (PRR)[41]. NOD1 activation in epithelial cells promotes CXCL1 secretion, leading to the recruitment of

neutrophils to the colon[41]. This activation of NOD1 is potentially important in the context of *C. difficile* infection, as NOD1$^{-/-}$ mice exhibit significantly higher mortality due to *C. difficile* infection than either their wild-type or NOD2$^{-/-}$ counterparts[41].

As *C. difficile* breaks through the intestinal epithelial cell layer, it encounters the mucosal immune environment, and continues to activate PRRs. *C. difficile* surface layer proteins (SLPs) activate Toll-like receptor 4 (TLR4) on various immune cell subsets[42,43]. Treatment of dendritic cells (DCs) with SLPs induces significant production of IL-12, IL-10, IL-23, and tumor necrosis factor (TNF)-α[42,43]. These dendritic cells also upregulate their expression of co-stimulatory molecules such as CD80, and can polarize naïve T cells towards a T helper (T_H)1, T_H2, or T_H17 phenotype depending on the exact cytokine composition[42,43]. Given that IL-12 polarizes T cells towards a T_H1 phenotype, many of the T cells that encounter SLP-treated DCs become potent producers of Interferon- γ (IFN-γ)[42,43]. TLR4$^{-/-}$ mice, similar to NOD1$^{-/-}$ mice, exhibit increased morbidity in response to *C. difficile* challenge compared to their WT counterparts, underlying the importance of generating a robust immune response[42]. Circling back to the type 17 immune response, SLPs also induce monocytes to produce IL-6 and IL-1β, further promoting an inflammatory response[43].

Type 1 immune responses characterized by the production of cytokines such as IL-12 and IFN-γ are known to be protective in the context of CDI. This is evidenced by the fact that a lack of type 1 innate lymphoid cells (ILC1s) is associated with increased mortality in murine models of acute CDI[44]. Transfer of ILC1s into mice lacking ILCs rescues their ability to resolve acute CDI[44]. It was further determined that ILC1s were the main producers of IFN-γ in the large intestine of mice with CDI, and the protection they imbue is highly reliant on secretion of this cytokine[44]. *C. difficile* infection also increases the production of IFN-γ by T cells[42,43,45]. The robust production of IFN-γ leads to high levels of this cytokine being found in the serum of CDI patients, particularly in patients with mild to moderate disease compared with patients that have severe disease[30]. Severe CDI was associated with a shift away from a type 1 IFN-γ response, and towards a type 17, IL-17A producing phenotype[30]. This would all seem to suggest that type 1 immune responses to CDI are protective and associated with reduced disease severity.

Type 17 cytokines including IL-6, IL-17A, and IL-23 are known to be increased in the serum of patients with CDI, and patients with severe infection have higher serum IL-6 and IL-23 than those with non-severe disease[30,46,47]. IL-6 can help promote $CD4^+$ T cell differentiation into T_H17 cells, and CDI patients with very high levels of serum IL-6 are more than 7 times less likely to survive their infection[46]. Furthermore, transfer of T_H17 cells, producing IL-17A, into naïve mice that are later infected with *C. difficile* leads to increased CDI severity[46]. IL-23, not IL-17A, is known to be involved in neutrophil recruitment to the colon during CDI in mice[48]. Mice deficient in IL-23 therefore exhibit reduced recruitment of neutrophils to the colon during CDI[48]. These IL-23 deficient mice exhibited reduced morbidity and mortality during CDI[47]. This would seem to suggest that type 17 responses involving neutrophils are pathogenic in the context of CDI. This is not entirely the case however, as depletion of neutrophils in murine CDI models, or neutropenia in human patients with CDI, are associated with increased mortality[49,50]. Type 17 responses therefore exist in a delicate balance of protective and pathogenic potentials during CDI.

Colonization by *C. difficile* is prevalent in neonates shortly after birth, though by the time they are toddlers, the colonization rates are reflective of those found in adults[51]. However, symptomatic colonization is uncommon in neonates[51]. It is postulated that this may be due to differences in their ability to recruit neutrophils to the intestinal tract, or a lack of receptors for toxin A[51]. This early life colonization is predicted to be the reason that some adults have anti-TcdA and anti-TcdB antibodies in their serum, even if they are no longer carriers of the bacteria[51,52]. Compared with CDI-free patients currently on antibiotics, CDI patients tend to have lower serum levels of pre-existing anti-TcdB IgA antibodies[52]. During CDI, patients who produce higher levels of anti-TcdA IgM and IgG antibodies are less likely to experience recurrent infection, as are patients with higher levels of TcdB-neutralizing antibodies[53,54]. The outcome of CDI is intricately tied to both pre-existing humoral responses and immune responses mounted over the course of the infection.

1.1.4 Disease Symptoms and Outcomes

The symptoms associated with CDI can vary greatly depending on the severity of disease, though frequent diarrhea is a hallmark symptom leading to the exploration of a CDI

diagnosis[55]. Mild CDI is often accompanied by a low grade fever, abdominal cramping, and elevated white blood cell counts[55]. With the progression to severe CDI, there is an increase in abdominal cramping and tenderness, fever grade, and the degree of leukocytosis[55,56]. Severe and complicated CDI, also called fulminant CDI, can be accompanied by hypotension, paralytic ileus, toxic megacolon, and increased risk of bowel perforation[55,56]. Due to the influx of neutrophils, tissue damage by toxins, and general inflammation, microabscesses can develop along the intestinal epithelium[57]. The collection of debris and cells can also form pseudomembranes, which cover the luminal surface of the large intestine (pseudomembranous colitis)[57]. As CDI progresses, the size and intestinal coverage of the pseudomembranes increases[57]. While only 3-8% of CDI patients will typically progress to fulminant infection, this degree of disease can require surgical intervention to remove and bypass the damaged sections of the intestines[55].

CDI can be diagnosed based on the clinical symptoms and an accompanying test verifying the presence of *C. difficile* toxins in the stool[55]. Once CDI has been confirmed, use of the antibiotic the patient was already taking is ceased[56]. Typically, metronidazole and vancomycin are recognized as the first-line treatments for the first episode of CDI[56,58]. Vancomycin is preferable however, due to the lower efficacy of and increasing drug resistance to metronidazole[11,14,58]. It has also been shown that vancomycin is more efficacious than metronidazole for the treatment of severe CDI[58]. Another antibiotic, fidaxomicin, shows similar efficacy to vancomycin in resolving CDI[58]. Treatment with fidaxomicin results in a recurrence rate of only 16-20% compared with the 25-36% recurrence rate tied to vancomycin[56,58]. The high cost of fidaxomicin has unfortunately been a significant barrier to widespread use of the drug against CDI[56].

Recurrent CDI (rCDI) occurs when a patient experiences another episode of CDI within 2 months of their previous CDI[13]. In a vicious cycle, as the patient experiences more episodes of rCDI, their risk of further episodes of rCDI also increases[13]. In addition to initial antibiotic choice, a variety of other factors can contribute to the risk for recurrence. In particular, the risk of rCDI is known to increase in patients older than the age of 65[13]. Additionally, the use of proton pump inhibitors and the resulting increase in gastric pH can leave patients vulnerable to another CDI episode[13]. Finally, failing to mount or

mounting aberrant immune responses can contribute to an increased risk for rCDI[53,54,59]. The production of high anti-toxin antibody titers during CDI makes patients less likely to experience another episode[53,54]. Interestingly, a common allele of IL-8, which features a mutation in the promoter, is tied to both the increased risk for initial CDI, and a higher risk for rCDI[59].

The mortality rate associated with CDI is somewhat difficult to calculate. While some studies have reported fatality rates of 6-30% from CDI, these rates may be inaccurate[60]. When a panel of physicians was asked to classify how strongly CDI contributed to the deaths of patients from Ontario hospitals based on summarized clinical information and official cause of death, they determined that 67% of the deaths in CDI patients were strongly contributed to by CDI[60]. In contrast, this same study also examined the rates of death attributed to CDI based on death certificate information, in which CDI was the primary cause of death in only 5% of patients[60]. Given that the death certificate can only list one cause of death, the agreement on CDI fatality rates was vastly different between physician panels and death certificates[60]. While physician panels may be more accurate, very few studies have implemented this laborious procedure to estimate CDI death rates[60]. Alternatively, some studies use death within 30 days of CDI diagnosis as a proxy to measure deaths contributed to by CDI[9,60]. In this case, one large scale study reported the death rate within 30 days of CDI diagnosis as approximately 1-9% depending on where infection was acquired[9].

1.2 Fecal Microbiota Transplantation

1.2.1 Principles of FMT

Fecal microbiota transplantation (FMT) involves the administration of commensal gut bacteria from healthy donor stool into the intestinal tract of a recipient with microbial dysbiosis[61]. This is thought to supplement a healthy microbiota composition in the colon by fixing or even replacing the perturbed existing microbiota composition[61,62]. The newly introduced donor microbiota can persist for months after FMT, and brings in the critical diversity the perturbed microbiota was lacking[62,63]. FMT also serves to reduce intestinal inflammation associated with microbial dysbiosis[64]. Administration of FMT into mice

with colitis has been shown to decrease the production of pro-inflammatory cytokines such as TNF-α and IL-1β by intestinal lamina propria mononuclear cells[64]. In contrast, FMT increased the colonic levels of IL-10, which was critical for the protective effects FMT exerted[64]. The restoration of the healthy intestinal microbiota even promotes the regeneration of the damaged epithelial cell layers[65]. As our understanding of the role the microbiota plays in various facets of health and disease becomes clearer, many conditions tied to microbial dysbiosis may benefit from this intervention[61].

In order to obtain the microbiota for FMT, potential fecal matter donors must first be screened to ensure safety. Donor exclusion criteria are normally obesity, age >65, presence of metabolic disorders, irritable bowel syndrome (IBS), use of antibiotics within the past 6 months, or diarrhea in the last 6 months, amongst other things[61]. The microbiota is intricately linked to our health, and mouse studies have shown that giving an FMT sourced from an IBS patient to a mouse can lead to perturbed intestinal function[66]. With this in mind, caution must be taken to ensure an atypical and potentially harmful microbiota composition is not transferred to the recipient. If donors make it through the initial screening, they undergo serological tests and fecal matter screening for various pathogens. Detection of viral, bacterial, or parasitic pathogens is also grounds for exclusion[61]. Given the costs of the diagnostic tests and the multitude of potential donors who do not pass them, it can cost around $15,000 USD to find a single donor for the FMT fecal material[67]. Following screening, the donor must provide a stool sample of at least 50 g to the clinic within one month[61]. The stool is diluted in saline, thoroughly mixed in, and filtered for use within 4 hours, or stored at -80°C for later use[61].

There are a variety of methods to administer the FMT into the intestinal tract of a recipient. Methods that fall into the category of lower gastrointestinal (GI) tract routes include fecal enemas and colonoscopy[68]. While colonoscopy use allows precise delivery of the FMT to the affected sites in the GI tract, it comes with inherent risks due to the use of anesthesia and the risk of bowel perforation[68]. FMT can also be administered from the upper GI tract using capsules or a nasogastric tube[68]. The capsule method is a relatively recent addition and involves wrapping the stool in multiple capsule layers to allow it to survive the harsh acidic environment of the stomach[68]. Despite the diversity in

administration methods, the efficacy of FMT is similar between them[68]. This therefore leaves more room for patients to make decisions about which method they are comfortable with, in conjunction with their doctor's consideration of which methods are feasible and safe for the patient[68].

FMT is widely considered a safe and efficacious method[58,61,69]. The most common side effects following FMT can include transient diarrhea, bloating, nausea, and cramping[69]. While fever has also been reported, this has not been severe[69]. In terms of infectious complications following FMT, a review of 109 publications by Baxter and Colville concluded that 4 patients out of 1555 did develop bacteremia following FMT[69]. One of these patients however had a history of bacteremia and comorbidities that likely increased their intestinal permeability, raising the question of if they were unusually susceptible to such a condition[69]. Use of colonoscopy to administer FMT also carries risk, as perforations and aspiration while under sedation have occurred during the procedure, though these complications are not due to FMT so much as the method used to administer it[69]. The overall safety of FMT therefore makes it a viable option to explore in the treatment of conditions related to microbial dysbiosis.

1.2.2 FMT to Treat Antibiotic-Refractory *C. difficile* Infections

Antibiotic-mediated perturbation of the healthy intestinal microbiota is a known risk factor for CDI[1,2]. CDI patients therefore have reduced gut microbial diversity, with an overrepresentation of phyla that differ from the healthy phyla composition[63]. Given that FMT is used to reverse microbial dysbiosis, it has gained favor as an effective treatment for rCDI after first-line antibiotics fail[61,67]. The antibiotics are used up until a few days before the FMT, to eradicate as much *C. difficile* as possible and increase the efficacy of treatment[61]. When used to treat rCDI, the success rate of FMT is between 83-94%, giving hope for patients that struggle with rCDI for extended periods of time[58,61]. Within a week of treatment, the CDI symptoms typically subside, as the healthy microbiota makes an inhospitable environment for the pathogen and as the intestinal tract begins to heal[4–7,61,65].

The successful elimination of CDI by FMT is associated with the return to a healthy microbiota composition[63]. In Europeans on a Western diet rich in lipids and animal

protein, this healthy microbiota is primarily composed of Bacteroidetes and Firmicutes[70]. Alternatively, in Hadza hunter-gatherers, the microbiota shows enrichment of Proteobacteria, highlighting how there is not one set "healthy microbiota"[70]. When FMT is performed using a donor on a western diet, it helps to repopulate Firmicutes and Bacteroidetes spp., increasing the bacterial diversity found in the recipient's intestinal tract[63]. In contrast, removing the diversity aspect of FMT by treating CDI with a few bacterial strains, in the form of probiotics, has been largely unsuccessful[71]. It is possible that the probiotics thus far have just not captured the most critical bacterial species or metabolites that aid in the success of FMT. Conversely, even if the bacterial species were identified and packaged as a probiotic, the hurdle of promoting stable engraftment remains[62]. It has also been suggested that focusing on a particular species for therapeutic value can go wrong when it fails to consider that the species would have its function modulated by the recipient's remaining intestinal microbiota[72]. Given the inter-patient variability in the remaining intestinal microbiota, it is not likely that a specific combination of limited microbial species will effectively treat CDI in all patients[72]. Rather, using an algorithm designed with ecology principles in mind may enable assembly of personalized probiotic compositions specific to individual patients in the future[72].

For now, it seems that the diversity of bacteria provided by FMT is needed to effectively cure rCDI and allow for the persistence of the new healthy microbiota[62]. While the role the microbiota plays in the resolution of CDI is becoming clearer, how the immune system contributes to the response to FMT and potentially to the subsequent resolution of CDI is still unclear. Different immune cell subsets likely work in concert to respond to FMT. With our growing knowledge of mucosa-associated immune cell subsets, we may be able to narrow in on the conductors that orchestrate this response and are affected by FMT treatment. The immune system could act as a therapeutic target to boost the efficacy of lower-dose FMT, thus reducing the financial burden associated with FMT and increasing the accessibility of the treatment.

1.3 MAIT Cells

1.3.1 Introduction to MAIT Cells

It all began in 1993 when Porcelli et al. observed that $CD4^{-}CD8^{-}$ α/β T cells were enriched for a particular T cell receptor (TCR) rearrangement[73]. This rearrangement was characterized by Vα7.2 joined to JαIGRa14 (which would later become known as Jα33)[73]. These $CD4^{-}CD8^{-}$ α/β T cells also used a limited repertoire of Vβ chains, leading the authors to wonder if this overrepresented TCR from multiple donors could utilize a nonpolymorphic molecule for antigen-presentation[73]. Years later these cells would acquire a name: mucosa-associated invariant T (MAIT) cells. MAIT cells are a subset of innate-like T lymphocytes that earned their name by residing primarily in mucosal tissues and the liver[74–77]. MAIT cells are also present in the peripheral blood, making up 1-10% of peripheral blood T cells[76,78]. Although MAIT cells were discovered as $CD4^{-}CD8^{-}$, it is now known that human MAIT cells are primarily $CD8^{+}$, with $CD4^{-}CD8^{-}$, $CD4^{+}$, and $CD8^{+}CD4^{+}$ making up smaller portions of the MAIT population[78]. The MAIT cell semi-invariant TCR primarily exhibits the Vα7.2-Jα33 rearrangement but can also utilize Vα7.2-Jα12 and Vα7.2-Jα20 rearrangements[78]. The TCR-α chain then predominantly pairs with a limited selection of TCR-β chains, typically Vβ2 or Vβ13[78].

MAIT cell surface expression of various markers is also unique compared with their conventional T cell counterparts. MAIT cells largely express the C-type lectin family member CD161, which is typically seen on natural killer (NK) cells[77]. In relation to other T cells, MAIT cells display an effector memory phenotype[77]. This phenotype is characterized by MAIT cell expression of $CD45RO^{+}CCR7^{-}CD62L^{lo}$, where CD45RO is a memory marker and $CCR7^{-}CD62L^{lo}$ speaks to an effector rather than central memory phenotype[77,79]. MAIT cells are also known to express a wide range of chemokine receptors including CCR9, CCR5, CXCR6, and CCR6, which suggests preferential homing to mucosal surfaces such as the intestines[77,78]. In keeping with the mucosal homing phenotype, MAIT cells largely express the α4β7 integrin, which is known to aid in recruitment to the intestinal tract particularly upon microbial challenge[76,80,81]. Additionally, MAIT cells exhibit high expression of the IL-18 receptor and the IL-12 receptor, allowing them to respond to threats in a TCR-independent manner[77,82]. This is

particularly useful in the context of viral infections, which do not produce ligands that the MAIT TCR recognizes[83].

1.3.2 Vitamin B Metabolites and the MHC Class I Related Protein

MAIT cells further diverge from their conventional T cell counterparts in that they do not recognize peptides presented in the context of major histocompatibility complex (MHC) molecules. Rather, the MAIT cell TCR recognizes vitamin B metabolites of bacterial or fungal origin, as well as certain drugs, presented on the MHC class I related protein (MR1)[84,85]. MR1 is monomorphic and evolutionarily conserved, in addition to being ubiquitously expressed in many human tissues[86,87]. Despite the presence of MR1 within many cell types, surface expression of MR1 is low in the absence of infection[84]. This is postulated to be due to low levels of antigens, which may be needed for MR1 to fold and make it to the surface in higher numbers[84,87]. Also critical to the assembly of MR1 is β2-microglobulin, a feature shared with MHC class I molecules[88].

A reservoir of unfolded MR1 molecules is present in the endoplasmic reticulum (ER) of many cell types[88]. The binding domain of MR1 has solvent exposed regions, as well as hydrophobic and charged residues, making it unable to bind peptides or lipids[84]. Certain vitamin B metabolites however, such as the vitamin B9 derivative 6-formyl pterin (6-FP), are perfectly suited to nestle into the highly conserved antigen binding cleft of MR1[84]. Binding of 6-FP to MR1 utilizes hydrophobic interactions, as well as the formation of a Schiff base between Lys43 of MR1 and the formyl group of 6-FP[84]. While 6-FP is nearly completely buried in the MR1 molecule, and is unable to activate MAIT cells, vitamin B2 (riboflavin) derivatives do not face the same problem[84]. The different structure of riboflavin derivatives allows for more of the ligand to be exposed and contact the MAIT TCR[84]. The bacterial riboflavin synthesis intermediate 5-amino-6-D-ribitylaminouracil (5-A-RU) can avoid enzymatic reactions to produce riboflavin, and instead react with glycolysis intermediates to form 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)[89,90]. Despite being unstable, 5-OP-RU can be efficiently captured by MR1 and form a Schiff base with Lys43[90]. From here, MR1 can move to the surface of the cell, and activate MAIT cells using the vitamin B2 intermediate[89].

