Metagenomic etiology and therapy in pediatric gastrointestinal inflammation

PhD thesis

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INTRODUCTION

Dietary influences may have an impact on microbiome composition and host immune responses, thereby modulating susceptibility toward inflammatory bowel diseases (IBDs): Crohn disease (CD) and ulcerative colitis (UC). The incidence of IBD peaks in young adulthood indicating that pediatric environmental exposures may be important in the etiology of this disease group.

Decreased consumption of dietary fibers, such as cellulose, has been proposed to promote the emergence of IBDs. It is also known that intestinal microbes are recognized to play an etiologic role in the development of CD and UC. However, it is not known if transient fiber consumption during critical developmental periods may prevent consecutive intestinal inflammation. Dietary ω-6 fatty acids have been associated with UC in prospective studies. However, the critical developmental period

when ω-6 consumption may induce UC is not known.

Complex bacteriotherapy, such as fecal microbiota transplantation (FMT), is an emerging therapeutic modality for recurrent *Clostridium difficile* infection (CDIF) and also in IBD. However, the complex mechanism behind FMT is unknown.

HYPOTHESES

1. There is a direct link between nutrition, microbiome and host response.
2. Postnatal exposure to different nutrients (such as cellulose or fat) has transient or persistent effect on intestinal homeostasis and predispose to the development for intestinal inflammation.

We studied the effects of transient dietary cellulose supplementation on dextran sulfate sodium (DSS) colitis susceptibility during the pediatric period in mice. Additionally, the effects of transiently

increased ω-6 consumption during pediatric development on subsequent DSS-induced acute murine colitis were also examined.

3. Complex bacteriotherapy, such as FMT provides treatment and/or the resolution of symptoms for patients suffering in CDIF and IBD.

Furthermore, we established phase 1 clinical trials for the treatment of CDIF and UC pediatric patients by FMT. We examined the consequent microbial changes in our pediatric cohorts following FMT.

METHODS

Nutritional supplementation in mouse model

C57BL/6J mice were used to study the dietary effects of fiber (12.5% vs. 2.5% cellulose) and fat (40% vs. 12% caloric content corn oil/linoleic acid, ω-6 fatty acid) supplementation. Mice were transiently treated between postnatal day (P) 30 and P90/P120 to examine the transient

nutritional effects. Dextrane sulfate sodium was used to induce colitis. Pooled cecal contents were used to transplant microbiome composition into SWGF germ-free mice to examine the effect of dietary altered microbiome.

Flow cytometry and *in vivo* chemokine blocking

Flow cytometry and *in vivo* chemokine blocking was used to understand the underlying immunological pathways of Cxcr5-CXCL13.

ELISA (Enzyme-linked Immunosorbent Assay)

Sera of 12 ulcerative colitis (UC), 11 Crohn's disease (CD) and 10 healthy controls (C) were used for quantification of circulating CXCL13 levels by ELISA.

Fecal microbiota transplantation protocol

We developed a protocol for a standardized and safe donor recruitment, screening and stool preparation. The donor screening and process was approved by the US Food and Drug Administration.

Pediatric CDIF patients received filtered, frozen-thawed fecal preparation from a single, screened, standardized or a self-designated donor through colonoscopy, followed by enema or nasogastric consecutive FMT, if clinically indicated. The CDIF patients received the therapy only one time (or a second time if there was no clinical improvement in their condition.

UC patients received a sequential therapy of more FMTs: Initial colonoscopy and FMT treatment (Day 1): At the time of colonoscopy, an assessment for macroscopic colitis using the Mayo classification was performed. Biopsies were obtained from the rectosigmoid and cecum in an ascending fashion for routine histopathology and research purposes. Following mucosal sampling, subjects underwent FMT with 250 ml of thawed stool preparation, 1/3 of which was endoscopically administered into the terminal ileum and 2/3 into the right colon as targeted site.

Subsequent FMT Treatments: The duration of FMT therapy was planned to be 12 weeks initially. Days 2 through 14: Subjects came to the ambulatory clinic daily for clinical symptom evaluation and fecal retention enema administration (60-250 ml rectally [as tolerated] with retention for at least 30 minutes). Days 15 through 28: Enemas were given 3 times a week on weeks 3 and 4 of the protocol. Days 29-84 (2 to 3 months): Enemas were given weekly for a total of 3-8 weeks (less than 8 secondary to the cessation of the protocol according to the FDA mandate).

