INFLAMMATORY BOWEL DISEASE

Unfermented β-fructan Fibers Fuel Inflammation in Select Inflammatory Bowel Disease Patients

Heather K. Armstrong,[1,2,3] Michael Bording-Jorgensen,[1,2] Deanna M. Santer,[4] Zhengxiao Zhang,[1,5,6] Rosica Valcheva,[1,5] Aja M. Rieger,[7] Justin Sung-Ho Kim,[8,9] Stephanie I. Dijk,[1,10] Ramsha Mahmood,[3] Olamide Ogungbola,[4] Juan Jovel,[1] France Moreau,[11] Hayley Gorman,[11] Robyn Dickner,[1,2] Jeremy Jerasi,[1,2] Inderdeep K. Mander,[1,2] Dawson Lafleur,[1,2] Christopher Cheng,[1,2] Alexandra Petrova,[2] Terri-Lyn Jeanson,[3] Andrew Mason,[1,5] Consolato M. Sergi,[12] Arie Levine,[13] Kris Chadee,[11] David Armstrong,[8] Sarah Rauscher,[8,9,14] Charles N. Bernstein,[3] Matthew W. Carroll,[2] Hien Q. Huynh,[2] Jens Walter,[1,15] Karen L. Madsen,[1,5] Levinus A. Dieleman,[1,5] and Eytan Wine[1,2,10]

[1]Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, Edmonton, Alberta, Canada; [2]Department of Pediatrics, University of Alberta, Edmonton, Alberta, Canada; [3]Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada; [4]Department of Immunology, University of Manitoba, Winnipeg, Manitoba, Canada; [5]Department of Medicine, Division of Gastroenterology, University of Alberta, Edmonton, Alberta, Canada; [6]College of Food and Biological Engineering, Jimei University, Xiamen, Fujian, China; [7]Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada; [8]Department of Chemical and Physical Sciences, University of Toronto Mississauga, Mississauga, Ontario, Canada; [9]Department of Physics, University of Toronto, Toronto, Ontario, Canada; [10]Department of Physiology, University of Alberta, Edmonton, Alberta, Canada; [11]Department of Microbiology, Immunology and Infectious Disease, University of Calgary, Calgary, Alberta, Canada; [12]Anatomic Pathology Division, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada; [13]Pediatric Gastroenterology Unit, Wolfson Medical Center, Tel-Aviv University, Holon, Israel; [14]Department of Chemistry, University of Toronto, Toronto, Ontario, Canada; and [15]APC Microbiome Ireland, School of Microbiology, and Department of Medicine, University College Cork, Cork, Ireland

BACKGROUND & AIMS: Inflammatory bowel diseases (IBD) are affected by dietary factors, including nondigestible carbohydrates (fibers), which are fermented by colonic microbes. Fibers are overall beneficial, but not all fibers are alike, and some patients with IBD report intolerance to fiber consumption. Given reproducible evidence of reduced fiber-fermenting microbes in patients with IBD, we hypothesized that fibers remain intact in select patients with reduced fiber-fermenting microbes and can then bind host cell receptors, subsequently promoting gut inflammation. **METHODS:** Colonic biopsies cultured ex vivo and cell lines in vitro were incubated with oligofructose (5 g/L), or fermentation supernatants (24-hour anaerobic fermentation) and immune responses (cytokine secretion [enzyme-linked immunosorbent assay/meso scale discovery] and expression [quantitative polymerase chain reaction]) were assessed. Influence of microbiota in mediating host response was examined and taxonomic classification of microbiota was conducted with Kraken2 and metabolic profiling by HUMAnN2, using R software. **RESULTS:** Unfermented dietary β-fructan fibers induced proinflammatory cytokines in a subset of IBD intestinal biopsies cultured ex vivo, and immune cells (including peripheral blood mononuclear cells). Results were validated in an adult IBD randomized controlled trial examining β-fructan supplementation. The proinflammatory response to intact β-fructan required activation of the NLRP3 and TLR2 pathways. Fermentation of β-fructans by human gut whole microbiota cultures reduced the proinflammatory response, but only when microbes were collected from patients without IBD or patients with inactive IBD. Fiber-induced immune responses correlated with microbe functions, luminal metabolites, and dietary fiber avoidance. **CONCLUSION:** Although fibers are typically beneficial in individuals with normal microbial fermentative potential, some dietary fibers have detrimental effects in select patients with active IBD who lack fermentative microbe activities. The study is publicly accessible at the U.S. National Institutes of Health database (clinicaltrials.gov identification number NCT02865707).

Keywords: Dietary Fibers; Microbiome; IBD; Fermentation.

Digestible carbohydrates are degraded in the small intestine; nondigestible carbohydrates (fiber and resistant starch) are fermented by colonic microbes.[1] Fermentation of dietary fibers produces gases, lactate, and short chain fatty acids (SCFAs),[2,3] with multiple beneficial physiological effects,[3] but fibers have also been shown to be harmful in select situations.[4,5] The beneficial potential for fermentable fibers in inflammatory bowel diseases (IBD) is demonstrated by low SCFA production, especially in

ulcerative colitis (UC), linked to absence of SCFA-producing microbes.[6] Administration of β-fructan fibers improved mild UC, associated with increased SCFA (butyrate) production.[2,7] However, this generally positive effect of fibers related to fermentation and SCFA production seems to have overshadowed potential detriments, as many patients with IBD describe sensitivity to fiber consumption[8]; ignoring or not understanding this process can lead to avoiding nondigestible fibers altogether through exclusion diets.[9–11] Such exclusion diets can improve symptoms but may deprive patients of the benefits of fibers, which are especially important in IBD.[2]