Empty MR1 is stabilized by ER chaperone proteins in a conformation ideal for ligand binding[91]. Once MR1 binds its ligand of exogenous origin in the ER, it can undergo a conformational change to become fully folded[88]. Binding of the ligand can serve to neutralize the positive charge of Lys43, and this change allows MR1 to move to the cell surface[88]. In the absence of exogenous ligands however, some MR1 does still make it to the cell surface[88]. In this case, MR1 does not require the transporter associated with antigen processing (TAP), calreticulin, or tapasin[92]. Instead, MR1 will associate with the Invariant chain, then traffic to the endosome and associate with HLA-DM, a pathway akin to that taken by MHC class II molecules[92]. In the late endosome, it was predicted that MR1 may switch out the Invariant chain for an endocytic ligand, allowing its movement to the cell surface[92]. In the absence of ligands of pathogenic origin, MR1 can therefore move to the surface with an endogenous antigen in tow. Given that some bacterial pathogens are intracellular, and do not effectively shed MR1 ligands into the extracellular environment, it is possible that this pathway allows MR1 to sample endosomes for pathogenic threats[93]. It has additionally been shown that MR1 presentation of antigens from intracellular pathogens can utilize different mechanisms than presentation of small exogenously added antigens (such as 6-FP)[93]. It would therefore seem that MR1 can utilize multiple pathways to acquire ligands and display them to MAIT cells.

1.3.3 MAIT Cell Development and Transcriptional Profile

MAIT cell development occurs in the thymus, with MAIT cells being positively selected by $CD4^+CD8^+$ thymocytes (double positive thymocytes)[94]. These double positive thymocytes express high levels of MR1, and can efficiently present vitamin B metabolites[94,95]. MAIT cell development in the thymus can be divided into three different stages[94]. In the first stage, MAIT cells are the most immature, and characterized by a $CD27^-CD161^-$ phenotype[94]. When MAIT cells move into the second stage, they become $CD27^+$ and can enter the periphery[94]. As the MAIT cells transition from stage 2 to stage 3, they then increase their expression of the transcription factors retinoid-related orphan receptor gamma t (RORγT), T-Box transcription factor (T-bet), and promyelocytic leukemia zinc finger (PLZF)[94]. Stage 3 MAIT cells are further characterized by

expression of the IL-18 receptor, and a surface $CD27^{pos\text{-}lo}CD161^{+}$ phenotype[94]. These stage 3 MAIT cells are fairly rare in the thymus and have a weak cytokine response to stimulation when they are found there[94]. Conversely, stage 3 MAIT cells from the blood can effectively produce cytokines in response to stimuli, demonstrating that MAIT cells continue to mature in the periphery[94]. This maturation may be due to interaction with ligands of microbial origin[94]. MAIT cells also expand in the periphery, particularly in fetuses and young children, as they strive to reach adult MAIT cell frequencies[77,96].

Interestingly, a small number of MAIT cells are present in germ-free mice indicating that MR1 may be able to present unknown self-antigens to allow limited MAIT cell survival[95]. This also supports the discovery that human fetuses do possess MAIT cells[96]. These MAIT cells display a predominantly immature phenotype in the thymus but shift to a mature phenotype upon movement to peripheral tissues such as the intestine[96]. This mature phenotype was characterized as $CD45RO^{+}CCR7^{-}CD62L^{-}$ with high expression of the IL-18 receptor and acquisition of the transcription factor PLZF[96]. This draws similarities to the MAIT cell developmental pathway mapped in young and adult human tissues. Some of the differences in the conclusions of the human fetal MAIT cell study, compared with the study identifying MAIT cell developmental stages, were likely due to their use of high CD161 expression to help identify MAIT cells[96]. As the more recent MAIT thymic development paper indicated, CD161 expression is not largely seen on MAIT cells in the earlier stages of development so these subsets would've been missed in the analysis. Nonetheless, the presence of mature MAIT cells in the periphery of the human fetuses would still be reliable.

Given that immature MAIT cell subsets do not express CD161, and that the use of surrogate markers such as CD161 is ineffective for $CD4^{+}$ MAIT cell subset identification, there has been a push for a more reliable staining procedure to identify MAIT cells[78,96]. While using antibodies against Vα7.2 in conjunction with surrogate markers can be effective for identifying MAIT cells, it raises the risk of missing MAIT cell subsets or including non-MAIT T cells featuring the Vα7.2 rearrangement[78]. With this in mind, researchers have developed MR1 tetramers which can specifically identify MAIT cells for analysis via flow cytometry[78]. These MR1 tetramers consist of four MR1 molecules

linked together and conjugated to a fluorochrome. The MR1 molecules are loaded with 5-OP-RU and can bind the MAIT cell TCR. As a control, MR1 tetramers are loaded with 6-FP, a molecule that would not bind the MAIT TCR. This is now the gold standard for MAIT cell identification and has allowed deeper exploration into the unique phenotype and capabilities of MAIT cells[94].

One such unique feature of MAIT cells is their transcriptional profile, which is critical in promoting their rapid responses and can be used to classify potential cytokine skew upon stimulation. PLZF is not found in conventional T cells, and aids in the rapid response of MAIT cells by suppressing naïve T cell genes and inducing effector genes[97,98]. RORγT is considered a master regulator of type 17 responses, including IL-17 production, and is generally highly expressed in mature human MAIT cells[76–78,99]. T-bet, a master regulator of type 1 responses associated with production of cytokines like IFN- γ, seems to be expressed at variable levels in human MAIT cells[76,78,95,99]. In mice, MAIT cells are either RORγT$^+$ or T-bet$^+$, and are aptly called MAIT-17 or MAIT-1 respectively[78,98]. In humans however, both transcription factors can simultaneously be expressed in mature MAIT cells, making the MAIT-1/MAIT-17 delineation less clear[76–78,99]. Despite expressing RORγT, blood MAIT cells primarily secrete IFN- γ upon stimulation[76]. MAIT cells from the female genital mucosa, however, are biased towards production of IL-17, indicating that tissue location and not just transcriptional profile can dictate how MAIT cells respond[76].

A current point of contention in the field is the existence of a type 2-skewed MAIT cell population (MAIT-2). In conventional T_H2 CD4$^+$ T cells, the transcription factor GATA binding protein 3 (GATA3) acts as an activator of type 2 cytokine production, such as IL-4, IL-5, and IL-13. MAIT cells however have not been found to express GATA3 at notable levels[78,100]. Interestingly, in older adults, peripheral blood MAIT cells were found to be capable of producing more IL-4 than MAIT cells from younger adults[101]. Additionally, upon prolonged stimulation *in vitro*, human MAIT cells can produce IL-13, IL-4, and IL-5[100]. It has been posited that chronic stimulation of MAIT cells might occur *in vivo* when tumors breakdown epithelial barriers and allow increased interaction between the immune system and commensal bacteria[100]. This has not been definitively

proven but draws parallels to the intestinal damage observed during CDI. Given that MAIT cells do not express GATA3 at notable levels, if at all, the mechanisms behind their type 2 cytokine production remain elusive. T follicular helper cells (T_{FH}) are known to produce IL-4 without dependence on GATA3, and it is therefore possible that MAIT cells also use a GATA3 independent pathway[102].

1.3.4 TCR-Dependent Activation

The MAIT cell TCR interacts with MR1 in a manner similar to that employed by conventional T cell TCRs interacting with MHC molecules[103]. The MAIT TCR therefore adopts an orthogonal docking position, which it uses when recognizing ligands presented by MR1[103]. While MAIT cells can employ different Jα chains, all of them still interact with MR1:antigen complexes in the same way[103]. Although the limited repertoire of Vβ chains utilized by the MAIT TCR does not require specific residues for MR1:antigen interaction, these Vβ chains can slightly affect the angle of the interactions[103]. Nonetheless, presentation of folate derivatives by MR1 does not serve to activate the majority of MAIT cells[84,103]. Conversely, MR1 makes different interactions with riboflavin derivatives, allowing exposure of their ribityl tail to interact with the MAIT cell TCR and activate MAIT cells[103]. Given the propensity of MAIT cells to respond to riboflavin derivatives, these derivatives are often employed for *in vitro* and *in vivo* TCR-dependent stimulation of MAIT cells. In particular, 5-OP-RU and the related molecule 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) are extremely effective MAIT cell agonists[103].

Human MAIT cells largely express CD8, though they can also express CD4 or neither CD4 nor CD8 (double negative)[78]. This would seem to indicate that MAIT cells do not always require a coreceptor to be functional. In fact, double negative MAIT cells can still respond robustly to bacterial antigens by producing proinflammatory cytokines[104]. CD4 normally stabilizes TCR:MHC class II interactions by interacting with the β chain of MHC class II. MR1 does not have a β chain, but instead has an α chain that associates with β2 microglobulin[86]. Thus, CD4 could not interact with MR1 in a conventional manner, and the importance of CD4 on MAIT cells is still a point of curiosity. In contrast, CD8 stabilizes interactions with MHC class I, which is also comprised of an α

chain associated with β2 microglobulin. While human MR1 does not appear to express the residues that allow classical MHC I to associate with CD8, this does not rule out interaction altogether[86]. It has been found that blocking CD8 on MR1 restricted T cell clones interferes with their ability to respond to bacterially infected cells[105]. Furthermore, the same was observed for blocking CD8 on human $CD8^{+}CD161^{++}$ $V\alpha7.2^{+}$ T cells[104]. While the role of CD8 on MAIT cells is still being explored, it would appear that CD8 may aid in MAIT cell responses, though it is not a requirement for their function.

Once activated via TCR signaling, human MAIT cells can produce a variety of cytokines as well as cytotoxic effector molecules. MAIT cells from the human female genital mucosa produce IL-17, IL-22, IFN-γ, TNF-α, and granzyme B upon stimulation with *E. coli*[76]. MAIT cells from the peripheral blood in contrast produce more granzyme B, IFN-γ, and TNF-α in response to *E. coli*[76]. Similarly, upon stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, anti-CD3/CD28 beads, or 5-OP-RU pulsed cell lines, human blood MAIT cells produced IFN-γ, granzyme B, and TNF-α[77,106]. PMA/ionomycin also induced a degree of IL-2 and IL-17 production, as well as production of IL-4 by MAIT cells from the elderly[77,101]. PMA/ionomycin mimics the downstream cascade following TCR stimulation, resulting in a response like that induced by conventional TCR stimulation[107]. The aforementioned experiments did not sort MAIT cells to stimulate them alone but instead included the other immune cells that would have been present in the tissue of origin. When MAIT cells are sorted, and they alone are incubated with anti-CD3/CD28 beads, their production of IFN-γ and TNF-α is rapid but short lived[108]. In contrast, upon anti-CD3/CD28 stimulation of peripheral blood mononuclear cells (PBMCs), containing the MAIT cell population, robust and sustained cytokine production is noted[108]. This would seem to suggest that fully activating MAIT cells requires other accessory signals, such as cytokines or engagement of cell surface co-stimulatory molecules[108,109].

1.3.5 TCR-Independent Activation

Upon infection, macrophages, monocytes, and DCs can release IL-12 and IL-18[110–112]. These cytokines are noted as a primary source of MAIT cell TCR-independent activation, given that MAIT cells constitutively express the high affinity IL-12 and IL-18

receptors[77,82,109,113]. Once activated by IL-12 and IL-18, human MAIT cells can rapidly produce IFN-γ, granzyme B, and perforin[108,114]. Recently, it has also been noted that IL-15 and type 1 IFNs can synergize with IL-12, IL-18, or both, to elicit effector responses from MAIT cells[83,108]. This process of activation independent of TCR signaling is particularly useful in the context of viral infections, as viruses do not have their own riboflavin synthesis pathway. It also positions MAIT cells as therapeutic targets that can participate in antiviral responses towards antigenically dissimilar viruses.

In recent years activation of human MAIT cells has been seen in the context of hepatitis C virus (HCV), influenza A viruses (IAV), and dengue virus[83]. MAIT cells isolated from individuals infected with any of the aforementioned viruses expressed notable levels of granzyme B, corroborating *in vitro* studies[83]. Furthermore, activating human MAIT cells using HCV-infected macrophages and treating infected cell lines with the resulting supernatant suppressed viral replication[83]. While MAIT cell antiviral responses to HCV have been noted *in vitro*, the role of MAIT cells in the context of IAV infection has been explored *in vivo*. When $MR1^{-/-}$ mice, which lack MAIT cells, were infected with IAV they experienced significantly higher morbidity and mortality than their WT counterparts[115]. This could be reversed by adoptively transferring MAIT cells into the $MR1^{-/-}$ mice before infection with IAV[115]. Knockout of IFN-γ in the MAIT cells used for the adoptive transfer again reduced the protective effect, highlighting the importance of IFN- γ production by MAIT cells in the antiviral response[115]. Elucidating if MAIT cells are protective in human IAV infections is a more complicated task. While correlation does not imply causation, hospitalized patients who succumbed to severe IAV infection had lower MAIT cell numbers than those that recovered[116]. The influence of cytokines on MAIT cell activation is not limited only to viral infections but can also synergize with TCR signaling to potently activate MAIT cells[106,108]. MAIT cells therefore have multiple modes of activation that contribute to broad antimicrobial immunity (Figure 1).

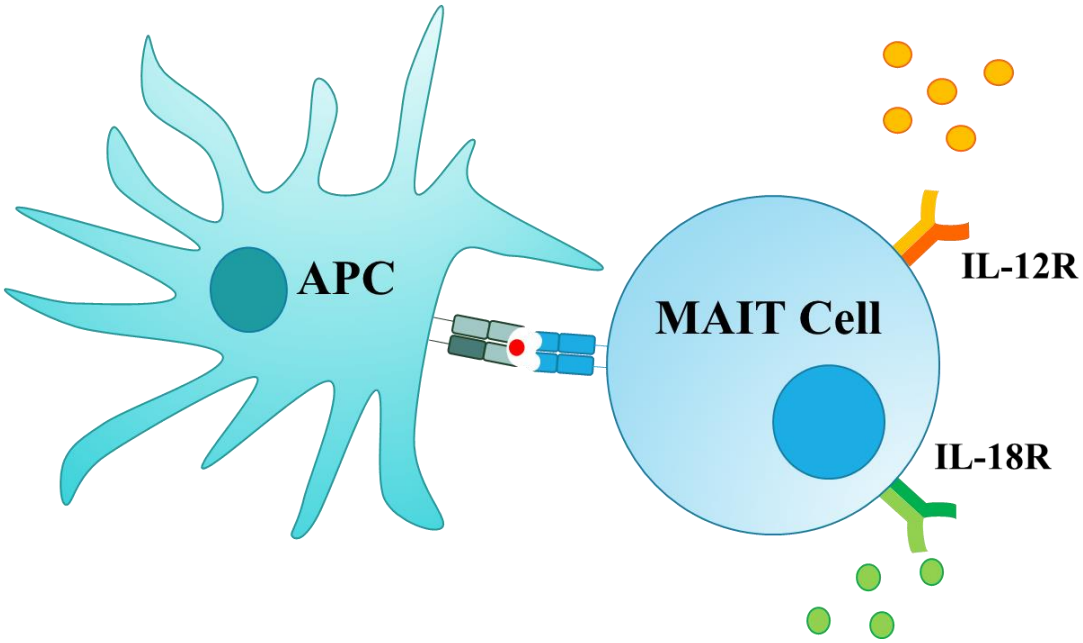


Figure 1. MAIT Cells Can Be Activated in Both TCR-Dependent and TCR-Independent Manners. Human MAIT cells possess a semi-invariant TCR typically using the Vα7.2-Jα33 rearrangement, though the alpha segment can also join with Jα12 or Jα20[78]. This semi-invariant TCR does not recognize peptides in the context of classical MHC molecules, but instead recognizes microbial vitamin B metabolites in the context of MR1[84]. Upon infection, cells such as antigen-presenting cells (APCs) can upregulate their surface expression of MR1, now bound to ligand[88]. During infection these APCs can also produce IL-12 and IL-18[110–112]. MAIT cells express IL-12 and IL-18 receptors, allowing them to be activated in a TCR-independent manner[77,82,109,113]. These two activation pathways are not mutually exclusive, and can work together to synergistically activate MAIT cells in the context of infection[106,108].

1.3.6 Activation and Exhaustion Marker Expression by T Cells

Upon activation, MAIT cells upregulate cell surface expression of markers associated with this activated state. These markers are not only indicative of activation but functionally contribute to enhancing activation and modulating T cell responses. One such marker, commonly associated with activation and tissue retention of T cells, is CD69[117]. This protein can fine tune the differentiation of conventional T cells into T_{regs} or T_H17 cells[117]. Intestinal MAIT cells, MAIT cells stimulated *in vitro*, and MAIT cells during infection *in vivo* can all express CD69[74,82,109,115,116]. Similarly, MAIT cells can also upregulate expression of the activation marker CD38 upon stimulation[83,99]. CD38 upregulation occurs on many immune cell types in response to inflammation, and CD38 possesses enzymatic activity[118]. The enzymatic activity of CD38 ultimately culminates in the mobilization of calcium, and increased T cell activation through the nuclear factor of activated T cells (NFAT) pathway[118]. Thus, activated MAIT cells can express markers both indicative of and contributory to their activation state.

Typically, the immune system works to clear infection and therefore the source of antigens. During persistent infections however, T cells receive repeated TCR stimulation. Repeated TCR stimulation over long periods of time pushes T cells beyond the state of activation, into a state of exhaustion. This exhausted state is characterized by the upregulation of inhibitory exhaustion molecules on the cell surface, as well the hindrance of a functional response[119]. One of the most well studied exhaustion markers is programmed cell death protein 1 (PD-1). The PD-1 gene has multiple regulatory elements, which possess binding sites for transcription factors activated by TCR signaling, such as NFAT[120]. When PD-1 binds to its ligands, either PD-L1 or PD-L2, it triggers a cascade of events leading to removal of phosphate groups from signaling molecules associated with the TCR[121]. These signaling molecules then lose their ability to transmit the TCR stimulation signal, resulting in impaired responses by the T cell[121]. PD-1 expression has been detected on MAIT cells in the context of bacterial and viral infections, and was associated with impaired cytokine production in response to stimulation[122,123]. Other markers of T cell exhaustion include B and T lymphocyte attenuator (BTLA), V-type immunoglobulin domain-containing suppressor of T cell

activation (VISTA), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), lymphocyte activation gene 3 (LAG3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and the co-inhibitory molecule cytotoxic T-lymphocyte antigen-4 (CTLA4). The molecular mechanisms of action associated with the molecules are not completely elucidated, but many of them have been found on MAIT cells from various disease states[122–125].

1.3.7 Antimicrobial Activity of MAIT Cells

In addition to the effects elicited by robust MAIT cell cytokine production, MAIT cells can also employ cytotoxic molecules to directly kill bacterially infected cells. Prior to activation, MAIT cells express granzyme A and granzyme K, which predominantly promote inflammation[126]. Once activated, MAIT cells can rapidly upregulate perforin and granzyme B[126]. These MAIT cells then degranulate, indicated by surface expression of the degranulation marker CD107A, and directly kill bacteria exposed cell lines[126,127]. Blocking MR1 can abolish this MAIT cell mediated cytotoxicity, indicating that TCR stimulation and recognition of target cells are required[126]. The cytotoxic effect of MAIT cells against *E. coli* exposed cells could be further boosted if PBMCs containing the MAIT cells were recently exposed to *E. coli*[126]. Similarly, exposure to IL-7 prior to encountering bacterially infected cells led to greater production of granzyme B and IFN-γ by MAIT cells[99]. These pre-armed MAIT cells could then detect even lows levels of bacteria, and directly kill bacterially infected cells[99]. Interestingly, though MAIT cells isolated from HIV-infected patients generally exhibit an ineffective cytotoxic response to stimuli, pre-exposure to IL-7 could partially restore their cytotoxic potential as well[99]. So, while resting MAIT cells can kill infected cells upon activation, pre-activation of MAIT cells can enhance this killing and may pose a therapeutic avenue to restore MAIT cell cytotoxicity in diverse disease contexts.