During the IND study (P001-P006, this protocol does not involve the first 3 patients from the pilot study), monthly enema was provided up to a year.

As supportive care, UC patients were allowed to take 4 mg (2 tablets of over the counter Imodium) loperamide by mouth 15-30 minutes prior to enema treatments to help retain the preparation.

Response and progression was monitored by PUCAI during the protocol. The clinical symptoms survey was performed prior to each enema delivery and a disease progression table was recorded for each enrolled patient. Additionally, adverse events were monitored throughout the protocol.

Metagenomic studies

Microbial studies were initiated on murine colonic mucosal samples and stool samples of pediatric patients with CDIF and UC. The fecal microbiome was characterized using 454 pyrosequencing of the bacterial *16S rRNA* gene on samples. Community DNA was extracted from each specimen using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). Barcoded universal primers 357F (5'-CCTACGGGAGGCAGCAG-3') and 926R (5'-CCGTCAATTCMTTTRAGT-3') were used to amplify the V3V5 region of the bacterial *16S rRNA* gene. Each library construct was then

processed and purified for 454 sequencing. Sequencing was performed on the Roche GS FLX 454 sequencer (454 Life Sciences, Branford, CT, USA).

Sequence data was parsed by barcode and quality filtered using QIIME (version 1.8.0). Sequences were pooled and assigned to operational taxonomic units (OTUs) at a similarity cut off of 97% using an open-reference OTU picking strategy employing the UCLUST algorithm and the GreenGenes13.8 reference database in QIIME. The data set was screened for potential chimeras using the ChimeraSlayer algorithm, and all potential chimeras identified among the *de novo* OTUs were excluded from downstream analysis. Identities were assigned to each OTU using the Ribosomal Database Project Classifier, with the Greengenes reference database (version 13.8) serving as its training set. Microbial data analysis involved the evaluation of richness, calculation of diversity

indices, including the Shannon diversity index and unweighted UniFrac distance measures. The results from the microbiome characterization of the donor were compared to the patient's microbiome prior and after transplantation. The murine study characterized the microbiome alteration upon different dietary changes.

Statistics

Nonparametric, two tailed Mann-Whitney-tests were utilized for richness calculation; parametric, two-tailed Student's T-test and Spearman correlation calculations were used in the analyses of OTU number changes and group comparisons. The statistical significance was declared at $p<0.05$. The significance of microbial data was corrected with Bonferroni. Error bars represent standard error of the mean (SEM). Prism4.03 software was used for statistical calculations.

RESULTS

Cellulose supplementation stimulated substantial shifts in the colonic mucosal microbiome. Several bacterial taxa decreased in relative abundance (Coriobacteriaceae $p=0.001$), and other taxa increased in abundance (Peptostreptococcaceae $p=0.008$ and Clostridiaceae $p=0.048$). Some of these shifts persisted for 10 days following the cessation of cellulose supplementation. The changes in the gut microbiome were associated with transient trophic and anticolitic effects 10 days following the cessation of a cellulose-enriched diet, but these changes diminished by 40 days following reversal to a low cellulose diet.

Upon temporary high ω-6 fat diet, mice transiently became obese then rapidly lost this phenotype. Interestingly, mice were protected against DSS colitis 40 days after ω-6 consumption. The transient high ω-6 induced protection against

colitis was fat type- and dietary reversal-dependent and could be transferred to germ-free mice by fecal microbiota transplantation. We also detected decreased numbers of Cxcr5(+) CD4(+) T cells in the mesenteric lymph nodes (MLNs) of transiently ω-6 fed mice. Further experiments revealed that anti-chemokine ligand (Cxcl)13 (the ligand of Cxcr5) antibody treatment decreased DSS colitis severity, implicating the importance of the Cxcr5-Cxcl13 pathway in mammalian colitis. Consecutively, we found elevated CXCL13 concentrations (CD: 1.8-fold, p=0.0077; UC: 1.9-fold, p=0.056) in the serum of untreated pediatric IBD patients. The human serologic observations supported the translational relevance of our findings.