Although research has intensified on the role of fiber fermentation in IBD, and β-fructan fibers in particular have been gaining attention for their prebiotic potential (promoting growth of "beneficial microbes"),[7,12] the role of microbiota and fiber fermentation processes, and whether they are beneficial or detrimental, remains poorly understood. Structurally, dietary fibers (Supplementary Table 1) and cell wall components of microorganisms (eg, fungal β-[1,3]glucans) are polymers of more than 3 sugars (oligofructose [FOS] $\sim$8 sugars; grain β-D-glucan $\sim$3 sugars) and ranging up to 50 to 100 sugars (inulin; fungal β-(1,3) glucan), which can vary in their degree of polymerization (DP), branching, solubility, and interactions with host cells. Immune response to polysaccharides on the surface of fungal cells suggests a possible link between whole unfermented fibers and inflammation.[13,14] β-(1,3)glucan on the surface of fungi (eg, zymosan, curdlan) interacts with immune cells (eg, macrophages), inducing proinflammatory antifungal immunity via Dectin-1 and TLR2.[13,14] Similarly, β-fructan fibers (inulin and FOS) induce TLR-mediated inflammatory pathways.[15,16] This led us to hypothesize that in patients with reduced fiber-fermenting microbes (eg, IBD), dietary fibers could remain intact, interact with host cell receptors, and promote gut inflammation. Here we demonstrate that unfermented dietary β-fructans induce proinflammatory cytokine secretion in select patients with IBD, mediated by microbial functions. Our data suggest that select fibers may be detrimental in individuals lacking fermentative microbes (eg, IBD, other chronic illnesses, antibiotic use), with increased opportunity for interactions between host immune cells and luminal contents (due to increased immune cells and disrupted epithelial barrier). These same fibers provide health benefits in individuals with high fermentative potential.

Materials and Methods

Complete methods are available in the supplementary materials.

Consent and Ethics Approval

Consent/assent was obtained from patients/guardians; approved by the University of Alberta Health Research Ethics Board (Study IDs Pro00023820 and Pro00092609), Edmonton, AB, Canada. Blood donors consented for isolation of human cells (Study ID Pro00046564). The randomized control trial (RCT) protocol was approved by Ethics Board at the University of Alberta (Study ID Pro00041938) and Natural Health

WHAT YOU NEED TO KNOW
BACKGROUND AND CONTEXT
Inflammatory bowel diseases are overall beneficially impacted by dietary fibers that are fermented by colonic microbes. However, not all fibers are alike, and patients report intolerance to fiber consumption.
NEW FINDINGS
Unfermented dietary β-fructan fibers induced proinflammatory cytokines in a subset of inflammatory bowel disease patient samples, via activation of the NLRP3 and TLR2 pathways; inflammation was reduced via fermentation by microbes.
LIMITATIONS
Fiber purity was confirmed, yet microbial contaminants may be present. Mucosal microbes were collected, which are less affected by bowel preparation; however, luminal microbiota are also important in fiber fermentation.
CLINICAL RESEARCH RELEVANCE
Patients describe a sensitivity to dietary fibers; however, detrimental effects have been largely overlooked to date. Our data support further clinical investigations of the detrimental effects of specific dietary fibers and support progression of personalized dietary fiber interventions designed to increase consumption of fibers that are safe for an individual, while avoiding detrimental fibers.
BASIC RESEARCH RELEVANCE
Although fibers are typically beneficial in individuals with normal microbial fermentative potential, some dietary fibers have detrimental effects in select patients with active inflammatory bowel disease who lack fermentative microbe activities. Here we show for the first time that unfermented β-fructan fibers induce inflammation via TLR2 and NLRP3 pathways.

Directorate at Health Canada. The study is publicly accessible at the U.S. National Institutes of Health database (clinicaltrials.gov identification number NCT02865707).

Patient Criteria and Sample Collection

Patients aged 3 to 18 years, with histologic and endoscopic confirmed Crohn disease (CD) or UC, or non-IBD controls undergoing colonoscopy for symptoms suspected to be IBD, confirmed as normal (Supplementary Table 1). Patient sample collection scheme provided (Supplementary Figure 1) is explained in depth in the Supplementary Methods section.

Cell Lines and Reagents

Cultures were incubated (37°C, 5% CO_2) and maintained as described in the Supplementary Methods. Cell lines included human THP-1 macrophage (Supplementary Methods) and T84 cells (Dulbecco's modified Eagle's medium/F12, 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate). Peripheral blood mononuclear cells (PBMCs) were isolated from non-IBD individuals with Lymphoprep Density Gradient Medium (STEMCELL Technologies, Vancouver,

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Canada). All cultures were treated with 250 μL of fiber or fructose (Sigma-Aldrich, St Louis, MO) prepared in phosphate-buffered saline and 5% culture media (Supplementary Table 2). Inhibition studies used YVAD (50 μM), Glyburide (200 μM), MCC950 (1 μM), or TL2-C29 (75 μM) for 1 hour.

Ex Vivo Culture of Patient Biopsy Tissues

Biopsy tissues collected from noninflamed regions during colonoscopy were dissected into 1-mm^3 pieces and cultured in duplicate (Supplementary Methods) with 250 μL fiber solution (phosphate-buffered saline, fiber, 5% Dulbecco's modified Eagle's medium/F12 [5% fetal bovine serum], 100 U/mL penicillin/100 μg/mL streptomycin) at 37°C for 24 hours. Supernatants were collected for enzyme-linked immunosorbent assay (ELISA) and biopsies were transferred to lysing matrix D bead beat tubes (MPbio) with 500 μL TRIzol and stored at -80°C for RNA isolation.

ELISA

Supernatants were centrifuged at 14,000g for 10 minutes to remove debris. Secreted interleukin (IL)-1β was measured following the manufacturer's protocol (R&D Systems, Minneapolis, MN). Sample/standard were added in duplicate; absorbance was measured at 450 nm with 540 nm; correction calculated using GraphPad Prism (LaJolla, CA). Alternatively, multiplex ELISA (Mesoscale Discovery, Rockville, MD) examined secretions from biopsies, PBMCs, and cell cultures following manufacturers protocols.

Flow Cytometry

Biopsy tissues were prepared for flow cytometry as described in the Supplementary Methods. Cells were acquired on an Attune NxT flow cytometer (BVRY configuration, ThermoFisher Scientific, Waltham, MA). Data analysis was completed using FlowJo V9 (BD Biosciences, San Jose, CA). Cells were gated based on forward scatter (FSC)/side scatter; single cells were gated on FSC-A/FSC-H; immune cells were gated for CD45. Cell populations were analyzed using cell type–specific markers. All gate boundaries were set using FMO controls.