The antimicrobial protective activity of MAIT cells has also been observed *in vivo*. In addition to the protective effects exerted by MAIT cells upon IAV challenge in mice, MAIT cells are also important for the clearance of bladder infections in these animals[115,128]. Once mice were infected by bacteria in the bladder, MAIT cells were recruited and led to greater bacterial clearance than was observed in $MR1^{-/-}$ mice[128].

Given that MAIT cells are found in urine samples from patients with urinary tract infections, it has been proposed that MAIT cells may display a similar function in humans[128]. Akin to what was observed in bladder infections, MR1$^{-/-}$ mice fare worse than their MAIT competent counterparts upon infection with *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, and *Legionella longbeachae*[129–131]. In the case of *Legionella longbeachae* infection, protection could be boosted by increasing lung MAIT frequencies through instillation of 5-OP-RU prior to infection[130].

As mentioned in section 1.3.4, elucidating the exact role of MAIT cells in the context of human disease is more of a challenge. Most data are based on correlation between disease states and MAIT cell activation or frequencies, which cannot prove MAIT cell protective or pathogenic potential. Hospitalized IAV infected patients that recovered displayed higher MAIT cell frequencies than those who did not recover[116]. In patients experiencing IAV, HCV, or dengue virus infections, MAIT cells expressed activation markers[83]. MAIT cell activation during HCV infection correlated with improved outcomes, whereas the converse was observed during dengue virus infection[83]. During oral *Shigella dysenteriae* vaccination, patients who responded to the vaccine by producing IgA were also noted to possess activated MAIT cells, whereas MAIT cells from non-responders were not in a state of activation[127]. Again, in human cholera-infected patients, MAIT cells displayed an activated phenotype[132]. Furthermore, increases in MAIT cell frequencies over the course of infection correlated with class-switched antibodies against *Vibrio cholerae*[132]. MAIT cells therefore may be involved in the responses to diverse pathogens encountered in the human body, especially those that find themselves in MAIT cell rich tissues.

Given the propensity for MAIT cells to become activated in humans experiencing bacterial exposure in the intestinal tract, researchers are beginning to explore if MAIT cells may be involved in the response to *C. difficile*. While not explored *in vivo*, it has been shown *in vitro* that MAIT cells can respond to *C. difficile* in both a TCR-dependent and TCR-independent manner[82]. In response to *C. difficile*, MAIT cells displayed an activated phenotype characterized by CD69 expression, and quickly produced IFN-γ, granzyme B, and perforin[82]. Not only did MAIT cells produce granzyme B, they could

also release their cytotoxic granules, as indicated by surface expression of CD107A[82]. Intriguingly, MAIT cell responses were strongest against a hypervirulent strain of *C. difficile*, which induces greater cytokine production by APCs[82]. The response to the hypervirulent strain was stronger than the responses to less virulent strains that produced higher levels of riboflavin, demonstrating the importance of accompanying cytokine signaling for potent MAIT cell activation[82]. Given the mucosal tissue location of MAIT cells, coupled with their bacterial metabolite reactivity, it is possible they may also respond to *C. difficile in vivo* during infections.

MAIT cells are known to be found within the intestinal lamina propria and intraepithelial lymphocyte compartments in humans[74,133]. These MAIT cell populations exhibit surface expression of the activation marker CD69, as well as the exhaustion markers PD-1, LAG-3, and the co-inhibitory molecule CTLA4[133,134]. These MAIT cells were also capable of producing higher levels of IFN-γ, granzyme B, IL-17A, and TNF-α upon stimulation compared to blood MAIT cells[134]. It has therefore been posited that MAIT cells in the gut may receive some degree of constant stimulation from the healthy intestinal microbiota, and that the expression of exhaustion markers may be an attempt to dampen their responses[134]. Furthermore, the cell free supernatant from some commensal species dampens MAIT cell responses to bacterial and TCR-dependent stimulation[135]. This could be used to hinder MAIT cell responses to commensal species in healthy conditions, a facet that would be removed during CDI. Multiple prominent genera of commensal bacteria, such as *Bacteroides*, *Escherichia*, and *Faecalibacterium*, are known to include commensal species or strains capable of riboflavin production[136–139]. Many of these species could putatively signal to MAIT cells, and some have even been shown to trigger MAIT cell activation *in vitro*[139]. The fecal slurry used for FMT thus contains a variety of bacteria and bacterial metabolites that could serve to activate MAIT cells in the intestinal tract, or even recruit MAIT cells from the periphery to respond to the stimuli. Furthermore, in the case of irritable bowel disease, MAIT cell frequencies are reduced in the blood but elevated in the inflamed intestinal tissue[133]. The inflammation induced by *C. difficile* infection, and the subsequent influx of microbial metabolites associated with FMT, could feasibly elicit similar MAIT cell migration.

1.4 Rationale, Hypothesis, and Specific Objectives

Despite the increasing use of FMT, and the developing understanding of the role the microbiota plays in the resolution of CDI, the interaction of FMT with the immune system has not been largely explored. Given that MAIT cells are bacterial metabolite-reactive and home to mucosal tissues, I posited that they are in a perfect position to respond to the influx of microbes and microbial metabolites associated with FMT. Interestingly, no one has yet examined human MAIT cells in the context of FMT to treat rCDI although FMT may tap into the innate biological function of these cells. With this in mind, I set out to examine the phenotypic and functional changes in peripheral blood MAIT cells of human rCDI patients pre- and post-FMT. This pilot study therefore sought to elucidate the impact of FMT on the immune response by MAIT cells, in addition to broadening our understanding of this poorly understood T cell subset.

It was hypothesized that FMT would result in altered MAIT cell frequencies and mucosal homing marker expression in the peripheral blood, potentially due to MAIT cell movement to the intestinal tract. It was also predicted that following FMT, MAIT cells would display altered levels of activation and exhaustion markers, increased proinflammatory cytokine production, and an altered MAIT-1/17 skew. My objectives therefore fell into two overarching aims:

1. To examine the frequency and mucosal homing marker expression of MAIT cells pre-FMT, compared with 1-week and 1-month post-FMT in rCDI patients. Alteration in these factors could act as a proxy indicating MAIT cells migrate through the intestinal tract.

2. To investigate the impact of FMT on MAIT cell phenotypes and functional capacity.

    i. Elucidation of MAIT cell activation and exhaustion marker expression pre- and post-FMT. FMT may activate MAIT cells and push them to the point of exhaustion, or conversely could rescue MAIT cells from a state of exhaustion.

ii. Exploration of cytokine production by MAIT cells pre- and post-FMT, to determine if FMT alters either the production capacity or the skew of which cytokines are made.

iii. Examination of MAIT-1 and MAIT-17 transcription factor skew pre- and post-FMT to determine if FMT changes the polarization of MAIT cells.

Chapter 2

2 Methods

Some of the listed procedures began with FMT patient #4 or #5. Due to COVID-19 lockdowns, we had time to consider informative additions to the study. When patient enrollment began again, we implemented these additions to further understand the changes occurring in the MAIT cell population associated with FMT.

2.1 Human Ethics Approval and Data Collection

All experiments performed using human blood were done in accordance with our Research Ethics Board (REB) approved protocol #114353 (Appendix 1). This protocol was approved through both Western University and Lawson Health Research Institute (ReDa ID: 7898). Patients enrolled in the study had recurrent *C. difficile* infections which had not been resolved by antibiotics. These patients finished their final course of antibiotics, typically vancomycin, 24 hours before coming to the clinic for their FMT procedure. Prior to the procedure, we drew 50 mL of blood into sodium heparin containing tubes, representing the patient's pre-FMT timepoint. The FMT would then be administered using either capsules or a fecal enema, the choice was up to the patient as both are efficacious. The enema route was given as two treatments one-week apart, while the capsules were given in a single clinic visit. Therefore, one week after whenever the final FMT treatment was given, we collected the 1-week post-FMT blood draw. This blood draw was again 50 mL. The patient then returned one month after their final FMT treatment for us to draw 50 mL of blood, representing their 1-month post-FMT timepoint.

The inclusion criteria for the study were consenting patients over 18 years of age, who were undergoing treatment at the Infectious Disease Care Program (St. Joseph's Health Centre, London) for recurrent *C. difficile* infections. The exclusion criteria for the study included patients on broad-immunomodulating drugs, those who had surgery within the last two weeks, and those with current or past cancer. Patient follow-up blood draws would also not be taken if the patient relapsed back to active *C. difficile* infection prior to the blood draw occurring. Unfortunately, a few patients were also lost for the one-month

follow-up during the initial COVID-19 lockdowns as we followed provincial and federal guidelines.

Patients were compensated for their participation as approved by the REB, to help cover any travel or parking costs associated with coming in for the blood draws. The letter of information and consent form given to each patient outlined the study goals in accessible language and informed them that they could withdraw from the study at any time without penalty (Appendix 2).

The data collection form for the study allowed us to collect information including gender, body mass index (BMI), and the date of birth of the patient as these could be useful in looking for correlations with different immune patterns (Appendix 3). Information regarding their comorbidities and recurrent *C. difficile* diagnosis/ history were also included, along with the administration method of FMT they selected. We also collected information on patient sex. A summary of patient characteristics is presented in Table 1.

Table 1. FMT Recipients' Characteristics

Patient Number	Sex	BMI	FMT Type	Samples Collected
Patient #1	Male	27.3	Capsules	Pre-FMT
Patient #2	Male	Not Available	Enema	Pre-FMT, 1-week post-FMT
Patient #3	Female	30.9	Capsules	Pre-FMT, 1-week post-FMT
Patient #4	Female	24.3	Capsules	All timepoints
Patient #5	Female	23.5	Capsules	All timepoints

2.2 Cell Culture and Stimulation Reagents

2.2.1 Cell Culture and Equipment

The C1R wild type (WT) cell line was gifted to us by Dr. Jose Villadangos (University of Melbourne, Australia). This cell line is of B cell origin and was transformed by Epstein-Barr Virus[145]. C1R WT cells express MR1 and can take up ligands like 5-OP-RU for presentation to MAIT cells. These cells were cultured in 175-cm^2 flasks (Thermo Fisher) in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 100 µg/mL streptomycin and 100 units/mL penicillin, 1x GlutaMax-I, and 10% FBS. The C1R WT cells were thawed four days prior to FMT patient sample arrival and reached close to confluence by the time the sample arrived.

All incubations took place in a HERACELL 150 incubator (Thermo) with 6% CO_2 at 37°C. All centrifugation was performed using an Allegra 6R centrifuge (Beckman Coulter) for 4 minutes at 456 x g and 4°C unless otherwise stated. All washes used this spin setting.

2.2.2 Stimulation Reagents

For *in vitro* stimulation conditions involving IL-12 and IL-18, recombinant human IL-18 (MBL) and recombinant human IL-12 p70 (Peprotech) were each used at a final concentration of 5 ng/mL.

5-A-RU was graciously provided by Dr. Olivier Lantz (Institut Curie, France). Two mM of 5-A-RU was mixed with 2 mM of methylglyoxal (Sigma) in the presence of dimethyl sulfoxide (DMSO, Sigma) and set at room temperature for 24 hours to produce 5-OP-RU. The 5-OP-RU was subsequently stored in aliquots at -80°C until needed.

2.3 *Clostridioides difficile* lysate generation

Clostridioides difficile strains were generously grown and provided to us by Dr. Jeremy Burton (Lawson Health Research Institute, Ontario). To protect donor identity for human isolates, the strains were given the de-identified designations of strain #1, strain #2, and strain #3. Each strain was diluted in Dulbecco’s phosphate-buffered saline (PBS, Sigma)

to an OD_{600} = 2 as verified by a SmartSpec 3000 spectrophotometer (Bio-Rad). Each strain was then subjected to 30,000 pounds per square inch (PSI) of pressure using a cell crusher (CF1 TS Series, Constant Systems Ltd.) to produce lysate. Dr. Carole Creuzenet (Western University, Ontario) allowed me to use her cell crusher, and her student Matthew Myles taught me how to operate it. Following the production of the lysate, the resulting solution was filter-sterilized through a 0.2 µm Nalgene filter (Thermo Fisher). The lysate was then aliquoted and stored at -80°C until needed.

2.4 Human Blood Processing

2.4.1 Peripheral Blood Mononuclear Cell Isolation

To isolate PBMCs from whole patient blood, the blood was first diluted 1:2 in PBS. Next, 15 mL of Ficoll Paque Plus (GE Healthcare) was put into the bottom of each SepMate Tube (StemCell). The diluted blood was slowly layered on top of the Ficoll. The tubes were then centrifuged at 1200 x g for 10 minutes at room temperature with the brake on. Once the spin was complete, plasma aliquots (6 x 1 mL) were collected from the top of the tube. The remaining liquid was transferred to a fresh 50 mL Falcon tube (FroggaBio) by inverting the SepMate tube for 2 seconds. This 50 mL Falcon tube was then spun for an additional 10 minutes at 460 x g, room temperature, with the brake on. The supernatant was aspirated, and the PBMCs resuspended for cell counting using a hemocytometer. The resuspension liquid was complete Roswell Park Memorial Institute medium (cRPMI). This was composed of RPMI 1640 (Gibco) with 1 mM sodium pyruvate (Gibco), 0.1 mM minimum essential medium non-essential amino acids solution (Gibco), 10 mM HEPES (Gibco), 100 µg/mL streptomycin and 100 units/mL penicillin (Gibco), 1 mM sodium pyruvate (Gibco), 1x GlutaMax-I (Gibco), and 10% heat inactivated fetal bovine serum (FBS, Gibco).

2.4.2 Freezing Cells

Two vials of cells each containing 5-10 million PBMCs were frozen for each patient, starting with patient 4. The PBMCs were spun down and resuspended in 90 % FBS + 10% DMSO at a concentration of 5-10 million cells/ mL. These vials were then placed in the Mr. Frosty (Thermo Fisher) which was moved to the -80°C freezer, as the Mr. Frosty

helps slowly bring the cells down to temperature. Once frozen, cells were transferred to storage in liquid nitrogen.

One to four million cells from each patient, starting with patient 4, were preserved in RNA Later for potential transcriptome analysis. The number of cells frozen depended on cell recovery from the patient's blood. The cells were spun down and resuspended in 5 parts RNA Later (Thermo Fisher). They were then placed in the 4°C fridge overnight. In the morning, the cells were again spun down, and the supernatant removed. The cell pellets were then stored in the -80°C freezer.

2.5 Cytofluorimetric Analyses

2.5.1 Equipment and Software

Flow cytometry was performed using a FACSCantoII (Becton Dickinson) located in the Haeryfar lab. The software that allowed data collection and control of cytometer settings was the FACSDiva software version 6 (Becton Dickinson). Compensation was performed for each panel using the OneComp eBeads (Thermo Fisher), with one tube made for each antibody or tetramer color present on the panel. To compensate for the viability dye, either 7-AAD (Thermo Fisher) or Fixable Viability Dye eFluor780 (FVD, Thermo Fisher), 300,000 cells from the patient sample were stained according to the protocol listed in section 2.5.2.

The FACSDiva software could then calculate and apply the compensation to each of the sample tubes prior to acquisition. Following data acquisition, FCS files were exported from FACSDiva and transferred onto a personal computer for analysis using FlowJo version 10.

2.5.2 Surface Staining

All washes in this and subsequent sections were performed using 200 µL of the listed buffer. All isotype controls were used at the same µg amount as the antibody they controlled for on a panel. MR1 tetramers, composed of four MR1 units linked to a fluorophore, loaded with 5-OP-RU were used to identify MAIT cells. The TCR of MAIT cells readily recognizes 5-OP-RU in the context of MR1, and this is a widely accepted

way to identify MAIT cells[78]. The negative control for the 5-OP-RU loaded MR1 tetramer is MR1 tetramer loaded with the vitamin B9 derivative 6-FP. The MAIT cell TCR largely does not stably bind 6-FP in the context of MR1[146]. Gating was therefore performed based on 6-FP and isotype controls, as appropriate. For tetramer gating strategy see Figure 2. For isotype gating strategy see Figure 3.

For cell surface staining, including tetramer staining, 0.5-1 million cells were pipetted into wells of a 96-well plate, then washed twice with PBS. FVD was diluted 1:1000 in PBS and 100 µL of this diluted dye was added to each of the wells. The cells were then incubated for 30 minutes at 4°C.

Following the incubation, the cells were washed twice with PBS + 2% FBS. Each well was then given the appropriate antibody cocktail, isotype control, or 6-FP tetramer control (Table 2) in 100 µL of PBS + 2% FBS. The plates were next incubated for 25 minutes at room temperature in the dark. Once the incubation was complete, the cells were washed twice with PBS + 2% FBS, and then resuspended in 150 µL of PBS + 2% FBS to read on the cytometer.

The only exception to receiving FVD was Activation Panel 1, which instead received 7-AAD viability dye due to color constraints based on the other antibody colors on the panel. 7-AAD was applied to Panel 1 after the surface stain, with 5 µL of 7-AAD being added per 100 µL of cells suspended in PBS + 2% FBS. These cells incubated for five minutes at room temperature and then were read on the cytometer.

Staining for the α4β7 integrin and CCR6 began with patient #5.

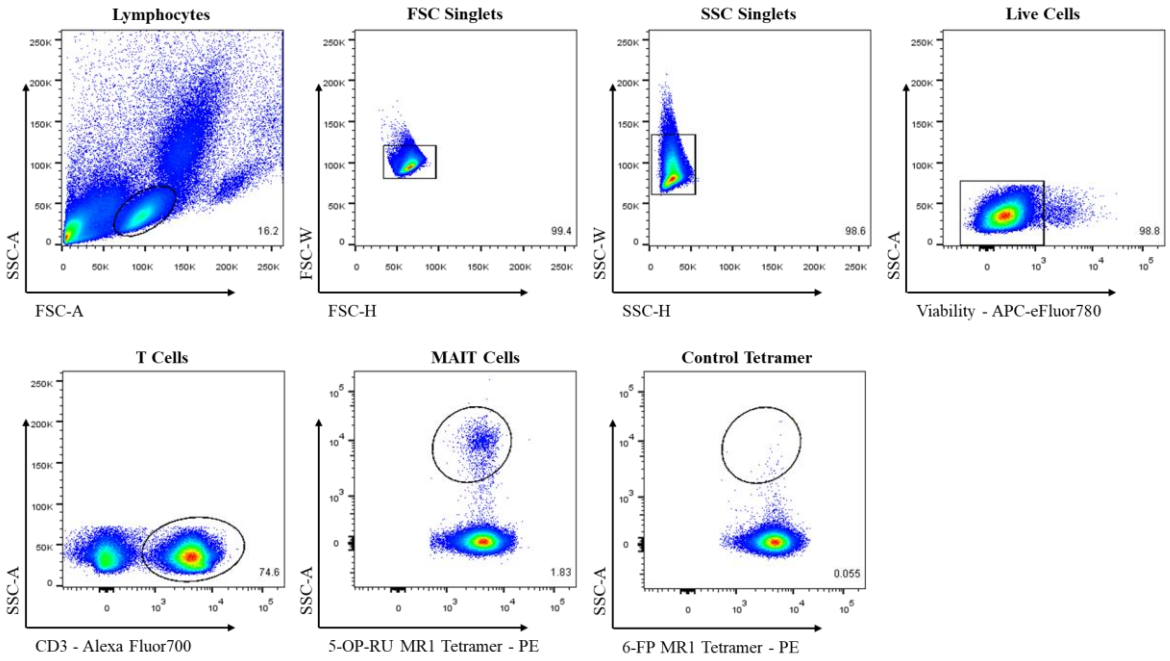


Figure 2. Gating Strategy to Identify MAIT Cells. Lymphocytes were gated on, then forward and side scatter doublets were excluded. Live cells were next identified, followed by T cells. MAIT cells were identified from the T cell population using MR1 tetramer loaded with 5-OP-RU. For reference, the negative control MR1 tetramer loaded with 6-FP is also included, as this was how gates were set. SSC indicates side scatter, FSC indicates forward scatter, the suffix -A denotes area, -H height, and -W width.