Based on our clinical trial findings, all 4 CDIF patients without underlying disease had resolution of their symptoms for more than 2 months following a single FMT. However, the

CDIF symptoms of patients complicated with co-morbidities had not been resolved upon single FMT.

Serial FMT transiently supported immunotherapy withdrawal in pediatric UC patients in our small pilot study (3 patients). However, our IND-linked extended study provided us controversial results. Only one patient remained in remission for more than 6 months. The results can be explained by the severity of UC patients disease. We found that the length of clinical remission was negatively correlated with mucosal disease activity at the initiation of FMT ($r=-0.845$, Spearman: $p=0.033$).

Adverse events were monitored during our extended study (101 FMT). Altogether, 17 adverse events were recorded mostly related to the anesthesia of colonoscopy, or related to upper respiratory infections. One time, fever was

presented specifically related to FMT, and probably to treatment failure and UC exacerbation.

Correlative metagenomic studies showed shifts in our pediatric CDIF and UC patients towards healthy pediatric microbiome composition with FMT. However, cessation of FMT in UC patients resulted in return to the original composition along with disease recurrence in those who transiently benefitted from our treatment protocol. The shift in microbiome was significantly larger in CDIF patients even upon single FMT compared to UC patinets following multiple FMT.

CONCLUSIONS

1. Fiber supplementation studies emphasize the transient protective effect of dietary cellulose in the mammalian large bowel and highlight the potential role of dietary fibers in amelioration of intestinal inflammation.
2. Transient high ω-6 diet during pediatric development in mice induced prolonged

protection against murine colitis, which associated with persistent colonic mucosal microbiome and lymphoid organ composition changes (Cxcr5-Cxcl13 pathway).

3. As a result of our translational studies, we found that FMT is safe in pediatric CDIF and UC patients.
4. CDIF patients without underlying disease had resolution of their symptoms for more than 2 months following a single FMT. However, the CDIF symptoms of patients complicated with co-morbidities had not been resolved upon single FMT.
5. Our results indicate that FMT is safer and more effective in UC patients who start the treatments with endoscopic remission (endoscopic Mayo score 0-1 without pseudopolyps). Indeed, endoscopic mucosal disease status at treatment initiation and clinical remission by FMT during withdrawal of

immunotherapy were negatively correlated in pediatric UC patients. We hypothesize that this may be related to an inhibition of the dynamic interaction between the gut microbiome and the colonic mucosa during active disease. Such inhibition would limit the success of microbiome based therapeutic interventions in UC during disease flares secondary to host unresponsiveness. Indeed, gene expression and gut microbiome associations have been shown to be decreased in clinically active UC patients compared to controls.

Also, we need to highlights the significances of our unique clincial study: Our protocol is the only immunotherapy withdrawal study of FMT in UC to date. This characteristic created a strong internal control for therapeutic efficacy of FMT within each patient compared to prednisone treatment. All of our patients were children or young adults without any co-

morbidity, making the recipients potentially ideal candidates for this experimental treatment. The majority of our patients had short disease duration (3-43 months) prior to FMT, which according to prior literuters may have led to an increase in FMT efficiency. The intensity of FMT therapy was considerably higher than other FMT trials to date.

6. Correlative metagenomic studies showed robust alteration in CDIF patients following even one FMT, and also a shift in UC microbiome following several FMT. The microbial changes were greater in CDIF compared to UC patients. Furthermore, the microbiome alteration was transient in UC. Cessation of FMT resulted in return to the original composition along with disease recurrence in those who short-termed benefitted from our treatment protocol. The majority of recipients received stool from a

single donor, limiting biologic variation on the donor side.

Our study underscores that transient dietary changes during critical periods of mammalian development can lead to the prolonged and concomitant modulation of the host-microbiome network relevant for intestinal inflammation. These findings may bare important implications for the nutritional developmental origins of IBD and promote the future development of novel preventive and therapeutic solutions for this disease group. Furthermore, complex bacteriotherapy, such as fecal microbiota transplantation provides the resolution of symptoms for pediatric CDIF and UC patients. Compound alteration of microbiome is seen following FMT, where patients microbiome moved towards the microbiome composition of healthy individuals.

RELATED PUBLICATIONS OF THE AUTHOR

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