Fructose Assay

Fructose concentration in unfermented fiber solutions was determined by fructose assay kit, following manufacturer's directions (Abcam, Cambridge, MA). To account for glucose interference, a series of fiber control solutions were prepared without fructose converting enzyme.

RNA Isolation and Gene Expression Analysis

RNA extraction was performed by Direct-zol microRNA prep kit (Zymo Research, Irvine, CA). Libraries were prepared using Superscript IV VILO Mastermix (ThermoFisher). Ex vivo biopsies and cell lines were analyzed by human chemokines reverse-transcriptase polymerase chain reaction (RT-qPCR) arrays (Origene, Rockville, MD, and Qiagen, Hilden, Germany) and validated by RT-qPCR using indicated genes (Supplementary Table 3) and were analyzed using CFX Manager Software V3.0 (Bio-Rad Laboratories, Inc.).

Trans-Epithelial Electrical Resistance

T84 cells were seeded on the apical side of a 12-mm, 0.4-μm Transwell (Corning, Corning, NY). Fibers were added to the apical wells and trans-epithelial electrical resistance was measured daily using a Millicell ERS voltohmmeter together with a STX1 electrode from World Precision Instruments (Sarasota, FL). The fiber-containing media was replaced every 2 days.

Molecular Docking of β-fructan (represented by Kestose-1) to TLR2 Heterodimers

We used 2 crystal structures containing TLR2 heterodimers for docking: TLR1-TLR2 (PDB ID: 2Z7X) and TLR2-TLR6 (PDB ID: 3A79). First, molecular dynamics simulations were carried out on these heterodimers. The simulations were used to provide an ensemble of conformations in solution to account for target flexibility.

Anaerobic Culture of Patient Intestinal Washes

Whole microbiota liquid cultures were obtained from patient intestinal washes, and immediately isolated and cultured anaerobically. Culture density was measured on a spectrophotometer, then back-diluted to an OD$_{600}$ of 1.0 and split into 2 equal samples. Microbe pellets were collected and resuspended in 10 mL no fiber (NF) or oligofructose (5 mg/mL) solution, supplemented with 5% BHI for 24 hours. Microbes were prepared for sequencing; supernatants were used as fermentation by-product solutions (SCFA gas chromatography) and pre-fermentation solution for incubation with THP-1 macrophage cells.

Gas Chromatography for Volatile Fatty Acids

SCFA concentrations were determined using volatile fatty acid analysis by gas chromatography. Samples containing 5% phosphoric acid were combined with 200 μL internal standard (isocaproic acid) in a gas chromatography vial and run on a 430-GC with flame ionization detection (Varian, Inc., Walnut Creek, CA) using a Stabilwax-DA fused silica column (Restek Corp., Bellefonte, PA, 30 m, 0.53 mm ID, 0.5 μm film), carrier gas helium 10 mL/min, injector/detector temperatures maintained at 250°C, and injection split 5:1. The injection volume was 1 μL. The oven was held for 0 minute at 80°C, then increased to 180°C at 20°C/min and held for 3 minutes for a total run time of 8 minutes, as determined using standard compounds and internal standard.

NGS Library Construction and Shotgun Metagenomics

Library construction and sequencing. Genomic DNA from aspirate washes was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) with additional steps described in the Supplementary Methods. Libraries were constructed using Nextera XT DNA Preparation Kit (Illumina Inc., San Diego, CA). Libraries were assayed on QIAxcel Fragment Analyzer System (Agilent, Santa Clara, CA) and quantified using Qubit Fluorometer. Multiplexed libraries were sequenced on a NovaSeq 6000 system (Illumina Inc.) using S2 flow cell at an average depth of 100 million reads per sample.

Bioinformatics analysis. Sequences were inspected with Fastqc and end read bases with quality scores <30 were trimmed with mcf-fastq allowing a 120 base pair minimal

trimmed length. Taxonomic classification was conducted with Kraken2 and metabolic profiling by HUMAnN2, as described in the Supplementary Methods.

Metabolomics

Metabolites were isolated from patient stool samples as directed by the Calgary Metabolomics Research Facility, University of Calgary.

Food Frequency Questionnaire

Food frequency questionnaire (FFQ), described in detail in the Supplementary Methods, was considered valid if completed within 90 days of specimen collection. Estimated caloric values were generated using the Canadian Nutrient File database. Estimated daily kilocalorie values were calculated for each patient and verified against patient age and weight. A fiber content database (Supplementary Table 4) was generated using published data on food fiber contents, used to calculate approximate daily intakes of inulin, oligofructose, pectin, and β-glucan (kilocalorie-adjusted by Willett residuals method). Spearman correlation with fiber content was analyzed in Stata 14 (StataCorp, College Station, TX).

Quantification and Statistical Analysis

Shotgun metagenomics data analysis. Row sequencing reads were deposited at the Short Reads Archive (SRA) NCBI database, publicly available under accession number PRJNA690735. Full data analysis methods are detailed in the Supplementary Methods. Separate random forest classifiers were independently trained on changes in fecal microbial composition and enzymes and area under the receiver operating characteristic curves (AUC-ROCs) were used to evaluate random forest classifier performance. Mann-Whitney U tests were performed between the response to oligofructose and its best predictors.

Statistical analysis. In addition to specific statistical methods described, groups were compared using paired Wilcoxon t-test (2-tailed) analysis, analysis of variance, or Kendall, depending on the relevant question, using GraphPad Prism. A P value of $<.05$ was considered significant in all cases and all error deviations are described by $\pm$SEM.