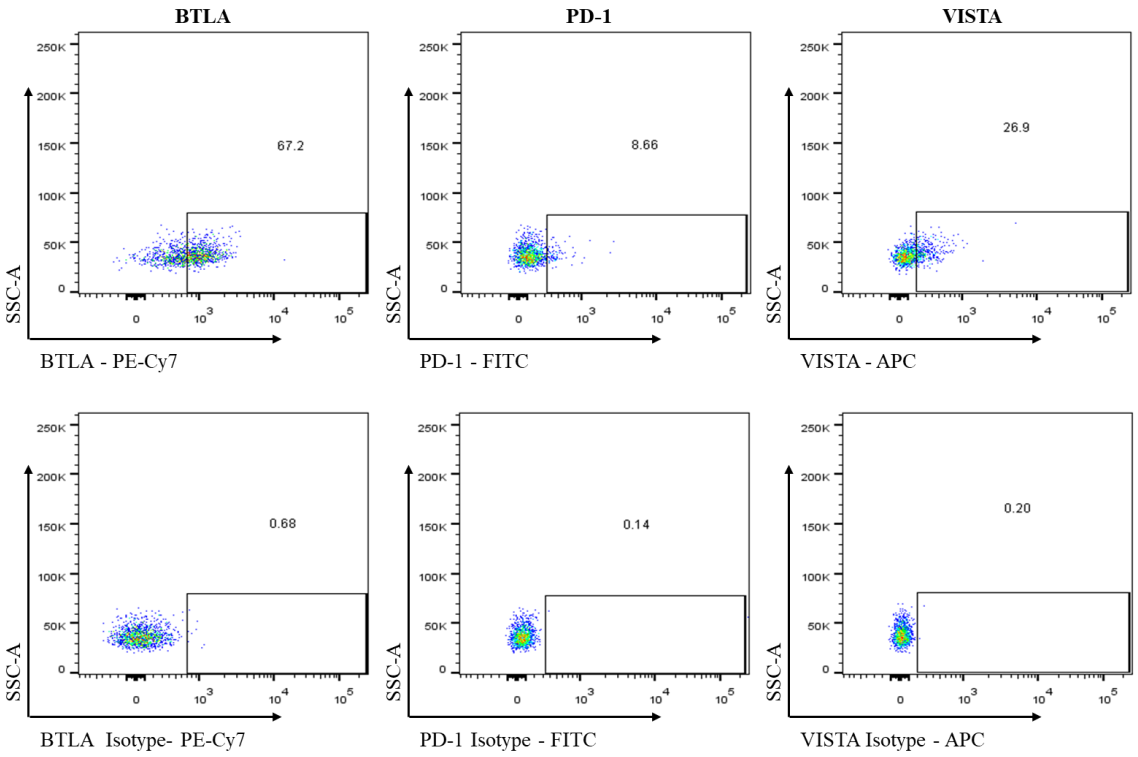


Figure 3. Gating Strategy for Surface Markers Based on Isotype Controls. Following gating on MAIT cells (Figure 2), the expression of the indicated surface markers on MAIT cells was determined based on the isotype control. The top row shows the antibodies which bind to the given marker, whereas the bottom row shows the isotype control matching each marker.

Table 2. Viability Dyes and Fluorophore-Labelled Antibodies Used

Target or Purpose	Manufacturer	Clone	Dilution or µg per test	Fluorophore	Panel used on
CD3	eBioscience	UCHT1	0.1	APC-eFluor780	Activation Panel 1
CD3	eBioscience	UCHT1	0.1	Alexa Fluor700	All panels except activation panel 1
CD4	eBioscience	RPA-T4	0.05	Alexa Fluor700	Activation Panel 1
CD8	eBioscience	SK1	0.025	FITC	Activation Panel 1
CD69	eBioscience	FN50	0.015	PE-Cyanine7	Activation Panel 1
CD38	eBioscience	HIT2	0.125	APC	Activation Panel 1
PD-1	eBioscience	MIH4	1	FITC	Exhaustion Panel 1
TIM3	Invitrogen	F38-2E2	0.03	APC	Exhaustion Panel 1
TIGIT	eBioscience	MBSA 43	0.125	PerCP-eFluor 710	Exhaustion Panel 1

BTLA	BioLegend	MIH26	0.75	PE-Cyanine7	Exhaustion Panel 1
CTLA4	eBioscience	14D3	0.03	PE-Cyanine7	Exhaustion Panel 2
VISTA	eBioscience	B7H5DS8	0.125	APC	Exhaustion Panel 2
LAG-3	eBioscience	3DS223H	0.0625	PE-eFluor 610	Exhaustion Panel 2
CCR6 (CD196)	eBioscience	R6H1	0.125	PerCP-eFluor 710	Exhaustion Panel 2
α4β7 integrin (LPAM-1)	R & D Systems	Hu117	0.5	Alexa Fluor 488	Exhaustion Panel 2
IFN-γ	eBioscience	4S.B3	0.1	PE-Cyanine7	Transcription Factor Panel, ICS Panel #1
TNF-α	eBioscience	MAb11	0.075	PE-eFluor 610	ICS Panel #1
IL-17A	eBioscience	eBio64DEC17	0.037	PerCP-Cyanine5.5	Transcription Factor Panel, ICS Panel #1
Granzyme B	Invitrogen	351927	Unlisted-used 5 µL per test	Alexa Fluor 488	ICS Panel #1
IL-13	eBioscience	85BRD	0.25	FITC	ICS Panel #2

IL-4	eBioscience	8D4-8	0.25	PE-Cyanine7	ICS Panel #2
IL-2	eBioscience	MQ1-17H12	0.0625	PE-eFluor 610	ICS Panel #2
RORγT	eBioscience	AFKJS-9	1	APC	Transcription Factor Panel
T-bet	BioLegend	4B10	1	FITC	Transcription Factor Panel
GATA3	eBioscience	TWAJ	0.03	PE-eFluor 610	Transcription Factor Panel
CD107A	Invitrogen	H4A3	Unlisted-used 0.5 µL per test	APC	ICS Panels 1, 2
MR1 Tetramer	NIH Tetramer Core Facility	N/A	0.65	PE	All panels
FVD	eBioscience	N/A	1:1000	eFluor 780	All except Activation Panel
7-AAD	eBioscience	N/A	1:20	Same Channel as PerCP-eFluor 710	Activation Panel

Abbreviations: In this table, APC denotes a fluorophore not an antigen-presenting cell.

2.5.3 Stimulation Conditions and Intracellular Staining

For *in vitro* stimulation of human PBMCs, cells were cultured in cRPMI. All stimulations lasted 24 hours in the incubator at 37°C with 6% CO_2. Prior to us acquiring C1R WT cells, the 5-OP-RU stimulation condition had used 5-OP-RU ligand alone (Patients #1, 2). For antibodies with unlisted concentrations on the product website (Table 2), either the recommended microliter amount or a microliter amount that had previously been verified to work in our setup was utilized.

First, the freshly isolated human PBMCs were resuspended in cRPMI and 1 million cells were added into the wells of a round-bottom 96-well plate. The stimulation conditions were as follows: C1R 5-OP-RU, IL-12 + IL-18, C1R 5-OP-RU + IL-12 + IL-18, and a 1:10 dilution of *C. difficile* strain #3. The appropriate media, 6-FP loaded MR1 tetramer, and isotype controls were included to help set gates for different markers and populations during analysis. For the wells receiving the C1R 5-OP-RU condition, 250,000 C1R WT cells were added to each well along with 5-OP-RU at a final concentration of 2 nM. For the IL-12 + IL-18 wells, each cytokine was added to a final concentration of 5 ng/mL. To prepare the 1:10 dilution of *C. difficile* lysate, each well received 20 µL of lysate. All wells were topped up with cRPMI to a final volume of 200 µL. Each of the aforementioned conditions with controls were repeated twice in the plate, as we had two separate staining panels for the intracellular cytokine staining (ICS) starting with patient #4.

Once the stimulations were added, the plate was placed in the incubator for 18 hours. At the 18-hour mark, 10 µg/mL of Brefeldin A (BFA, Sigma), 2 µM monensin (BioLegend), and APC-conjugated anti-CD107A (1:400) or the appropriate isotype were added to each well. BFA and monensin inhibit protein secretion and therefore allow cytokines to accumulate in the cells[147]. The plates were then returned to the incubator for another 6 hours.

Twenty-four hours after the stimulation had begun, the plate was removed from the incubator for staining. The cells were spun down, and the surface stains performed as previously described. The cells were then washed twice with PBS, and fixed using 100

µL IC Fixation Buffer (eBioscience). Cells were incubated in the fixation buffer for 30 minutes at room temperature in the dark. During this time, 10x Permeabilization Buffer (eBioscience) was diluted to 1x working concentration. Once the incubation was complete, cells were washed twice with the 1x Permeabilization Buffer. The cells were then stained using antibody cocktails for the desired intracellular markers (Table 2) in 100 µL of Permeabilization Buffer. This incubation was performed for 30 minutes in the dark at room temperature. Finally, the cells were washed twice with PBS + 2% FBS, before being resuspended in 150 µL of PBS + 2% FBS to read. Gating for the 24-hour stimulation was based on a media control, and a 6-FP control tetramer. An isotype was used to set the gate for CD107A.

For transcription factor staining, PBMCs were used without any *in vitro* stimulation. The surface stains were first performed using the methods outlined in section 2.5.2. During this time, 1 part Foxp3 Transcription Factor Fixation/Permeabilization Concentrate was added to 3 parts Foxp3 Transcription Factor Fixation/Permeabilization Diluent per the manufacturer's instructions (eBioscience). Once the surface staining was complete, the cells were washed twice with PBS and then resuspended in 200 µL of the Foxp3 Fixation/Permeabilization working solution. The cells were incubated in this solution for 30 minutes at 4°C, then washed twice with 1x Permeabilization Buffer (as mentioned above). Finally, the cells were stained using antibody cocktails for the desired intracellular markers (Table 2) in 100 µL of Permeabilization Buffer for 30 minutes at room temperature in the dark. When this was complete, the cells were washed twice with PBS + 2% FBS, before being resuspended in 150 µL of PBS + 2% FBS to read.

Staining for GATA3, IL-2, IL-4, and IL-13 began with patient #4.

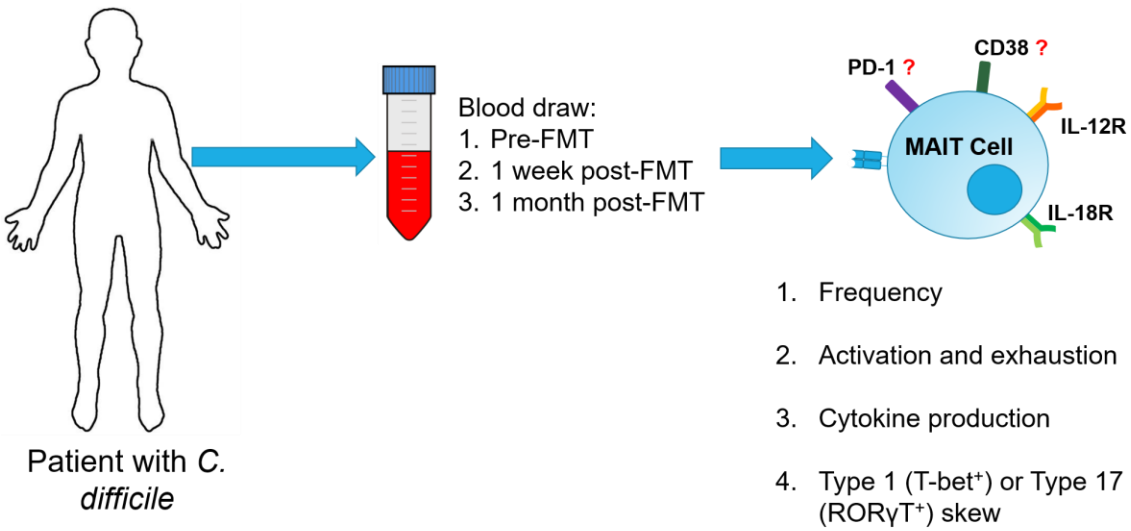


Figure 4. Schematic Overview of Sample Acquisition and Processing. Patients with rCDI completed their final course of antibiotics prior to coming into St. Joseph's hospital for their FMT. 50 mL of blood was drawn at three timepoints: prior to FMT, 1-week post-FMT, and 1-month post-FMT. From this blood, PBMCs were isolated. These cells were then stained to examine the MAIT cell frequency and MAIT cell expression of mucosal homing markers, activation and exhaustion markers, and Type 1 and Type 17 transcription factors using flow cytometry. PBMCs were also stimulated for 24 hours using a variety of stimuli, and MAIT cell cytokine production was assessed. The MAIT cell phenotypes and functional capacities were then compared across timepoints.

2.6 Statistical Analyses

Statistical analyses and graphs, with the exception of the principal component analysis, were produced using GraphPad Prism versions 8 and 9. Results were plotted with each circle of the same color representing a different patient at that timepoint, and lines connecting the dots for samples from the same patient over time. Being that the pre-FMT and 1-week post-FMT groups were the only ones with enough samples to calculate statistics, all comparisons were made between these groups. Normality was not assumed, considering the inherent variability in the population and the low n number.

2.6.1 R Code Process for Principal Component Analysis

The principal component analysis (PCA) was performed using RStudio and R x 64 4.0.4. Git was also needed to install the appropriate packages. The packages required for the analysis included devtools and ggbiplot. In order to make the correct argument modifiers in the R code, background was needed to understand what a PCA fundamentally did. To do this, the website Built In provided an explanation[148]. To code the PCA in R, a PCA coding tutorial from Datacamp was used as it helped lay the framework for what the code and adjustments meant[149]. For the code, see Appendix 4. The PCA was run using the frequency of MAIT cells expressing each marker from each patient pre-FMT, 1-week post-FMT, and 1-month post-FMT. The markers used in the analyses are shown in the figure at the end of each vector of the loading plot.

As the website Built In explained it, a PCA is reducing the dimensionality of the data so we can see its shape along the new axes (the principal components). The first thing I did to the PCA code was scale/ standardize my data. Since the PCA looks at variance, cell markers that had larger value ranges would be weighted more heavily and the importance of cell markers that changed but did so within a narrower range of values would be lost. Scaling the data puts all of these markers on the same scale. While this may sound counterintuitive, a fairly recent publication in Nature regarding methods to analyze multicolor flow cytometry data did explain the reasoning[150]. Variability in the expression of different markers can occur due to natural variability in the expression of these markers on cells, but it can also be contributed to by differences in the efficiency of the

fluorophores to convert the laser light into emitted light (quantum yield)[150]. Scaling the data therefore seems to be a widely accepted and practiced data pre-processing step, even for flow data which exists in the same units (in this case percentage). I also centered my data, which is the default argument in the R code and seems to align with what is accepted in the field. This setting centers the data around zero, giving a common point from which all points go[150,151]. Now because I was analyzing each FMT patient's expression of 13 different markers, each patient would have a point in 13-dimensional space that represented their expression of all the markers simultaneously. This is extremely hard to visualize. Briefly, the PCA therefore finds a new line (eigenvector of the covariance matrix) that maximizes the variance of the points around it, and accounts for a certain amount of the total variance of the dataset. This line becomes principal component one (PC1), the new x axis, and the FMT patient points will have a position along it. The next principal component accounts for the next highest amount of variance (PC2) and is perpendicular to PC1. It can therefore be the new y axis, and the patient data points will all have a position along it too. Differences are more meaningful along PC1 than PC2. The PCA keeps doing this until it has made enough lines to account for all the variance in the data. Realistically, the first two account for the majority of the variance in most cases, so just having a new graph in two dimensions (instead of including the other principal components as dimensions) is commonly used. The values along the new principal component axes don't really have a unit or meaning, since the principal component is kind of accounting for a combination of the initial variables.

The vectors on the plot that are each labelled with the marker they represent are collectively referred to as a loading plot[152]. The direction of each vector, and the marker it represents, shows how it influenced the principal components[152]. For instance, vectors that are more horizontal on the plot had more influence on the new X axis, PC1. Vectors that are more vertical on the plot had a greater influence on the development of PC2. If two vectors have a small angle between them, they are positively correlated, though not necessarily significantly so[152]. An angle closer to 180° in contrast suggests a negative correlation between the two markers[152].

Chapter 3

3 Results

3.1 MAIT Cell Frequency Does Not Reproducibly Change Following FMT

The first aim of my study was to determine if there is a change in the frequency of MAIT cells in the peripheral blood of patients pre-FMT compared with post-FMT. This could be used as a proxy to suggest potential MAIT cell migration to the intestinal tract in response to FMT. In order to address this aim, MAIT cell frequencies in patient PBMCs were assessed using MR1 tetramer staining. The frequency of MAIT cells, out of total $CD3^+$ cells, did not significantly differ in patients pre-FMT compared with 1-week post-FMT (Figure 5). Two patients exhibited a decrease in their frequency of MAIT cells in the peripheral blood 1-week post-FMT, whereas the other two displayed either stable or slightly increased MAIT frequencies. Therefore, there does not appear to be a consistent trend with respect to changes in MAIT cell frequencies following FMT. The variability in the frequencies of MAIT cells observed between patients was expected, considering the natural variability in MAIT cell frequencies in the population at large[76,78].

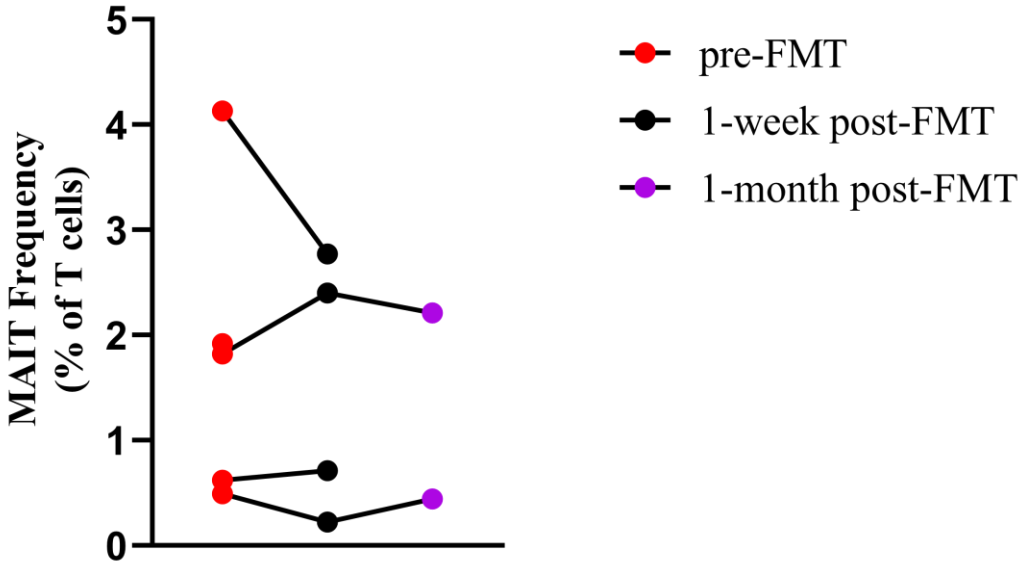


Figure 5. MAIT Cell Frequencies Do Not Reproducibly Change in Patients Pre- vs Post-FMT. Peripheral blood was collected from patients pre-FMT, 1-week post-FMT, and 1-month post-FMT as indicated. Peripheral blood mononuclear cells were isolated, stained, and analyzed using flow cytometry. Following live gating on $CD3^+$ events, the frequency of MAIT cells as MR1 $tetramer^+$ cells was determined. Lines connect samples from the same patient over time. Pre-FMT n=5, 1-week post-FMT n=4, 1-month post-FMT n=2. A Wilcoxon matched-pairs signed rank test, which was used to assess significant differences between the pre-FMT and 1-week post-FMT timepoints, yielded no statistically different results.