Results

β-fructans (Inulin and FOS) Induced Inflammation in Cell Models and IBD

To assess if select intact fibers (Supplementary Table 1) can stimulate a proinflammatory response, we used a human colonic tissue explant model (Supplementary Figure 1: outlines study design). To examine cell heterogeneity, we first defined specific immune cell types found in IBD and non-IBD colonic biopsies, demonstrating increased CD45+ cells in IBD biopsies (Supplementary Figure 2A). Next, we
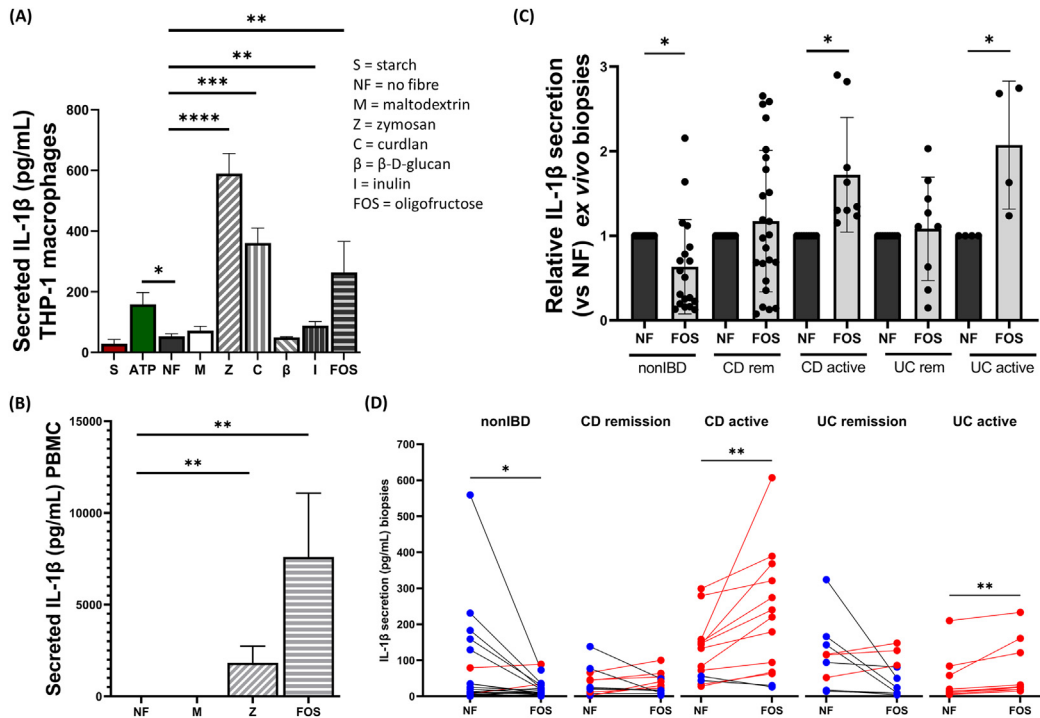

Figure 1. Unfermented dietary FOS induces a proinflammatory immune response in THP-1 macrophages, PBMCs, and patient biopsy tissues cultured ex vivo. ELISA for secreted IL-1β (marker of inflammation; supernatants) was performed in (A) THP-1 macrophages in response to starch (S), ATP, NF, maltodextrin (M), zymosan (Z), curdlan (C), oat β-D-glucan (B), inulin (I), or FOS, stimulated for 24 hours; (B) human PBMC in response to NF, maltodextrin, zymosan, and FOS; and (C and D) pediatric non-IBD (n = 19), CD (n = 33), and UC (n = 13) patient biopsies cultured ex vivo with NF or FOS (5 mg/mL) for 24 hours. Results are displayed as (C) fold-change in FOS/NF secretion for individual patients for ease of comparison, or (D) paired raw IL-1β secretions from biopsy tissues (decreased [blue] and increased [red] IL-1β secretion; FOS vs NF). *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

assessed response of immune cells to dietary fibers using THP-1-derived macrophages and primary PBMCs. Inulin and FOS, but not barley β-D-glucan, maltodextrin, or starch, induced IL-1β secretion by THP-1 macrophages, comparable to previously studied fungal β-(1,3)glucans (zymosan, curdlan; Figure 1A). Similar results were demonstrated in PBMCs from healthy donors (Figure 1B). To address the possibility that free fructose could drive this effect,[17] we determined the concentration of fructose within the fiber solutions and showed that IL-1β production was not increased by THP-1 macrophages with the same fructose concentrations (Supplementary Figure 2B). Leukocytes were confirmed to be present in the luminal mucus layer, particularly in patients with IBD, supporting the potential for direct physiological interaction with luminal fibers (Supplementary Figure 2C). Colonic biopsies from pediatric non-IBD (n = 19), CD (n = 33), and UC (n = 13) patients were cultured ex vivo with NF or FOS (5 mg/mL; 24 hours). FOS increased proinflammatory IL-1β secretion by a mean of 75% (CD active; $P < .05$) and 105% (UC active; $P < .05$) compared with NF, and to a lesser extent in biopsies from patients with IBD in remission (Figure 1C and D). Conversely, IL-1β was decreased by 40% ($P < .05$) in non-IBD control biopsies exposed to FOS.

β-fructans Induced Specific Inflammatory Pathways and Altered Epithelial Barrier

Human cytokine gene arrays of pediatric patient biopsies, cultured ex vivo with FOS or NF, identified broad FOS-induced proinflammatory pathways (eg, CX3CR1, IL8, IL1B, NFKB1), but the magnitude or direction varied, depending on IBD activity (Supplementary Figure 3A). Distinct gene expression footprints were identified in B cell, T cell, and macrophage cell lines (Supplementary Figure 3B). Top genes of interest in ex vivo biopsy response to FOS were identified (fold-change >1.5 vs NF; $P < .25$) and literature review of STRING analysis (ELIXIR) targets demonstrated that targets increased in response to FOS in pediatric IBD patient biopsies were linked to inflammation and epithelial barrier integrity (Supplementary Figure 3C).[18–27] Gene targets of interest (IL1β, CX3CL1, IL23A, NLRP3) were validated by RT-qPCR in 35 pediatric patient biopsies cultured ex vivo and various cell lines, cultured with fibers, demonstrating that proinflammatory markers were increased in active IBD patient biopsies in response to FOS compared with non-IBD, mostly driven by myeloid cells (Supplementary Figure 3D).

Pathways associated with the epithelial barrier (eg, IL-23, STAT3, CCL3) were examined by assessing fiber effects on different physical properties (Supplementary Table 1) in an in vitro epithelial monolayer model. We found fiber type–specific effects on epithelial barrier formation, possibly further affecting IBD pathogenesis (Supplementary Figure 4). Inulin Sigma (chicory root; $P < .0001$), inulin High Performance (chicory root; $P < .001$), and maltodextrin ($P < .01$) improved epithelial barrier formation, whereas β-D-glucan isolated from barley diminished barrier formation ($P < .0001$) in T84 intestinal epithelial cells; FOS

did not significantly alter barrier formation. Interestingly, inulin purity and polymerization appeared to relate to differences in its effect on barrier formation as we examined 2 inulin compounds, both sourced from chicory root (Supplementary Table 1); inulin High Performance (DP 25, 99.5%) significantly increased barrier formation compared to inulin Sigma (DP 12, 92%; $P < .05$).

To expand on fiber-mediated inflammatory effects, secreted cytokines associated with identified pathways (IL-1β, IL-23, MIP-1α, IL-5) were validated by multiplex ELISA (MesoScale Discovery; MSD) using supernatants from 40 pediatric patient ex vivo biopsy cultures (Figure 2A). To better examine the subset of patients observed to experience proinflammatory response to FOS, pediatric patients were defined as proinflammatory responders (IBD-R) or nonresponders (IBD-NR), based on ex vivo biopsy inflammatory response to FOS (IBD-R: IL-1β fold increase >1.1 vs NF defined as responder). We observed a significant increase in IL-1β, IL-23, and IL-5 secretion, but not MIP-1α in IBD-R, compared with IBD-NR (Figure 2A). We further validated these cytokines significantly increased in response to FOS in THP-1 macrophages (Figure 2B) and PBMCs (Figure 2C), compared with NF.

FOS Promoted Inflammation via the NLRP3 and TLR2 Pathways

As we had identified a number of NLRP3 pathway targets associated with response to FOS, we used NLRP3 inflammasome inhibitors (Ac-YVAD-cmk, glyburide, and MCC950) with positive control activator (ATP) or FOS in THP-1 macrophages to assess the role of NLRP3 (Figure 2D). We found that inhibition of NLRP3 significantly reduced IL-1β secretion in response to FOS (glyburide and MCC950) and ATP (YVAD and MCC950). We next treated THP-1 macrophages with a series of fibers, with or without MCC950 and showed that inhibition of NLRP3 significantly reduced proinflammatory response (IL-1β secretion) to zymosan, curdlan, and FOS (Figure 2E). In PBMCs, inhibition of NLRP3 also reduced proinflammatory response (IL-1β and IL-23 secretion) to FOS (Figure 2F). Prior research using basic molecular modeling suggests that TLR2 may serve as a receptor for β-fructans;[16] therefore, we conducted improved comprehensive docking prediction of the kestose-1 molecule, a precursor and structural representative of β-fructan, on the following heterodimers: TLR1-TLR2 (PDB ID: 2Z7X) and TLR2-TLR6 (PDB ID: 3A79) (Figure 2G and H).[28,29] A known ligand (Pam3CSK4) has been shown to bridge the ectodomains and stabilize the TLR1-TLR2 heterodimer through hydrophobic, hydrogen-bonding, and electrostatic interactions.[28] The interactions of Pam3CSK4 occur along the TLR1-TLR2 interface, containing the long and continuous lipid-binding site formed in conjunction with the TLR1 channel and the TLR2 pocket.[28] The region of the TLR1-TLR2 interface that forms hydrophilic interactions with Pam3CSK4 corresponded to the best predicted poses of kestose-1 (Figure 2G). For the TLR2-TLR6 heterodimer, a similar ligand (Pam2CSK4) interacts with the TLR2 pocket that induces dimerization.[29] Unlike Pam3CSK4, Pam2CSK4
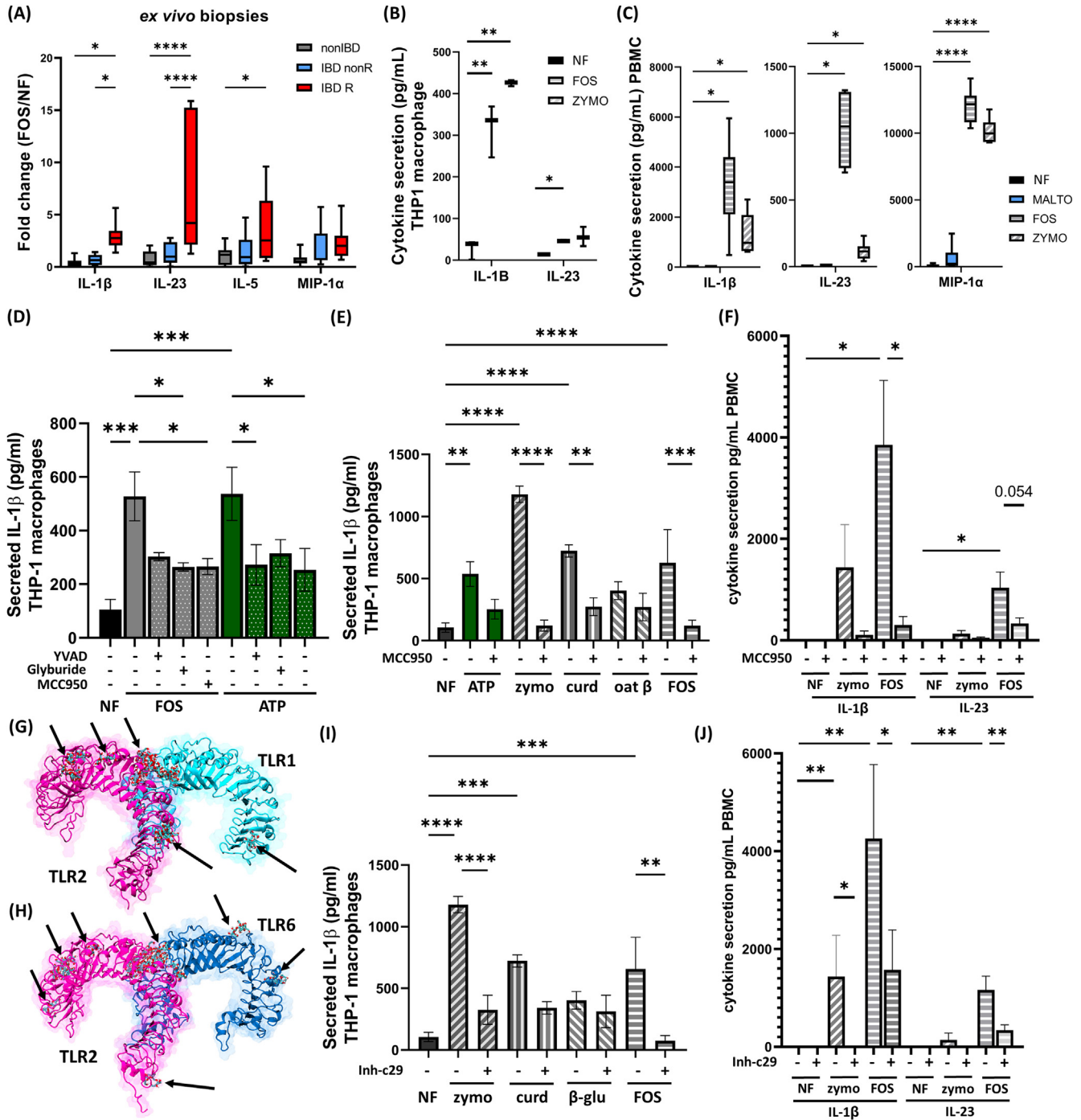