3.2 MAIT Cells' Expression of Intestinal-Homing Markers

The second aspect of Aim 1 was to examine MAIT cells' expression of intestinal homing markers pre- and post-FMT. Changes in the expression of these markers could further inform and strengthen interpretations of MAIT cell migration to the intestinal tract following FMT. Since this was a relatively recent undertaking, only one patient was examined for the expression of the intestinal homing markers CCR6 and the α4β7 integrin on MAIT cells[76–78,80,81] (Figure 6). In this patient, most MAIT cells expressed the α4β7 integrin, with a slight dip in the frequency of $\alpha4\beta7^+$ MAIT cells 1-week post-FMT. The same trend was observed for the geometric mean fluorescence intensity (gMFI) of the α4β7 staining on MAIT cells, indicating a decrease in the expression of this marker on a per cell basis. MAIT cell expression of CCR6, however, displayed a slightly different trend. Following FMT, the frequency of $CCR6^+$ MAIT cells decreased. Conversely, the gMFI for CCR6 trended towards an increase. Therefore, while less MAIT cells seemed to express CCR6 following FMT in this patient, the expression of CCR6 on a per cell basis trended in the opposite way. It is not possible to draw any strong conclusions from one patient however, though it will be interesting to see if this trend is replicated as more patients will enroll in this study in the future.

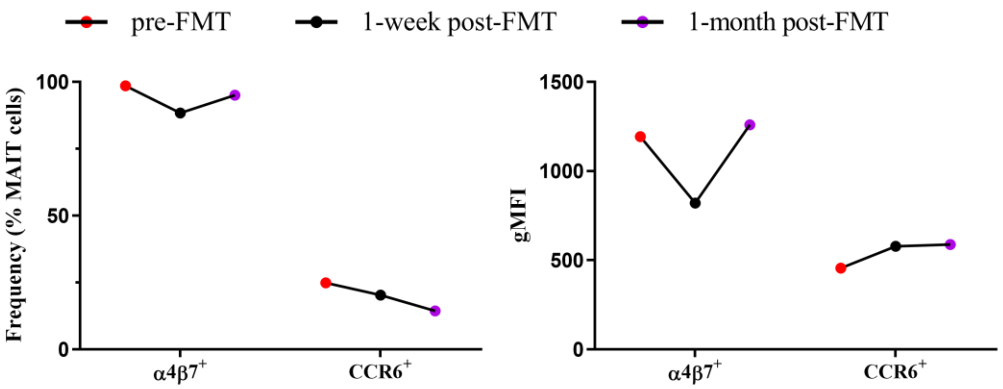


Figure 6. Variation in MAIT Cell Mucosal Homing Markers Pre- and Post-FMT. Peripheral blood was collected from a patient pre-FMT, 1-week post-FMT, and 1-month post-FMT. Peripheral blood mononuclear cells were isolated, stained, and analyzed using flow cytometry. Following gating on MAIT cells, the frequency of MAIT cells expressing each marker and the gMFI of the markers were determined. Lines connect samples from the same patient over time. Pre-FMT n=1, 1-week post-FMT n=1, 1-month post-FMT n=1. Statistical significance could not be assessed from only one patient.

3.3 MAIT Cells Display Altered Levels of Activation and Exhaustion Markers Following FMT

To address the second Aim of the study, expression of activation and exhaustion markers on MAIT cells was examined pre- and post-FMT. The frequency of MAIT cells expressing the exhaustion markers BTLA, PD-1, and VISTA appeared elevated in pre- and post-FMT patients (Figure 7A). 1-week post-FMT, the frequency of $BTLA^+$ or $PD\text{-}1^+$ MAIT cells trended towards a decrease for most patients (Figure 7A). While the gMFI of BTLA in pre- and post-FMT patient MAIT cells did not display a consistent trend, the expression of PD-1 on a per cell basis remained largely unchanged between the timepoints (Figure 7A). In contrast to what was observed for the exhaustion markers BTLA and PD-1, the frequency of MAIT cells expressing VISTA 1-week post-FMT trended towards an increase, as did the gMFI of staining for VISTA (Figure 7A). Given the low number of patients enrolled and the disparate responses observed at the 1-month post-FMT timepoint, it is difficult to draw strong conclusions from what happens to the MAIT cell exhaustion then. However, it appears that 1-week post-FMT, the frequency of phenotypically exhausted MAIT cells trends towards a decrease in most patients, though some exhaustion markers are more persistent. While the exhaustion markers TIM3, LAG3, TIGIT, and the co-inhibitory molecule CTLA4 were also examined on MAIT cells pre- and post-FMT, the expression of these markers was not notable in most patients (Figure 7A, C).

In conjunction with determining the expression of exhaustion markers on MAIT cells pre- and post-FMT, MAIT cells' expression of the activation markers CD38 and CD69 was also examined. Most patients displayed either a plateau or decrease in $CD38^+$ or $CD69^+$ MAIT cells at 1-week post-FMT and 1-month post-FMT compared with the pre-FMT timepoint (Figure 7B). The same trend was found when comparing the 1-week and 1-month post-FMT timepoints (Figure 7B). The aforementioned trend was not observed on a per cell basis, where phenotypes were more variable, and no apparent trend emerged (Figure 7B). Similar to what was observed with the frequency of MAIT cells expressing exhaustion markers, post-FMT MAIT cells may slightly move away from their formerly activated state.

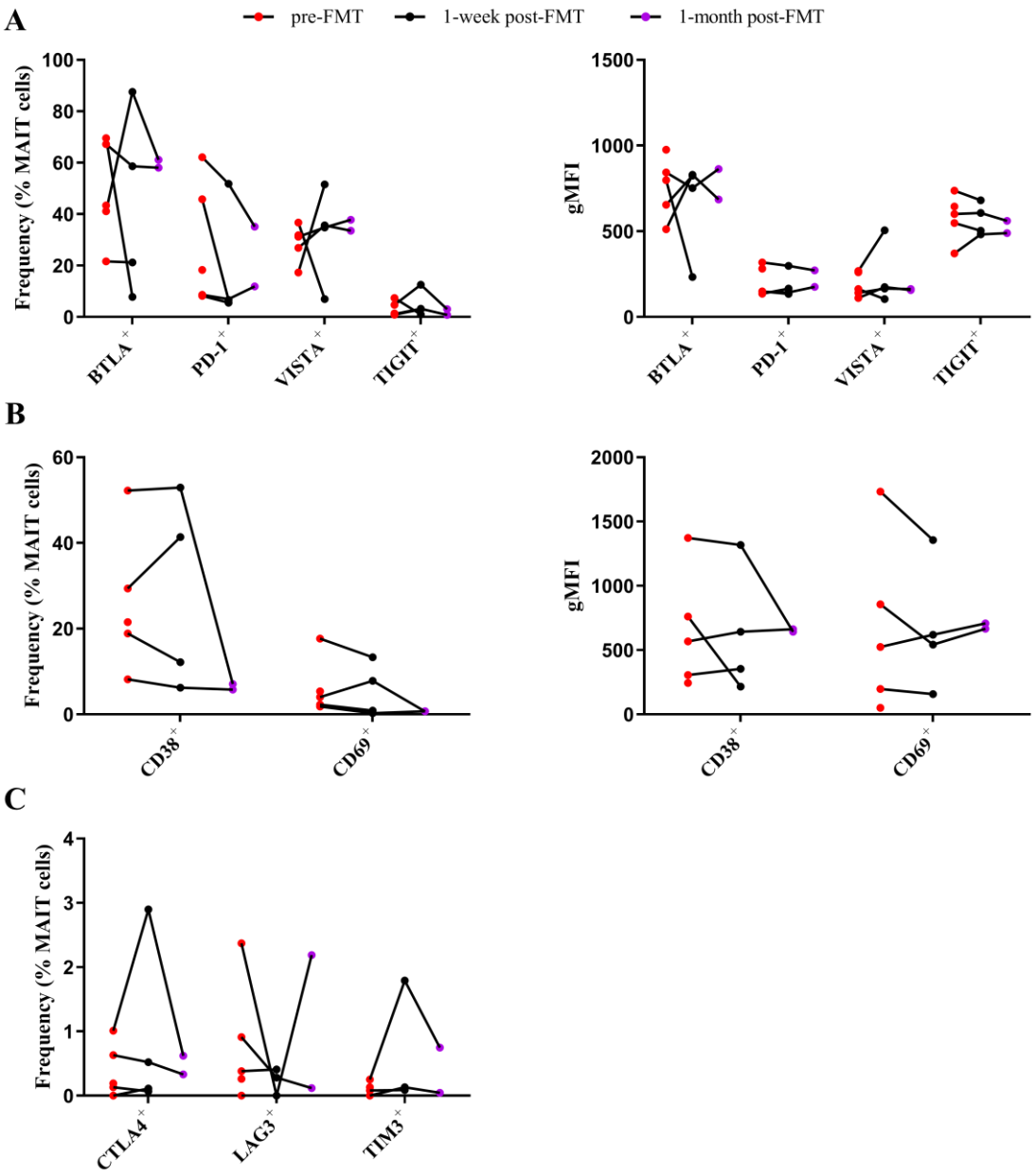


Figure 7. MAIT Cells' Expression of Activation and Exhaustion Markers Changes Following FMT. Peripheral blood was collected from patients pre-FMT, 1-week post-FMT, and again 1-month post-FMT. Peripheral blood mononuclear cells were isolated, stained, and analyzed using flow cytometry. Following gating on MAIT cells, the frequency of MAIT cells expressing each marker and the gMFI of the markers were determined. Lines connect samples from the same patient over time. Pre-FMT n=5, 1-week post-FMT n=4, 1-month post-FMT n=2. Statistical significance was examined

using multiple Wilcoxon matched-pairs signed rank tests with corrections for multiple comparisons using the Holm-Šídák method. No statistically significant changes were observed.

3.4 FMT May Help Restore MAIT Cells' Cytokine Production Capacity

Continuing to address the second Aim of our study, I sought to determine if FMT changes not only the cytokine-producing capacity, but also the skew of which cytokines are produced, by MAIT cells. Given the phenotypic exhaustion observed on the surface of the MAIT cells, determining their cytokine producing capacity also gave further insight into how functionally exhausted they were. First, I attempted to stimulate PBMCs, containing MAIT cells, from two pre-FMT patients using 5-OP-RU ligand alone (Figure 8A). This ligand is known to be able to activate MAIT cells in a TCR-dependent manner[103]. We did not observe cytokine production by the FMT patient MAIT cells in response to 5-OP-RU ligand (Figure 8A). Riboflavin derivatives such as 5-OP-RU are inherently unstable and prone to cyclization, which likely hindered the use of the ligand alone to activate MAIT cells in culture[90]. In order to address this issue, moving forward C1R WT cells were also added to the culture with 5-OP-RU. This cell line expresses MR1 and captures antigens for presentation to MAIT cells, helping to solve the instability issue with rapid ligand uptake[91].

The use of C1R WT cells as APCs to present 5-OP-RU to MAIT cells was successful. In response to this largely TCR-dependent stimulus, MAIT cells from FMT patients were consistently able to produce the proinflammatory cytokine IFN-γ, the cytotoxic effector molecule granzyme B, and displayed surface expression of the degranulation marker CD107A (Figure 8B). Compared with pre-FMT, at the 1-week post-FMT timepoint, two out of three patients trended towards an increase in their frequency of granzyme B^+, IFN-γ^+, and $CD107A^+$ MAIT cells (Figure 8B). A similar trend was observed for the per-cell expression of IFN-γ and CD107A (Figure 8B). Responses to stimulation at the 1-month post-FMT timepoint were more divergent, and only the enrollment of more participants will allow the examination of a trend at this timepoint.

Stimulation of MAIT cells in PBMCs using the TCR-independent stimuli IL-12 + IL-18 elicited a similar effect to that seen using C1R WT cells + 5-OP-RU (Figure 8C). Compared with pre-FMT, MAIT cells both as a population and on a per-cell basis generally trended towards increased expression of IFN-γ, CD107A, and granzyme B 1-

week post-FMT (Figure 8C). The increase in IFN-γ production in particular was quite noticeable. Interestingly, the frequency of MAIT cells producing granzyme B, and the changes in granzyme B production following FMT, were so similar between two patients that their data points nearly completely overlap on the graph (Figure 8C). While drawing strong conclusions from the data from 2 patients is not possible, it is intriguing that these two patients both trended towards decreased IFN-γ production 1-month post-FMT compared with 1-week post-FMT.

Finally, PBMCs were stimulated with a combination of C1R WT cells + 5-OP-RU + IL-12 + IL-18 (Figure 8D). Activation of MAIT cells in a TCR-dependent or TCR-independent manner is not mutually exclusive, and the two stimulation modes can work in concert to synergistically activate MAIT cells[106,108]. As seen in Figure 8D, this stimulation elicited the highest production of IFN-γ by the pre-FMT MAIT cell population of any of the tested conditions. The frequency of MAIT cells producing IFN-γ or granzyme B continued on an upward trajectory at the 1-week post-FMT timepoint (Figure 8D). The production of IFN-γ on a per-cell basis 1-week post-FMT, compared with pre-FMT, followed the same trajectory. As was seen in the C1R WT cells + 5-OP-RU stimulation condition, 2 out of 3 patients also trended towards elevated expression of the degranulation marker CD107A, indicating the release of lytic granules. Based on the data obtained from each stimulation condition, it appears that 1-week post-FMT, compared with pre-FMT, more MAIT cells from the patients express IFN-γ and granzyme B. This fits with the trend towards a reduction in surface exhaustion marker expression by MAIT cells 1-week post-FMT (Figure 7).

Recently, we were gifted multiple human isolates of *C. difficile*, which were turned into lysates for use as a relevant stimulation condition. *C. difficile* can activate MAIT cells in both a TCR-dependent and a TCR-independent manner, though the strength of this activation can depend on both riboflavin production and the ability to elicit IL-12 and IL-18 production from surrounding PBMCs. So far, we have examined the response to a 1:10 dilution of *C. difficile* lysate by MAIT cells in the PBMCs of one patient (Figure 8E). In contrast to what was observed in the IL-12 and IL-18 containing conditions (Figure 8C, 8D), the frequency of MAIT cells that produced IFN-γ or granzyme B in

response to lysate were reduced 1-week post-FMT compared with pre-FMT (Figure 8E). This patient was the same patient, however, that exhibited a decreased frequency of IFN-γ and CD107A expressing MAIT cells in response to C1R WT cells + 5-OP-RU 1-week post-FMT compared with pre-FMT (Figure 8B). The decreased production of IFN-γ and granzyme B did rebound at the 1-month post-FMT timepoint (Figure 8B, 8E). The response to *C. difficile* lysate by this patient therefore more closely mirrored their TCR-dependent reaction than their TCR-independent one.

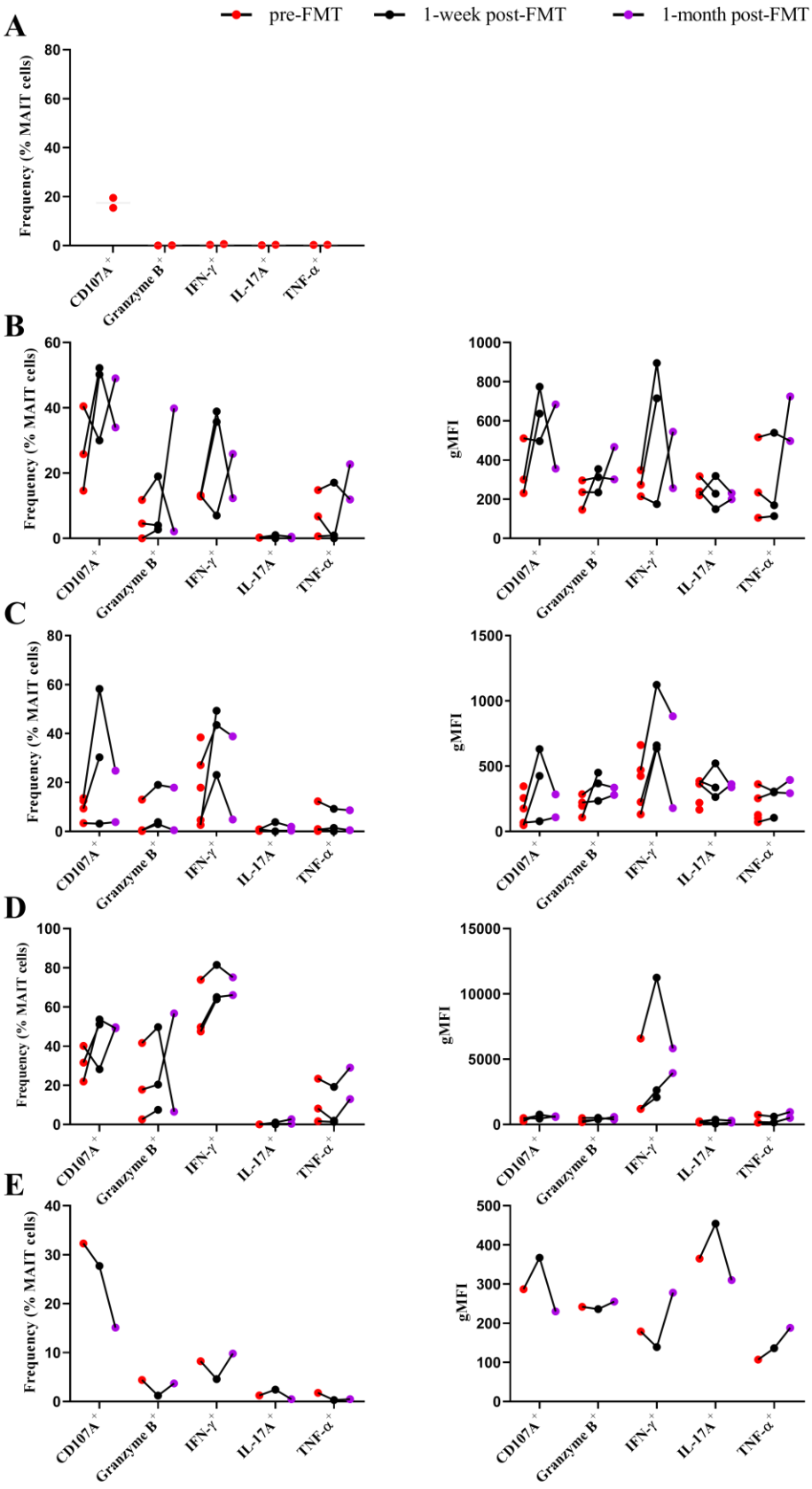

pre-FMT
1-week post-FMT
1-month post-FMT
A
B
C
D
E
Frequency (% MAIT cells)
gMFI
CD107A+
Granzyme B+
IFN-γ+
IL-17A+
TNF-α+

Figure 8. MAIT Cells' Cytokine-Producing Capacity Trends Towards an Increase Following FMT. Peripheral blood was collected from patients pre-FMT, 1-week post-FMT, and 1-month post-FMT. Peripheral blood mononuclear cells were isolated and stimulated for 24 hours with 5-OP-RU ligand alone (A), C1R WT cells + 5-OP-RU (B), IL-12 + IL-18 (C), IL-12 + IL-18 + C1R WT cells + 5-OP-RU (D), or a 1:10 dilution of *C. difficile* lysate strain #3 (E). Cells were then stained for intracellular expression of the listed cytokines and cytotoxic effector molecules and analyzed by flow cytometry. Lines connect samples from the same patient over time. (A) pre-FMT n=2. (B, D) pre-FMT n=3, 1-week post-FMT n=3, 1-month post-FMT n=2. (C) pre-FMT n=5, 1-week post-FMT n=3, 1-month post-FMT n=2. (E) pre-FMT n=1, 1-week post-FMT n=1, 1-month post-FMT n=1. Statistical significance was examined using multiple Wilcoxon matched-pairs signed rank tests with corrections for multiple comparisons using the Holm-Šídák method. No statistically significant changes were observed.