Figure 2. A prototypical proinflammatory response is driven through NLRP3 and TLR2. Multiplex ELISA (MSD) was used to measure secretion of proinflammatory cytokines in response to FOS (5 mg/mL) vs NF in (*A*) ex vivo biopsy secretions, in non-IBD (n = 12), IBD nonR [CD nonR (n = 6), UC nonR (4)], and IBD R [CD R (n = 9), and UC R (n = 5)] patient biopsies, (*B*) THP-1 macrophages, and (*C*) PBMCs (n = 6). (*D*) ELISA of secreted IL-1β was performed in supernatants from THP-1 macrophages treated with NF, FOS, or ATP control with the addition of the NLRP3 inhibitors Ac-YVAD-cmk (50μM), glyburide (200μM), or MCC950 (1μM). Effect of inhibition of NLRP3 (MCC950) on cytokine section in response to indicated fibers was measured by MSD in (*E*) THP-1 macrophages and (*F*) PBMCs. (*G*) TLR1(cyan)-TLR2(magenta) heterodimer with top 35 docked kestose-1 (represents β-fructan) poses (eg, *arrows*) aligned and overlaid on the TLR1-TLR2 (PDB ID: 2Z7X) structure represents all poses with a predicted binding free energy < –6 kJ/mol and maximum –7.48 kJ/mol. (*H*) TLR2 (*magenta*)-TLR6 (*blue*) heterodimer with the top 52 docked kestose-1 poses (eg, *arrows*) aligned and overlaid on the TLR2-TLR6 (PDB ID: 3A79) structure represents all poses with a predicted binding free energy < –6 kJ/mol and maximum –7.39 kJ/mol. Effect of inhibition of TLR2 (inh-c29; 75μM) on cytokine section in response to indicted fibers was measured by MSD in (*I*) THP-1 macrophages and (*J*) PBMCs. *P < .05, ** P < .01, *** P < .001, **** P < .0001. IBD rem, remission; Bc, B cell; Tc, T cell; Mφ, macrophage. Proinflammatory responders (IBD-R: IL-1β fold increase >1.1 vs NF; ELISA, Figure 1*A*) or nonresponders (IBD-NR).
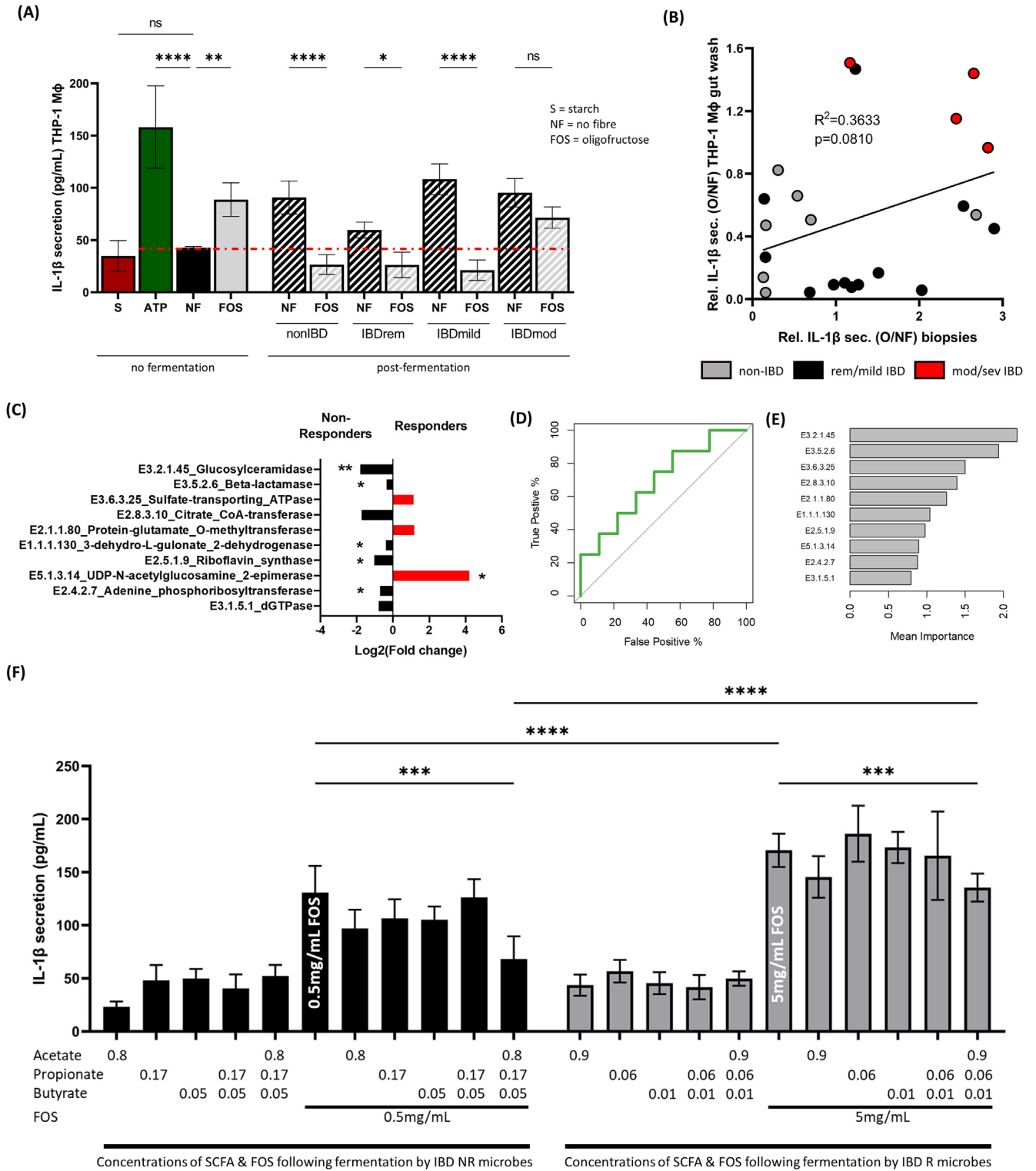
Figure 3. IBD patient microbial consortia fermentation function correlates with fiber-mediated immune response. (*A*) Pediatric non-IBD (n = 8), IBD remission (n = 4), IBD mild (n = 6), or IBD moderate/severe (n = 4) patient intestinal wash samples were collected during colonoscopy; total microbe wash was incubated with NF or FOS to create a whole microbe fermentation solution from each patient. These solutions were then incubated with macrophages and compared with NF (*red dot line*) or FOS alone by measuring IL-1β secretion by ELISA. (*B*) Results from non-IBD (*gray*), remission/mild IBD (*black*), and moderate/severe IBD (*red*) biopsies and washes calculated as the ratio of FOS/NF for each patient were compared and statistically evaluated by Kendall ranking. (*C*) Relative enzyme abundance in biopsy FOS responder (n = 8) and nonresponder (n = 9) cohorts determined by metagenomics. (*D*) ROC-AUC and random forest classification demonstrate an ability of enzyme abundance to predict response to FOS in patient biopsies. (*E*) Random forest analysis identifies the top 10 enzymes that contribute the most to the fermentation effect. (*F*) THP-1 macrophages were cultured with SCFA (acetate, butyrate, propionate) or in combination with FOS at levels following fermentation by responder microbiota (5 mg/mL) or nonresponder (0.5 mg/mL). * $P < .05$, ** $P < .01$, *** $P < .001$.

does not extend into the TLR6 channel. Again, the best predicted poses of kestose-1 for the TLR2-TLR6 heterodimer are found near the TLR2 pocket, but not within the TLR6 channel (Figure 2H). Inhibition of TLR2 in THP-1 macrophages significantly reduced proinflammatory response (IL-1β secretion) to zymosan and FOS (Figure 2I), whereas in PBMCs inhibition of TLR2 significantly reduced the FOS-induced proinflammatory response (IL-1β and IL-23 secretion; Figure 2J). Together, these findings support a direct interaction between β-fructan and TLR2 driving proinflammatory response through the NLRP3 inflammasome pathway.

FOS Fermentation Reduces Inflammation

To examine the link between microbiota composition or function and fermentation, we collected whole microbiota colonic intestinal mucosal washes from pediatric non-IBD patients and patients with IBD during colonoscopy. Washes were cultured anaerobically with NF or FOS for 24 hours, followed by centrifugation to remove microbes; supernatants were collected. THP-1 macrophages were then incubated with these supernatants, or with NF or FOS alone. Unfermented FOS and ATP (positive control) increased IL-1β (Figure 3A; left panel). NF supernatants, which included the natural fermentation products of patient microbes, increased IL-1β secretion (right panel). Although microbes were removed by centrifugation, these post-fermentation supernatants likely contained secretions from microbe cultures, which could increase IL-1β. Supporting our hypothesis, fermentation of FOS with whole intestinal wash microbes from non-IBD or IBD remission/mild patients reduced IL-1β secretion in macrophages, but microbes from patients with IBD with active disease (biopsies were collected from normal-appearing bowel) did not (Figure 3A; far right), likely due to an impaired ability of the microbial community to ferment FOS. A positive correlative trend was found between IL-1β secreted from FOS-treated patient biopsies (shown in Figure 1C) and IL-1β secreted from THP-1 macrophages, treated with matching patient microbe fermentation supernatants ($R^2 = 0.3633$; $P = .08$; Figure 3B), further supporting that changes in microbe-mediated fermentation drive the proinflammatory response to FOS.

We next examined production of SCFA (eg, acetate, propionate, butyrate) in the patient whole microbiota fiber fermentation supernatants described previously. Acetate levels in fermentation supernatants (Supplementary Figure 5A) correlated positively with THP-1 macrophage secretion of IL-1β in response to fermentation supernatants in both non-IBD patients and patients with IBD-R, but not in IBD-NR. Propionate and butyrate negatively correlated with THP-1 macrophage secretion of IL-1β in response to FOS fermentation supernatants in both non-IBD and IBD-R. This links FOS fermentation and SCFA production in prevention of inflammatory response to dietary fibers. The amount of fiber remaining in these fermentation supernatants was also evaluated, demonstrating a near-complete breakdown of β-fructan (FOS and inulin) following fermentation with microbes from non-IBD patients (average 0.5 mg/mL

remaining), vs approximately 50% average fermentation following fermentation with microbes from responder patients with IBD (average 2.5–5 mg/mL remaining; Supplementary Figure 5B).

Shotgun metagenomics of intestinal washes (those used for fermentation cultures) demonstrated an expected level of variability among pediatric patients (Supplementary Figure 6). At the phylum level, Firmicutes were decreased in CD (29.72% vs 39.66% in non-IBD); Actinobacteria were increased in UC (14.19% vs 5.44% in non-IBD; $P < .05$). Patients with active CD displayed increased Proteobacteria (38.25% vs 4.12% in non-IBD; $P < .05$). At the species level (Supplementary Table 2), *Parabacteroides distasonis* was lower in CD (0.14%; $P < .05$) and UC (0.37%; $P < .05$), compared with non-IBD (2.00%). *Bacteroides stercoris* was also reduced in CD (0.03% vs 1.27% in non-IBD; $P < .05$).