3.5 FMT-Elicited Skewing of MAIT Cells' Transcriptional Profile

MAIT cells are predominantly viewed as falling into two subsets: the type 1 skewed MAIT-1 cells, and the type 17 skewed MAIT-17 cells. In mice this delineation is clear; MAIT cells are either MAIT-1 or MAIT-17[78,98]. In humans however, MAIT cells more commonly display high levels of the type 17 transcription factor RORγT, and a sliding scale in their expression of the type 1 transcription factor T-bet[76–78,99]. It was therefore unsurprising that the frequency of RORγT$^+$ MAIT cells pre-FMT was high (Figure 9). Following FMT, different patients displayed divergent phenotypes in their per-cell expression of RORγT. Despite this, at the 1-week post-FMT timepoint, most of the patients trended towards a decrease in their frequency of RORγT$^+$ MAIT cells (Figure 9). This corresponded at least in part with a shift towards a phenotype where MAIT cells expressed neither RORγT nor T-bet. The significance of this shift remains to be determined, but it did lead us to explore a recently reported MAIT cell phenotype.

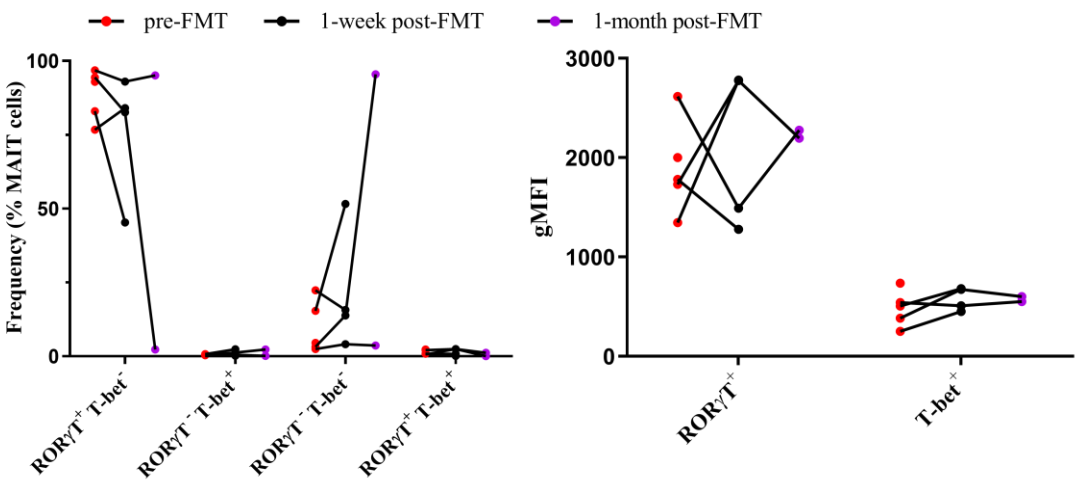


Figure 9. MAIT Cells Move Away from MAIT-17 and MAIT-1 Phenotypes Following FMT. Peripheral blood was collected from patients pre-FMT, 1-week post-FMT, and 1-month post-FMT. Peripheral blood mononuclear cells were isolated and stained to determine frequency of MAIT cells expressing each transcription factor profile using flow cytometry. Lines connect samples from the same patient over time. Pre-FMT n=5, 1-week post-FMT n=4, 1-month post-FMT n=2. Statistical significance was examined using multiple Wilcoxon matched-pairs signed rank tests with corrections for multiple comparisons using the Holm-Šídák method. No statistically significant changes were observed.

3.6 Exploration of MAIT-2 Cells

Given the movement of MAIT cells away from a transcriptional MAIT-1 or MAIT-17 phenotype following FMT, we decided to explore if these MAIT cells were becoming type 2 skewed. First, fresh MAIT cell expression of the prototypic type 2 transcription factor GATA3 was examined (Figure 10A). GATA3 was not largely expressed by the MAIT cells of the tested patients (Figure 10A). While transcription factor expression helps to identify the skew of MAIT cells, production of cytokines is also informative to determine if cells are functionally skewed. To this end, I stimulated MAIT cells with IL-12 + IL-18 to activate them in a TCR-independent manner, C1R WT cells pulsed with 5-OP-RU to activate them in a largely TCR-dependent manner, or a combination of all three stimuli to potently activate the MAIT cells. I also included a stimulation condition using *C. difficile* lysate, which can activate MAIT cells in both a TCR-dependent and TCR-independent manner. In all of the aforementioned stimulation conditions, MAIT cells did not display notable expression of the type 2 associated cytokines IL-4 or IL-13 (Figure 10B-E). The MAIT cells also did not display notable production of the T cell proliferation cytokine IL-2 (Figure 10B-E). Therefore, in the examined FMT patients, MAIT cells which express neither a MAIT-1 nor a MAIT-17 phenotype are not becoming MAIT-2 cells.

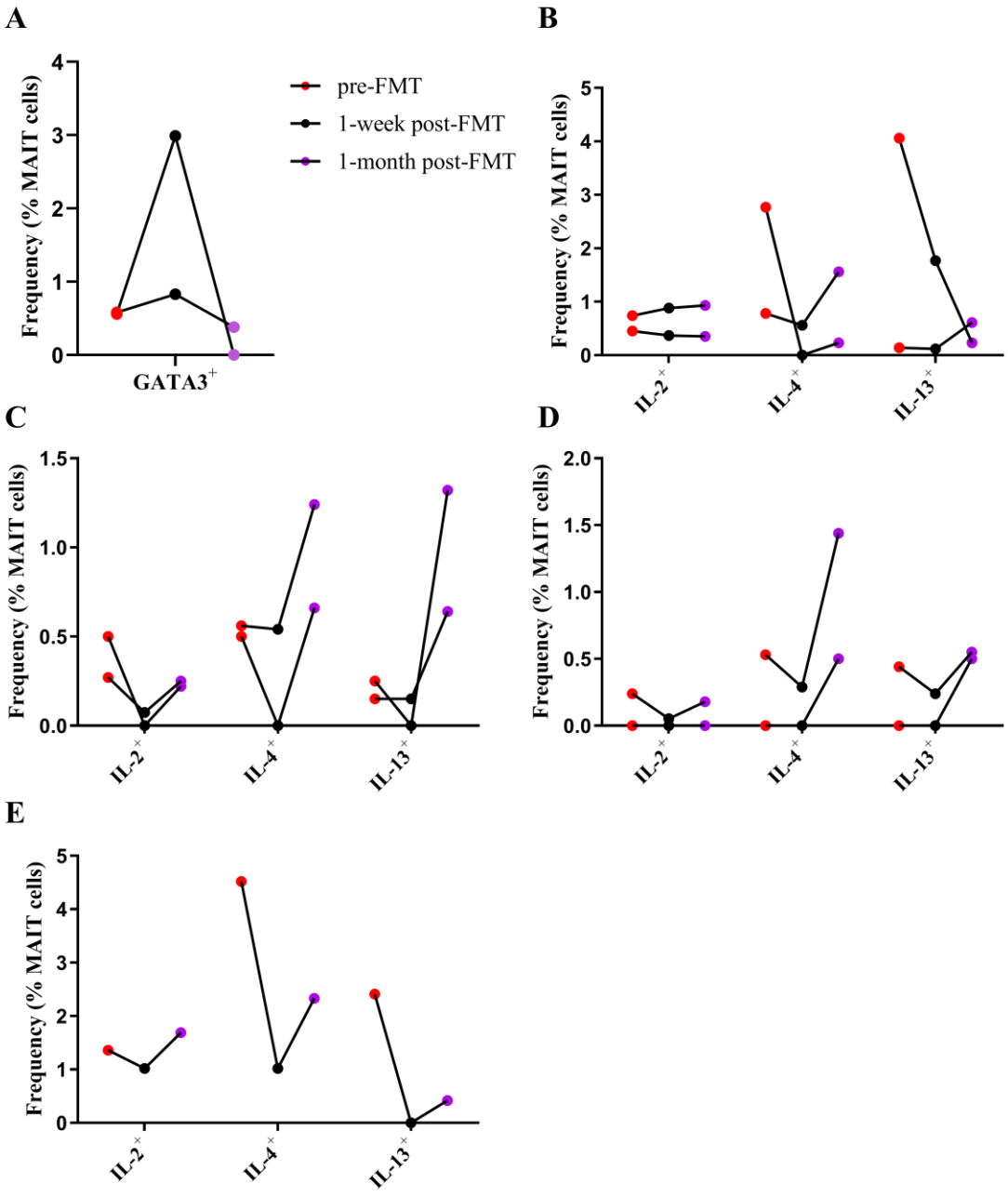
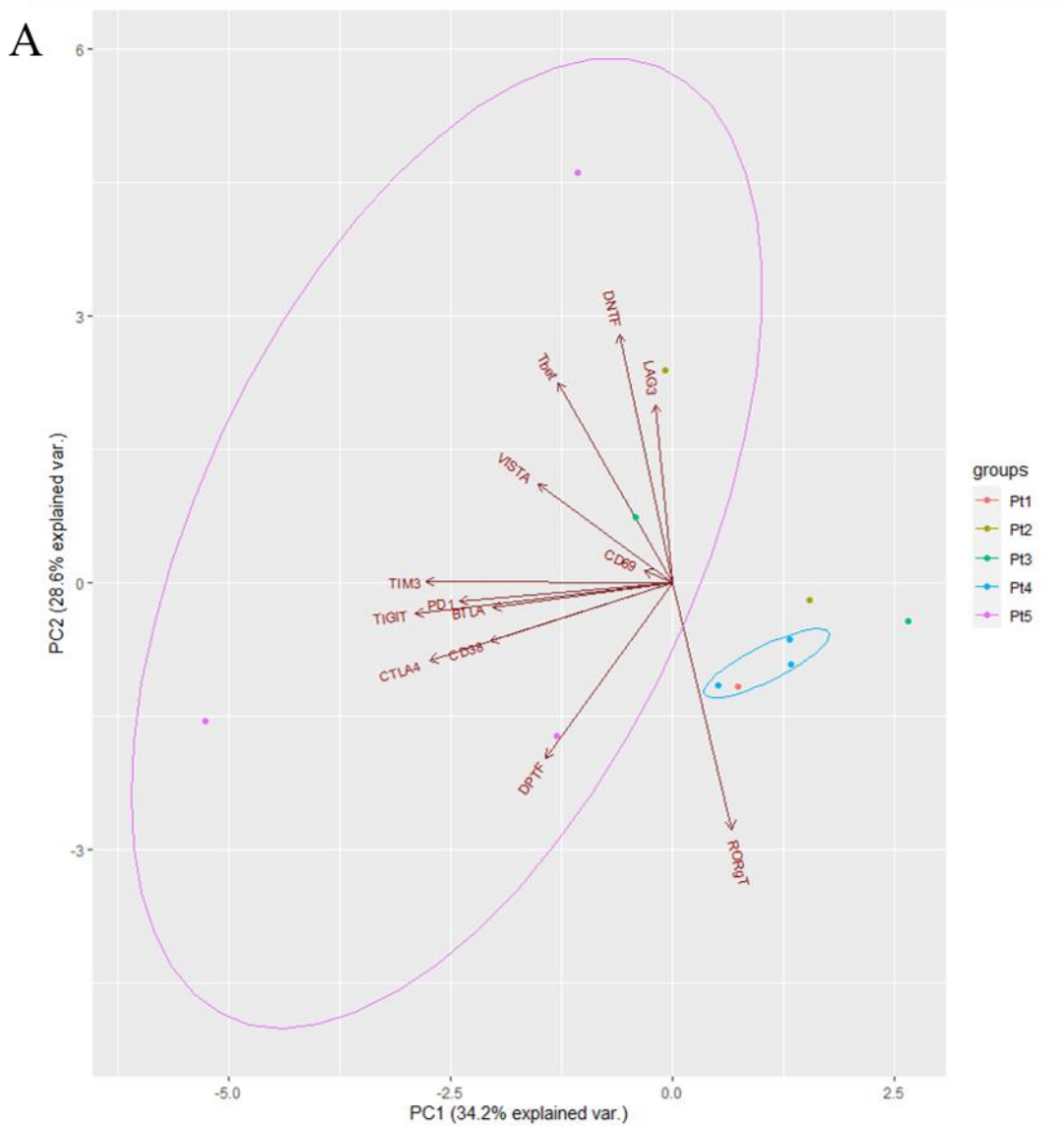


Figure 10. MAIT Cells from FMT Patients Do Not Display a MAIT-2 Phenotype. Peripheral blood was collected from patients pre-FMT, 1-week post-FMT, and 1-month post-FMT. Peripheral blood mononuclear cells were isolated, stained for expression of the transcription factor GATA3, and analyzed via flow cytometry (A). Peripheral blood mononuclear cells were also stimulated for 24 hours with IL-12 + IL-18 (B), C1R WT cells + 5-OP-RU (C), IL-12 + IL-18 + C1R WT cells + 5-OP-RU (D), or 1:10 dilution of

C. difficile lysate strain #3 (E). Cells were then stained for intracellular expression of the listed cytokines and interrogated using flow cytometry. Lines connect samples from the same patient over time. Pre-FMT n=2, 1-week post-FMT n=2, 1-month post-FMT n=2 (A-D). Pre-FMT n=1, 1-week post-FMT n=1, 1-month post-FMT n=1 (E).

3.7 Principal Component Analysis of MAIT Cell Phenotypes

The data obtained from the FMT patients were multiparametric, making it somewhat difficult to see how the samples from different timepoints, or the samples from the same patient over time, might be similar. To visualize the overall structure of the data, linear dimensionality reductions were completed in the form of a principal component analysis. The vectors on the plots in Figure 11 each represent the influence of the marker listed on them on the PCA. Lines going left or right indicate the markers had more influence on principal component 1 (PC1), whereas lines going up and down indicate influence on PC2. Being that PC1 accounts for more of the variance in the dataset, differences along this axis are more meaningful. In Figure 11A, the plot was created with groupings based on FMT patients. The same color represents samples from the same patient over time, with an ellipse drawn for patient samples in the same color as their points. Samples from the same patient over time did not strikingly cluster with one another, with the exception of patient 4. This indicates a degree of dissimilarity for surface marker and transcription factor expression, even within the same patient, over the course of pre- and post-FMT treatment. The next question to be answered, if a patient's MAIT cell phenotype was changing over time, was if samples from the same timepoint relative to FMT may be similar. To this end, clustering was also performed based on the timepoint a sample was taken, using the same markers from Figure 11A (Figure 11B). While 1-week post-FMT patient samples appeared unalike, pre-FMT samples did display loose clustering and thus some similarity. Therefore, despite all the differences between FMT patients, their pre-FMT MAIT cells may display a similar overall profile after fighting rCDI.


A
6
3
0
-3
PC2 (28.6% explained var.)
-5.0
-2.5
0.0
2.5
PC1 (34.2% explained var.)
DNTF
LAG3
Tbet
VISTA
CD69
TIM3
TIGIT
PD1
BTLA
CTLA4
CD38
DPTF
RORgT
groups
Pt1
Pt2
Pt3
Pt4
Pt5

B

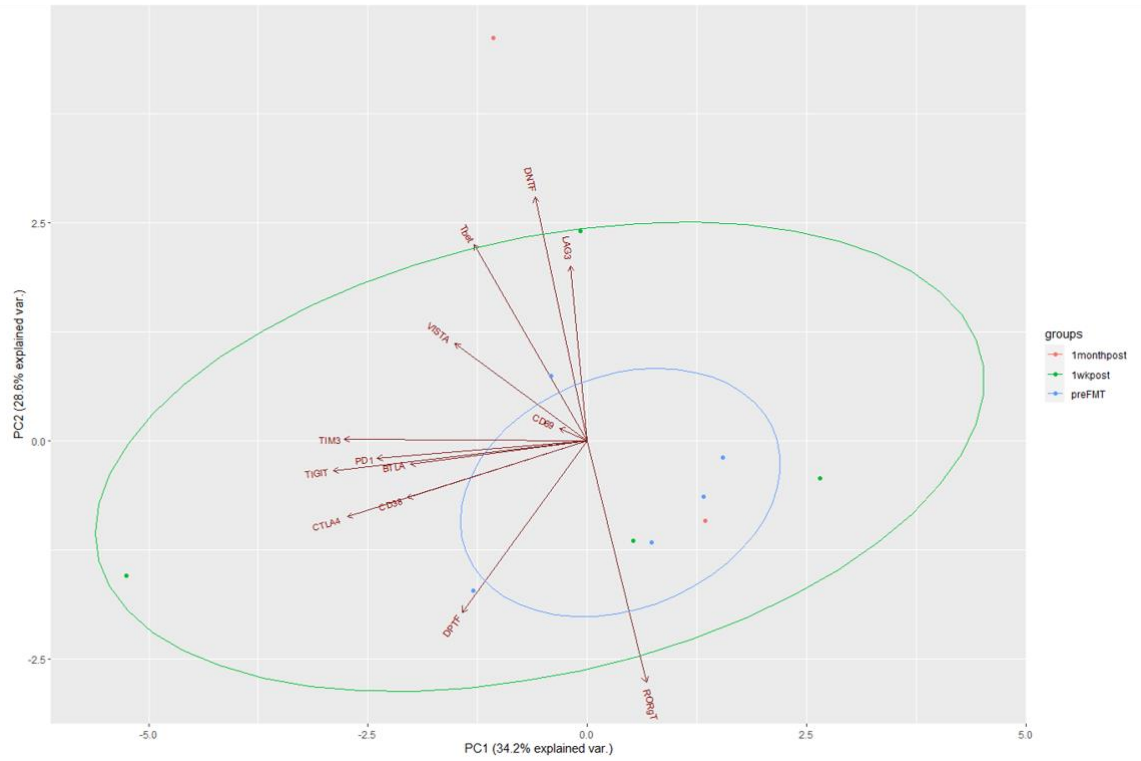


Figure 11. Principal Component Analysis Based on MAIT cell Surface Marker and Transcription Factor Expression. Peripheral blood was collected from patients pre-FMT, 1-week post-FMT, and 1-month post-FMT. Peripheral blood mononuclear cells were isolated, stained for activation, exhaustion, and transcription factor expression on MAIT cells, then analyzed using flow cytometry. The frequencies of MAIT cells expressing each of the 13 markers were loaded into RStudio and a principal component analysis (PCA) was performed. Each patient therefore had a point in 13-dimensional space representing their expression of all the markers simultaneously. Principal component 1 (PC1) is the line (eigenvector) passing through this space that maximized the variance of the points around it, becoming the new X axis. Principal component 2 (PC2) is perpendicular to PC1, and accounts for the next highest level of variance, becoming the new Y axis. The original points therefore have a position on each of these new axes. 10A. Features grouping by patient, with the same colors corresponding to the same patients. 10B. Features grouping by timepoint relative to FMT. Pre-FMT n=5, 1-week post-FMT n=4, 1-month post-FMT n=2. DNTF means double negative for both

RORγT and T-bet. DPTF means double positive for both RORγT and T-bet. Ellipses to show clustering were included for groups containing more than two data points.

Chapter 4

4 Discussion

4.1 MAIT Cell Recruitment to the Intestinal Tract

In the past, researchers have found decreased frequencies of peripheral blood MAIT cells in patients with inflammatory bowel diseases compared with healthy controls[133,153]. This corresponded with increased frequencies of MAIT cells in inflamed intestinal tissue compared with uninflamed tissue[133,153]. The blood MAIT cells did not display elevated expression of apoptotic markers, indicating the decreased frequency was not likely due to MAIT cell death[153]. While this does not prove MAIT cell recruitment to the intestinal tract, it has been postulated that in cases of intestinal inflammation, decreased MAIT cell frequencies may be due to intestinal recruitment[133,153]. Furthermore, it has been found that patients with bacterial sepsis have reduced levels of circulating MAIT cells compared to critically ill uninfected patients[154]. This decrease in MAIT cell count was less prominent in the case of septic patients with streptococcal infections, which MAIT cells do not typically respond to[154]. The authors therefore hypothesized that the decreased MAIT cell count could be due to MAIT cell recruitment to the site of infection[154]. With this in mind, I examined if the frequency of MAIT cells in the peripheral blood of FMT patients changed pre- vs post-FMT, potentially due to their recruitment to the intestinal tract (Figure 5). Not only can *C. difficile* infection elicit inflammatory conditions, FMT contains commensal bacteria species capable of riboflavin production and thus potentially MAIT cell activation[43,136–139]. In our patient cohort thus far, we did not see significant differences in MAIT cell frequencies pre- vs post-FMT. This would seem to suggest that if there is any recruitment of MAIT cells to the intestinal tract, it is transient or evened out by an equal circulation of tissue MAIT cells back into the blood. Given that FMT and CDI represent an infectious and microbial scenario, contrasting with the autoimmune inflammation seen in inflammatory bowel diseases, it is feasible that the kinetics of MAIT cell movement could differ. Similarly, sepsis is characterized by a dysregulated immune response, with the early phase characterized by excessive inflammation and the late phase associated with immunosuppression[155]. The kinetics of MAIT cell movement during sepsis could thus prove different from the kinetics of MAIT cell movement

surrounding FMT. As appropriate controls will be enrolled in the ongoing study, it will be determined if FMT patients in general have decreased overall MAIT cell frequencies.

Examining the frequency of MAIT cells in the peripheral blood can provide an initial indication that there may be recruitment of MAIT cells to other tissues, but it is not a definitive answer. One of the major limitations of my study is the use of blood MAIT cells as a proxy to explore the impact of FMT, which occurs in the gut, on the MAIT population. Nonetheless, MAIT cells are known to circulate through the body, and the blood has been used in the past as a lens through which the impact of intestinal perturbations on MAIT cells can be explored[133,153]. Furthermore, it would not be ethical to ask CDI patients to undergo colonic biopsies that are not standard of care for the sake of more relevant samples. To strengthen our understanding of MAIT cell circulation in FMT patients, we next examined their expression of the intestinal recruitment markers CCR6 and the α4β7 integrin, which they are known to express (Figure 6)[76–78,80,81]. Only one patient was examined for these markers so far, so strong conclusions cannot be drawn. While MAIT cells are commonly described as $CCR6^+$, less than half of the patient's MAIT cells expressed this marker[77,78]. It is possible that the majority of CCR6 expressing MAIT cells had already been recruited to the intestinal tract by *C. difficile* infection. It has been shown *in vitro* that *C. difficile* can lead to increased production of CCL20 by intestinal epithelial cells[156]. CCL20 is the ligand for CCR6, and thus could recruit CCR6 expressing cells from the periphery over the course of CDI[156]. Following FMT, the frequency of CCR6 expressing MAIT cells trended towards a decrease, whereas the expression of CCR6 on a per-cell basis trended upwards. This again could indicate that the $CCR6^+$ MAIT cells may be homing to another tissue, such as the colon, if verified in a larger cohort of patients.

The trend observed for MAIT cell expression of the α4β7 integrin did differ from that observed with CCR6 (Figure 6). Fitting with previous literature, the patient's MAIT cells had notable expression of the α4β7 integrin[76,80]. At the 1-week post-FMT timepoint, the patient had a reduced frequency and per cell expression of α4β7 by MAIT cells. If this trend were replicated in a larger patient cohort, it could indicate that MAIT cells are employing this integrin to migrate to the intestinal tract — hence the decrease in $\alpha4\beta7^+$

MAIT cells 1-week post-FMT. It has been previously shown that MAIT cells from pigtail macaques upregulate their expression of α4β7 following intrarectal infection with simian immunodeficiency viruses[80]. This increase did correlate with the viral load, and corresponded to an increased frequency of MAIT cells found in the rectal mucosa around the time of α4β7 upregulation[80]. While we did not observe an initial increase in the frequency of $\alpha4\beta7^+$ MAIT cells in FMT patients, it should be noted that pigtail macaque MAIT cells express lower levels of α4β7 than human MAIT cells do[80]. This left more room for the MAIT cells from these animals to noticeably increase α4β7 integrin expression. Despite this difference, MAIT cells from pigtail macaques are otherwise largely phenotypically similar to human MAIT cells, and expression of the α4β7 integrin would likely have a similar functional outcome[80]. Thus, examination of MAIT cell α4β7 integrin expression in patients pre- and post-FMT will be informative as a larger patient cohort is obtained.

One of the strengths of the study design was having the same patient provide the samples pre- and post-FMT to help reduce the confounding effect of natural variability in MAIT cell phenotypes. If different patients provided the pre- and post-FMT samples, it would be difficult to elucidate true changes in the MAIT cell population considering variability between people, and potential differences in *C. difficile* load or strains. The COVID-19 pandemic was a major hurdle to study recruitment, with the shutdowns of face-to-face research and non-essential procedures in hospitals, as well as the understandable hesitancy of patients to return to the hospital for FMT. Despite considerable efforts by the Haeryfar lab team and our collaborators, the low enrollment of this study thus far posed a major limitation to the ability to draw strong conclusions. In order to confirm the dominant trends related to changes in MAIT cell phenotypes pre- and post-FMT, more patients will have to be enrolled in the study.

As these patients are enrolled, a power analysis can then be conducted to determine how many should be recruited. Given that this was a pilot study, with no comparable available data, the effect size will need to be gauged in order to compute the power analysis. Additionally, healthy individuals would not be the appropriate control for our study. We will need to enroll individuals who are fighting CDI, but not scheduled to undergo FMT

at this time. Blood would be drawn from participants upon completion of an antibiotic course, lining up with a pre-FMT sample, and then again 1 week and 1 month later, matching up with the post-FMT timepoints. This would allow a tentative exploration of the influence of CDI and antibiotic use on the MAIT cell population, and how it changes over time. Aspects unique to the post-FMT timepoints could then be teased out. The argument could be made that individuals not scheduled for FMT may have lower bacterial burden, or lower episodes of recurrence. This is true and something to be considered, but these controls are the best available option to ensure everyone is getting the care they need and deserve.

In a scenario more reminiscent of a clinical trial, recruitment would be of individuals experiencing their first or second recurrence of CDI. Antibiotics, rather than FMT, are recommended for the treatment of a first recurrence and are also accepted for treatment of a second recurrence[56,61]. Thus, the recruited patients could be randomized to receive either the conventional antibiotic treatment or FMT[157]. Unlike in our current study design, randomizing patients to either condition would not deprive rCDI patients experiencing a greater number of recurrences of the FMT they need, and would be ethically acceptable[157]. This would also mitigate concerns regarding the antibiotic treated rCDI patients being less ill than those that receive FMT in our current study plan. The same sample collection timeline used in the current study could be used in this scenario.

The 1-week post-FMT timepoint was selected because FMT patients typically return to a more normal stool form and frequency by this point and display regeneration of intestinal epithelial cells with a trend towards decreased levels of an intestinal damage marker[61,65,140]. The decrease in the intestinal damage marker reached significance at the 1-month post-FMT timepoint[140]. Although multiple studies have used the 8-week post-FMT timepoint, the justification for this timepoint is not firm[141]. If FMT is going to fail, this failure is typically observed within the first month after the treatment[141]. The FMT national registry examines effectiveness of the treatment by observing the cure rate of CDI at this timepoint, making it a useful addition to our study[142]. Additionally, samples acquired 1-month post-FMT have shown that the diversity of the microbiota is higher in responding patients than non-responding ones at this timepoint[143]. Furthermore, a study of

FMT outcomes in people with IBS noted that responses were observed by this 1-month post-FMT timepoint, with increases in different genera associated with lower IBS severity scores and fatigue[144]. In the future, the REB may also be amended to allow an additional blood draw at the cessation of antibiotics. This would allow examination of how MAIT cells may change between then and the pre-FMT timepoint, as little is known about this period.

4.2 The Impact of FMT on MAIT Cell Phenotype and Functional Capacity

The second aim of our study was to investigate the impact of FMT on the phenotype and functional capacity of human peripheral blood MAIT cells. The first parameter examined was MAIT cell expression of activation and exhaustion markers pre- vs post-FMT. At the 1-week post-FMT timepoint, compared with pre-FMT, the frequency of MAIT cells expressing BTLA, PD-1, or the activation marker CD69, trended towards a decrease (Figure 7A, B). A similar trend was observed for the per-cell MAIT cell expression of CD69, while the per cell expression of PD-1 was largely stable (Figure 7A, B). In contrast to what was seen with these exhaustion markers, the frequency and per MAIT cell expression of the exhaustion marker VISTA trended upwards (Figure 7A). Overall, it therefore appeared that the MAIT cell population moved away from an exhausted surface phenotype at the 1-week timepoint following FMT.

The move away from a state of exhaustion, considering the influx of microbes and microbial metabolites associated with FMT, may initially seem counterintuitive. It is known, however, that within a week of FMT, the intestinal tract epithelial barrier heals and plasma markers of intestinal damage begin to decrease[61,65,140]. Thus, regeneration of intestinal epithelial cells and removal of *C. difficile* can reduce both the inflammatory state caused by the pathogen and the leakage of bacterial products across the formerly damaged barrier[29,43,158,159]. This could subsequently reduce the level of constant stimulation MAIT cells may be experiencing, and with it the signaling leading to production of exhaustion and activation markers[117,120,160]. With limited signaling for the production of new activation and exhaustion molecules, the existing markers would be subject to natural recycling and degradation with time[161]. The reduced frequency of

exhaustion markers expressed on MAIT cells 1-week post-FMT may therefore be mediated by the reduction in MAIT cell stimuli in the intestinal tract.

The reason for MAIT cell expression of the particular activation and exhaustion markers observed in this study is an outstanding question. At this time, the mechanisms of many of these markers, and how they then influence MAIT cell functional capacity, is unknown. Interestingly, VISTA, BTLA, and PD-1 have all been implicated in the negative regulation of IL-17A production by γδ T cells[162]. In particular, VISTA can also negatively regulate IL-17A production by conventional $CD4^+$ T cells, while not affecting their capacity to produce IFN-γ or TNF-α[162]. Furthermore, VISTA also reduces IL-7-mediated proliferation of γδ T cells[162]. Since the frequency of MAIT cells expressing VISTA trends toward an increase following FMT, while the frequency of $BTLA^+$ or PD-1^+ MAIT cells trends towards a decrease, these markers may represent fine tuning of the MAIT-17 response. Given that IL-17 walks a fine line between protection and pathogenicity in the context of CDI, tightly constraining this phenotype pre-FMT could serve as an attempt to reduce negative outcomes[46,49,50]. With the resolution of *C. difficile* infection, MAIT cells could be freed to use the type 17 end of their arsenal against different pathogens. Further adding to this concept, the activation marker CD69, found to be expressed on MAIT cells in this study, can also fine tune type 17 responses[117].

Exploration of MAIT cell expression of activation and exhaustion markers can give insight into their state and potential interaction with FMT. However, exhaustion is not based solely on cell surface phenotype, but also on functional capacity[163]. I therefore examined the functional capacity of MAIT cells from pre- and post-FMT samples, to elucidate if there were changes in the levels of cytokines produced. This also provided the opportunity to observe if the skew of the MAIT cells, in terms of which cytokines were produced, was altered following FMT. Regardless of whether the stimulation condition was TCR-dependent, TCR-independent, or both, 1-week post-FMT MAIT cell populations largely trended towards an increased production of IFN-γ and granzyme B compared with pre-FMT populations (Figure 8). The increased frequency of MAIT cells producing granzyme B was often coupled with an increased frequency of MAIT cells expressing the degranulation marker CD107A, indicating actual release of lytic granules.

The reduction in exhaustion marker expression observed on MAIT cells 1-week post-FMT would reduce the inhibitory signaling they receive, and thus allow a more potent response upon stimulation. Therefore, the increased cytokine producing capacity observed in the 1-week post-FMT MAIT cells, compared with the pre-FMT MAIT cells, further strengthens the idea that these cells may be moving away from a state of exhaustion.

It has previously been shown that the microbiota can tailor the phenotype and functional capacity of T cells[164,165]. In a murine cancer model, FMT was observed to influence the balance of exhausted stem cell-like and effector exhausted CD8 T cells[165]. Mice that displayed a response to anti-PD-L1 checkpoint therapy following FMT ("responder microbiota") exhibited a shift towards the effector exhausted CD8 T cell subset, characterized by production of IFN-γ and an elevated cytotoxic ability[165]. While this model presents a striking contrast to our own study, the major takeaway is that a healthy microbiota possesses the ability to fine tune aspects of T cell functional capacity[165]. Furthermore, when microbial dysbiosis is induced in a mouse model of tuberculosis, MAIT cell production of IL-17A is reduced compared with mice that possess a normal microbiota[164]. The mice with microbial dysbiosis also displayed impaired MAIT cell recruitment and greater bacterial burden[164]. Subsequent repopulation of the healthy microbiota reversed these perturbations in the MAIT cell compartment[164]. A similar phenomenon may occur following FMT, with the healthy intestinal microbiota serving to provide signals which restore MAIT cell function after the dysbiosis associated with CDI. The healthy microbial signaling, plus the reduction in exhaustion marker expression, are both probable players in this restoration of MAIT cell function. In the coming years we will look to sequence the slurry samples given to FMT patients in the study. This will allow us to explore if the abundance of certain bacteria, particularly riboflavin producers, is associated with altered MAIT cell phenotypes.

Peripheral blood MAIT cells are known to be biased towards the production of type 1 cytokines, such as IFN-γ[76]. This does not however negate the observed trend towards increased IFN-γ production upon stimulation in 1-week post-FMT MAIT cells compared

with pre-FMT MAIT cells. It does indicate that while MAIT cells may move away from a state of exhaustion following FMT, they do not move away from their expected cytokine skew given their location. Importantly, *C. difficile* infection is known to increase the production of IFN-γ by T cells, and production of this cytokine is protective against CDI[42–45]. Fitting with this, MAIT cells have been shown *in vitro* to upregulate their production of IFN-γ and granzyme B in response to *C. difficile*[82]. MAIT cells, particularly the 1-week post-FMT population displaying potent IFN-γ production, therefore possess the capacity to be amongst the contributors of IFN-γ to the cytokine milieu, aiding in the clearance of CDI.

Considering the production of IFN-γ and granzyme B observed by MAIT cells in response to *C. difficile* in a previous study, the response to *C. difficile* lysate we observed here may initially seem surprising (Figure 8E)[82]. Notably, the strain used in our study was a non-toxigenic isolate, while the strain in the previous study that elicited the strongest response by MAIT cells was toxigenic[82]. The previous study also noted that toxigenic strains lead to higher levels of IL-12 production by APCs than non-toxigenic strains do[82]. Additionally, the toxigenic strain induced a stronger response than a non-toxigenic strain that produced higher levels of riboflavin[82]. This would suggest that inducing cytokine production by surrounding APCs, rather than just production of MAIT cell TCR ligands, is important for a potent MAIT cell response. The mild response elicited by our non-toxigenic strain is likely due to mild, not strong, induction of IL-12 by APCs, and riboflavin production by this strain may another be a factor. This also fits with the observed trend in this patient's MAIT cell response to *C. difficile* lysate closely mirroring their C1R WT + 5-OP-RU TCR-dependent response (Figure 8B, E). In contrast, the patient's MAIT cell response to stimulations containing IL-12 and IL-18 showed the opposite trend, suggesting that these cytokines were not major contributors in the *C. difficile* stimulation condition (Figure 8C, E). The response by this this patient's MAIT cells to *C. difficile* lysate, although odd upon initial appraisal, does fit with their overall response pattern to different stimulations.

Continuing with our investigation into the impact of FMT on MAIT cell phenotypes, we next examined the MAIT-1 and MAIT-17 transcriptional breakdown pre- and post-FMT.

Human MAIT cells are generally known to express high levels of the type 17 transcription factor RORγT, while possessing a sliding scale in their expression of the type 1 transcription factor T-bet[76–78,99]. This does make the MAIT-1 and MAIT-17 delineation less clear than in mice, as mouse MAIT cells express one transcription factor or the other[78,98]. Considering that the RORγT$^+$ phenotype is favored by human blood MAIT cells, it was unsurprising that the majority of MAIT cells in pre-FMT samples were RORγT$^+$ (Figure 9). 1-week post-FMT however, 3 of the patients displayed reduced frequencies of RORγT$^+$ MAIT cells. This corresponded with an increased frequency of MAIT cells expressing neither RORγT nor T-bet, thus neither the prototypical MAIT-1 or MAIT-17 phenotype. This shift away from a RORγT$^+$ phenotype, and move towards a phenotype expressing neither transcription factor, continued for 1 patient at the 1-month post-FMT timepoint. Here, 95% of the patient's examined MAIT cells expressed neither RORγT nor T-bet. Since these MAIT cells did not fall into the expected MAIT-1 or MAIT-17 subsets, the meaning of this shift was a topic of curiosity and further exploration.

The existence of a MAIT-2 subset in humans is currently still under investigation. MAIT cells have not been found to typically express the type 2 transcription factor GATA3[78,100]. Despite the lack of GATA3 in MAIT cells, it has been shown that chronic stimulation of MAIT cells can elicit type 2 cytokine production[100]. In cases of intestinal pathology, such as colorectal cancer, the integrity of the intestinal barrier can be compromised[100]. This may allow the leakage of bacterial products across the barrier, and chronic inflammation and stimulation of immune cells[100]. With this situation in mind, researchers determined that prolonged *in vitro* stimulation of human peripheral blood MAIT cells could indeed elicit production of IL-13, IL-5, and IL-4[100]. Although the *in vitro* stimulation performed by the authors does not prove that such a response can be elicited *in vivo*, chronic stimulation due to intestinal inflammation and bacterial translocation is something that could feasibly occur during CDI. This led to the exploration of pre- and post-FMT MAIT cell expression of GATA3 and, upon stimulation, type 2 cytokines (Figure 10). Unfortunately, MAIT cells from both pre- and post-FMT samples did not display notable expression of type 2 cytokines or GATA3. The MAIT cell population that is neither

MAIT-1 nor MAIT-17 does not appear to be MAIT-2 either, leaving the significance of the shift an enduring question.

Given the atypical transcriptional shift observed in the MAIT cell population following FMT, we have considered other avenues to characterize them. We recently acquired RNA later and have been freezing cells from patient samples in it for later transcriptomics. Once we have a larger batch of samples they will be sent for sequencing. New sequencing papers are coming out quite frequently, so we may be able to find a published T cell dataset that displays a similar transcriptional profile to the post-FMT MAIT cells. Even if the skew displayed by the MAIT cells has not been previously explored, the consequences of this transcription factor shift may become clearer upon viewing the dominant upregulated transcripts and pathways. We have also been saving plasma samples from patients, to examine how riboflavin derivative levels in the blood may change pre- vs post-FMT, and if this correlates with MAIT cell phenotypes or FMT outcomes.

Finally, the overall similarity between samples from the same patient over time, or between different patients at the same timepoint was explored using a principal component analysis (Figure 11). When ellipses were drawn based on the patient, it appeared that the pre- and post-FMT samples from the same patient were generally dissimilar (Figure 11A). With the exception of patient 4, this could suggest a shift in the MAIT cell exhaustion marker and transcription factor profile following FMT. Enrollment of additional patients up to the 1-month post-FMT timepoint is required to confirm if this trend is representative. Grouping based on sample timepoint relative to FMT was more informative, as the patient pre-FMT samples did loosely cluster (Figure 11B). It would be interesting if further patient samples contributed to this same cluster structure, that despite all the variation in the population, pre-FMT patients may have a similar overall immune phenotype with respect to the examined parameters. Observing the overall effect of CDI and FMT on the MAIT cell phenotype is difficult when markers are examined individually. A PCA allows the compression of multiparametric data onto a new two-dimensional plot, while preserving as much of the data structure as possible. This will

therefore be a useful tool with continued patient enrollment to visualize overall changes in MAIT cell phenotypes.

4.3 Summary and Conclusions

In this study I have observed a trend towards decreased phenotypic and functional exhaustion of human blood MAIT cells following FMT to treat rCDI. One exhaustion marker, VISTA, showed the opposite trend in its expression, suggesting a degree of fine tuning of the MAIT cell response capacity. The trend towards an increased functional capacity by MAIT cells 1-week post-FMT was characterized by expression of the proinflammatory cytokine IFN-γ and the cytotoxic effector molecule granzyme B. Pre-FMT MAIT cells were primarily MAIT-17, as expected. Following FMT, however, MAIT cells in some of the patients displayed a transcription factor phenotype that was not characteristic of MAIT-1, MAIT-2, or MAIT-17. The implications of this shift, and the new skew of the MAIT cells, remains an area for further exploration. Considering this data, our working hypothesis is that FMT serves to reverse MAIT cell exhaustion following rCDI. As more patients are enrolled in the study, stronger conclusions can be drawn. The overall findings of this study and a summary of future directions can be seen in Figure 12.

This study is to our knowledge the first to examine longitudinal changes in human MAIT cell phenotypes and functions following FMT treatment for rCDI. This unique opportunity, to explore both the fundamental biology of MAIT cells and their place in a novel healthcare setting, is not one that is widely available. According to the Canadian Agency for Drugs and Technologies in Health, there are at least 7 centers offering FMT in Canada, with some able to treat up to 80 patients a year[166]. They also note considerable barriers to accessibility and availability of FMT[166]. Our geographical location and collaboration with Dr. Silverman at St. Joseph's Hospital therefore allowed us to examine a broad array of MAIT cell markers to begin to elucidate their role in the context of FMT. This broad phenotypic and functional characterization of MAIT cells paves the way for better understanding this unique cell subset and the immunology behind FMT.

If FMT corresponds to phenotypic and functional changes in the MAIT cell compartment, the next step would be to determine if MAIT cells are protective or pathogenic in the context of FMT. As the ligand presentation molecule MR1 is monomorphic, and therefore the same in everyone in the population, therapies which target MR1 and MAIT cells would be widely applicable to the population at large[86,87]. The use of MAIT cell activating or inhibitory ligands/ ligand producing bacteria, depending on if they are protective or pathogenic, could thus serve to supplement a lower FMT dose. Considering the costly and laborious process of FMT donor recruitment, stretching each donation to cover more patients would make the therapy more accessible[67]. This study lays the foundation for future exploration into harnessing the immunotherapeutic potential of MAIT cells.

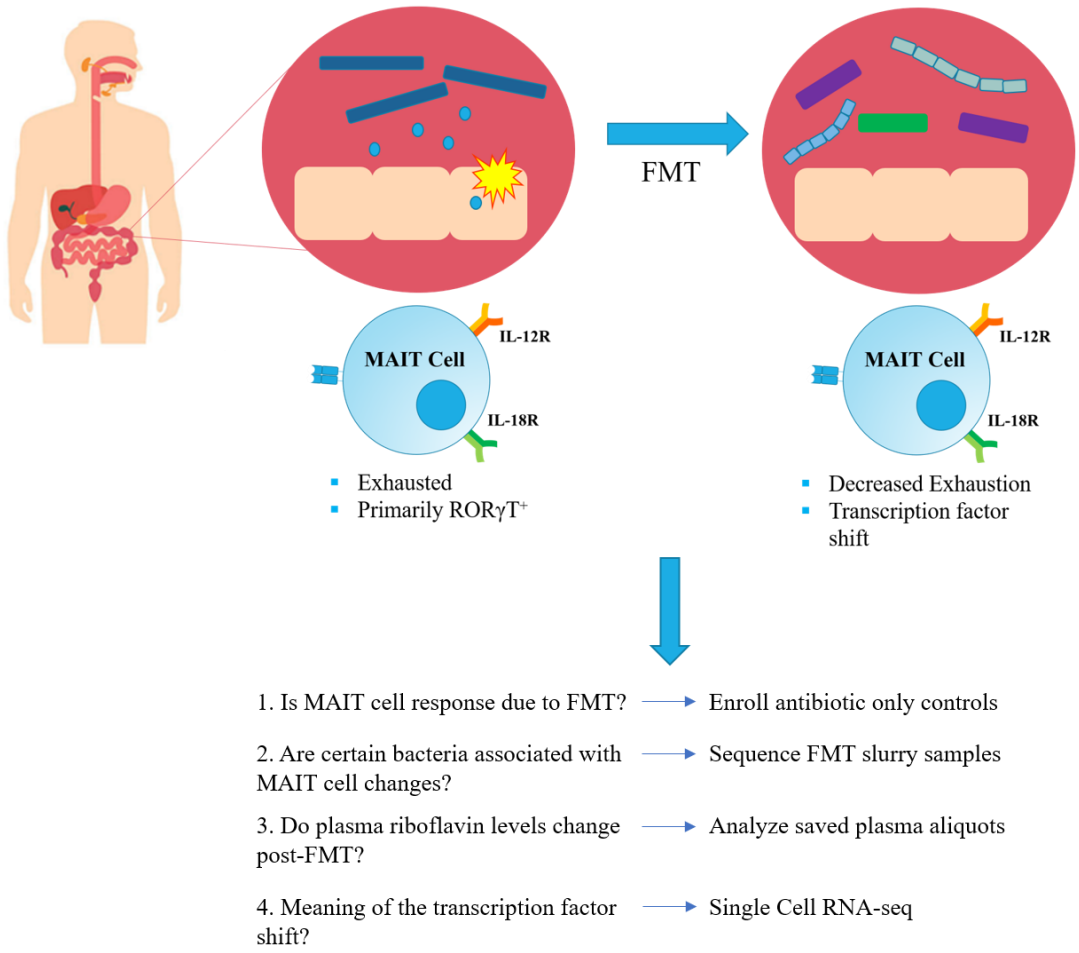


Figure 12. Alterations in MAIT Cell Compartment Post-FMT and Future Directions. Pre-FMT MAIT cells displayed notable levels of exhaustion markers and were primarily RORγT^{+}. 1-week post-FMT, the expression of multiple exhaustion and activation markers trended towards a decrease, and cytokine producing capacity trended upwards. MAIT cells also shifted towards a transcriptional phenotype that was not consistent with MAIT-1, MAIT-17, or MAIT-2. In addition to increased patient enrollment, controls receiving antibiotics alone for CDI will be recruited. Slurry samples from the FMT donors will be sequenced to determine bacterial composition, and if certain bacteria are associated with changes in the MAIT cell compartment. Similarly, plasma aliquots will be analyzed to examine if there are changes in the levels of riboflavin or riboflavin derivatives following FMT. Finally, the phenotype of the MAIT

cells that underwent a transcriptional shift will be evaluated using single cell RNA-seq in order to understand their purpose.

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Appendices

Appendix 1. REB Approval

Western Research

Date: 29 August 2019

To: Dr. Mansour Haeryfar

Project ID: 114353

Study Title: Examining the effect of fecal microbiota transplant for Clostridium difficile infection on antimicrobial T cells

Application Type: HSREB Initial Application

Review Type: Delegated

Full Board Reporting Date: September 17, 2019

Date Approval Issued: 29/Aug/2019

REB Approval Expiry Date: 29/Aug/2020

Dear Dr. Mansour Haeryfar

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
Data Collection Cdiff FMT WREM 114353	Other Data Collection Instruments	10/Jul/2019	v1
Protocol Research Plan 114353 v1 July 9 2019	Protocol	09/Jul/2019	v1
WREM 114353 Letter of Information and Consent FMT Control v2 August 20 2019	Written Consent/Assent	20/Aug/2019	v2
WREM 114353 Letter of Information and Consent FMT Enema v1 August 20 2019	Written Consent/Assent	20/Aug/2019	v1
WREM 114353 Letter of Information and Consent FMT Oral v1 August 20 2019	Written Consent/Assent	20/Aug/2019	v1

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Karen Gopaul, Ethics Officer on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Appendix 2. Letter of Information and Consent Form

Western Medicine&Dentistry
Schulich School of Medicine & Dentistry

St JOSEPH's HEALTH CARE LONDON

Letter of Information and Consent – Oral FMT Group

Project Title: Examining the effect of fecal microbiota transplant for *Clostridioides difficile* infection on antimicrobial T cells

Principal Investigator:
Mansour Haeryfar, Doctorate (Lab Medicine), M.Sc., Ph.D. **Departments of Microbiology & Immunology, Medicine, and Surgery** **Phone: 519**[redacted] **Email:** [redacted]
Co-Investigator:
Michael Silverman, MD, Department of Medicine

Sponsor/Funder Information:
This study is being sponsored by the Principal Investigator, Dr. Mansour Haeryfar, using Western University Funds provided to him for research.

Conflict of Interest:
There are no conflicts of interest to declare related to this study.

Invitation to Participate in this Research Study:
You are being invited to participate in a research study because you have symptoms suggestive of a recurrent *Clostridioides difficile* infection (RCDI). Your contribution in this study could help to improve the treatment of future RCDI patients. Participation in this study is voluntary. Please take the time to read the contents of this letter and ask any questions you need to ask before deciding to participate. Your decision to participate or not to participate will have no effect on your standard care.

Purpose of the Study:
The purpose of this study is to increase our understanding of powerful immune cells in the body and their role in RCDIs. These cells play an important role in protecting your body from infections and foreign invaders. In this study we will compare these immune cells in the blood at various time points. Time points will vary depending on which treatment(s) you have chosen to receive. Some participants in this study will have chosen to receive only antibiotics and some will have chosen to have antibiotics

followed by fecal microbiota transplant (FMT). We will determine if these immune cells can predict if a patient will suffer from more RCDIs or infections that are more difficult to treat. We will also determine the effect of FMT on the function of these immune cells. Please note that your treatment course is determined between you and your doctor and is in no way related to your participation in this study.

How Many People Will Take Part in This Study?

30 RCDI patients in total will be recruited at the Infectious Disease Care Program (St. Joseph's Hospital). It is expected that you will be in this research study for one year, most of this time will be to monitor your hospital health records for the occurrence of new CDIs. This study should take two years to complete. The results of this study are for research purposes only. Our findings will not be reported to the participants enrolled in this study. Our findings will not have any relevance to the health care of study participants.

What are the Study Procedures?

- All study group participants will be given antibiotics to treat the infection (this is standard of care for all RCDI patients). You will complete your antibiotic treatment and 24 hours after taking the last dose you will come to the Infectious Disease Care Program Clinic to receive your FMT treatment via the oral capsule route. Just before receiving the FMT treatment you will provide a 50ml blood sample which will be given to the Haeryfar research lab (Western University) for analysis.
- You will then come to the Infectious Disease Care Program Clinic to provide a second 50ml blood sample 7 days after you provide the first blood sample. This blood sample will also be given to the Haeryfar research lab (Western University) for analysis.
- You will then come to the Infectious Disease Care Program Clinic to provide a third 50ml blood sample one month after you provide the first blood sample. This blood sample will also be given to the Haeryfar research lab (Western University) for analysis.
- Please note that your decision to receive oral FMT treatment is made between you and your treating physician and is no way related to your participation in this study.
- Your first study sample will be taken during a standard clinic visit and will only take an extra 5 minutes of your time. The second and third study visits are not standard clinic visits and will require roughly 20 minutes of your time per visit.
- We will use data from your hospital chart and combine it with the results from experiments on your blood samples to understand the role of special immune cells in RCDIs. The research experiments will focus on:
    1. Measuring how many immune cells are in your blood after antibiotic treatment.
    2. Looking at whether these immune cells are functioning normally.

3. Trying to figure out whether a patient is likely to suffer from more RCDIs or infections that are more difficult to treat based on our immune cell research results.

Research Database:
We will collect blood samples and information from your hospital chart for research. If you agree to participate, data relating to you and your health history (age, past medical conditions), current condition (diagnostic data), and future infections will be copied from your hospital records to a separate computerized research database called REDCap. REDCap is a secure, web-based application for building and managing online databases. The REDCap database is hosted at the hospitals' data center and is administrated by Lawson Health Research Institute. Your name and hospital PIN will be removed and will be exchanged for a unique code that will link your research record and urine samples with your hospital chart. This link will allow the information in the research database to be verified, while continuing to protect your confidentiality and privacy. Study data will be also be stored on a computer (server) in a secure location at the Western University. Your biological samples will be used immediately, within 48 hours of collection. We will follow your hospital chart for up to one year to follow your infection outcome and record any future RCDIs. Data will be kept confidential and stored in a secure location for 15 years, according to Lawson Health Research policy. Access to all data will be secure and password protected. You may withdraw your sample(s) from the study at any time by contacting the principal investigator, Dr. Mansour Haeryfar at 519[redacted].

Risks of Participation:
The sample blood collections are not part of your standard of care and would be for research purposes only. You may experience some bruising at the site where blood is taken from, but this should resolve within a day or so. There is also a chance that you will experience some mild dizziness or light-headedness because of the blood draw. This risk is small due to the small amount of blood being drawn. There are risks associated with providing your health information and protection of privacy which are described below.

Benefits of Participation:
There are no direct benefits to participating in this study. Patients enrolled in this study will be receiving the standard of care. Your participation will have no impact on your care.

Confidentiality and Protection of Privacy:
All information in the database will be password protected, will not contain any personal identifiable information, and will only be accessed by members of the study team. All identifiable information collected for the study (name, partial date of birth, and hospital ID number) will be stored in a secure location and only the study investigator and study coordinator will have access to it. Your personal health

information (hospital chart) will only be accessed by the study coordinator for the defined length of the study follow-up (one year). If the results of the research are published or presented at scientific meetings, your name will not be used and no information that discloses your identity will be released or published.

Risks of Providing Health Information for Research Use:
As noted above, we have designed this project with multiple safeguards to protect your privacy and confidentiality. There is, nevertheless, a very small chance that your personal information could accidentally become known to employees of this institution or others who are not associated with this study. In this case information could potentially be used to discriminate against you socially, in insurance, employment or other areas.

Withdrawal of Consent:
Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the current study at any time with no effect on your future care. You do not waive any legal rights by signing the consent form. You may withdraw your consent from the study at any time by contacting the principal investigator, Dr. Mansour Haeryfar at 519-[redacted]. Once consent is withdrawn we will no longer access your personal health records to collect data. If you withdraw consent before your urine sample has been analyzed, the sample will be destroyed. Any study data collected up to the time of withdrawal of consent may still be used for study purposes.

Reimbursement:
You will be compensated $100 for each study visit you attend. This will cover costs related to your participation in this research study (e.g. travel, parking, meal). Should you choose to withdraw from the study you will keep all compensation for study visits you have already attended.

Representatives of Western University Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research.

The Quality Assurance and Education Officers from Lawson Health Research Institute (Lawson) may audit this research study for quality assurance purposes.

Questions and Help:

If you have any questions about the research or the study database, you may contact the **Research Coordinator: Crystal Engelage (519)[redacted] or the Principal Investigator: Dr. Mansour Haeryfar at 519[redacted].**

If you have any questions/concerns about your rights as a research participant or the conduct of this study, please contact: **St. Joseph's Health Care London Patient Relations Consultant at** [redacted]

This letter is for you to keep.

Western Medicine&Dentistry
Schulich School of Medicine & Dentistry

St JOSEPH's HEALTH CARE LONDON

Consent Form – Oral FMT Group

Examining the effect of fecal microbiota transplant for *Clostridioides difficile* infection on antimicrobial T cells

This study has been explained to me and any questions I had have been answered.

I know that I may leave the study at any time. I agree to take part in this study.

Printed Name of Participant	Signature of Participant	Date (DD-MON-YYYY)

My signature means that I have explained the study to the participant named above. I have
answered all questions.

Printed Name of Person Obtaining Consent	Signature of Person Obtaining Consent	Date (DD-MON-YYYY)

Appendix 3. Data Collection Form

Demographics

ID: _____

Gender: ____

Height: _____

Weight: _____

BMI (calculated):_____

Date of birth: _____ (mmm-yyyy)

Smoker: ____ current/former/no

Comorbidities

Diabetes: _____

Immunologic Disease (eg. Rheumatoid Arthritis, HIV): _____

Other Conditions: _____

RCDI Diagnosis Data

GDH test results: _____

C. difficile toxin PCR testing results: _____

Antibiotic prescribed before referral: _____

Dose and length of treatment: _____

Comments:_____
_____
_____
_____

Infectious Disease Care Program Data

Date of referral: _____

Antibiotic prescribed: _____

Dose and length of treatment: _____

FMT treatment: _____ yes or no

If yes: oral capsule _____ Date of FMT treatment: _____ (dd-mmm-yyyy)

or colonoscopy/enema _____ Date of FMT treatment #1: _____ (dd-mmm-yyyy)
Date of FMT treatment #2: _____ (dd-mmm-yyyy)

Infection Cleared: _____ yes or no

If no, what is the follow-up treatment
course:_____
_____
_____

Comments:
_____
_____
_____

Recurrence History

Recurrence: _____ no/yes/unknown

Date(s) of recurrence: _____ (dd-mmm-yyyy)

_____ (dd-mmm-yyyy)

_____ (dd-mmm-yyyy)

Details:_____
_____
_____

Notes:
_____
_____
_____
_____

Appendix 4. R Code for PCA Analysis

```
read.csv("FMTdata.csv")
FMTdata<-read.csv("FMTdata.csv")
rownames(FMTdata)<-FMTdata[ ,1]
#the above says to use column one as the row names
FMTdata.pca <- prcomp(FMTdata[,c(2:14)], center = TRUE,scale. = TRUE)
#skipped the first column because it is patient number and timepoint
library(usethis)
library(devtools)
library(ggplot2)
 library(plyr)
 library(scales)
 library(grid)
 library(ggbiplot)
 FMTtime<-c(rep("preFMT", 2), "1wkpost", "preFMT", "1wkpost", "preFMT", "1wkpost", "1monthpost", "preFMT", "1wkpost", "1monthpost")
 #the above assigns the correct timepoint identifier to each row
 FMTpt<-c("Pt1", rep("Pt2", 2), rep("Pt3",2), rep("Pt4", 3), rep("Pt5", 3))
 #the above assigns the correct patient number to each row
 #Var scale 2 makes resulting graph less compressed, easier to read labels
 ggbiplot(FMTdata.pca,ellipse = TRUE, obs.scale = 1,var.scale = 2,groups = FMTpt, varname.size = 3)
 ggbiplot(FMTdata.pca,ellipse = TRUE, obs.scale = 1,var.scale = 2,groups = FMTtime, varname.size = 3)
```

Curriculum Vitae

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