Random forest classification trained on microbial functions (enzyme abundances by metagenomics), correlating biopsy response to FOS with matching microbe composition and function, predicted response (IBD-R vs IBD-NR) to FOS (Figure 3C); ROC curve, indicated an acceptable diagnostic potential (ROC-AUC = 0.7; Figure 3D). Ten enzymes with the highest predictive value were identified (Figure 3E). Of those, Riboflavin synthase, Glucosylceramidase, β-lactamase, 3-dehydro-L-gulonate 2-dehydrogenase, and Adenine phosphoribosyltransferase were increased in the IBD-NR, whereas UDP-N-acetylglucosamine 2-epimerase was increased in IBD-R (Figure 3C). In contrast to function, microbial composition alone could not predict patient response to FOS, based on the random forest classification model (out-of-bag error rate >0.7). These data indicate that gut microbial function (not composition) predicts patient proinflammatory response to FOS, supporting our hypothesis that overall community function affects fiber fermentation and affects associated proinflammatory effects.

We were further able to support these findings by treating THP-1 macrophages with physiologically relevant concentrations of SCFA and FOS, identified in Supplementary Figure 5, following fermentation with IBD-NR (black) or IBD-R (gray) patient microbe cultures (Figure 3F). Significantly less IL-1β was secreted following treatment of THP-1 with the concentration of FOS remaining, following fermentation with IBD-NR microbes (0.5 mg/mL FOS), compared with IBD-R (5 mg/mL FOS). Addition of SCFA concentrations produced following FOS fermentation by microbes from either IBD-NR or IBD-R significantly dampened inflammatory effect (reduced THP-1 macrophage IL-1β secretion) of their respective FOS fermentation concentrations (IBD-NR 0.5mg/mL FOS; IBD-R 5mg/mL FOS). Only the combination of reduced FOS and specific production of SCFA from fermentation by IBD-NR microbes was able to entirely negate the proinflammatory effect of FOS.

Patterns of microbes presently known to be involved in fiber fermentation in published reports (Supplementary Table 3; eg, *Faecalibacterium, Roseburia, Bifidobacterium, Bacteroides*) were associated with disease states (Supplementary Figure 6D), supporting that clusters of microbes known to be essential for fiber fermentation and SCFA production are typically reduced or altered in patients
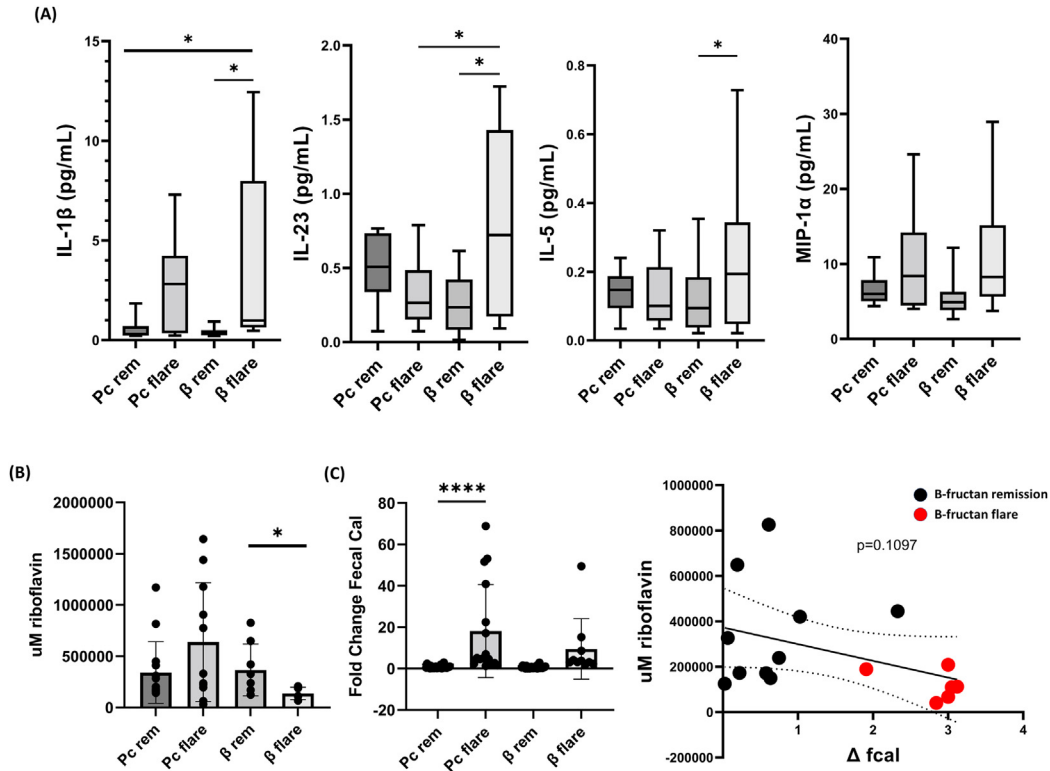
Figure 4. Proinflammatory response to consumption of β-fructans was confirmed in an RCT cohort. (*A*) Multiplex ELISA of UC patient biopsy lysates from a prebiotic RCT including placebo remission (Pc rem, n = 11), placebo flare (Pc flare, n = 10), β-fructan remission (β rem, n = 10), β-fructan flare (β flare, n = 7). (*B*) Riboflavin (baseline) and (*C*) fecal calprotectin (month 6 vs baseline) were measured in stool from a prebiotic RCT in patients with UC, including placebo remission (n = 11), placebo flare (n = 10), β-fructan remission (n = 10), β-fructan flare (n = 7), and were correlated against one another using linear regression modeling. *$P < .05$, ****$P < .0001$.

with IBD.[30] Taken together, metagenomics suggests that specific microbe community fiber fermentation functional deficiencies could explain the observed proinflammatory response to fiber in patients with IBD with active disease.

Proinflammatory Responses to β-fructan Confirmed in an RCT of Patients With UC in Remission

We recently completed an RCT, aimed to assess the efficacy of β-fructans (FOS and inulin) in preventing relapse in adult patients with UC in symptomatic remission.[12] Although our findings showed a positive impact of β-fructans in many patients in remission, significantly reducing the risk of biochemical relapse (defined as fecal calprotectin >200) compared with placebo, we also demonstrated that β-fructans (15 g/d over 6 months) could not prevent symptomatic relapses in all patients with UC in remission; in fact, 31% of individuals in the β-fructans group vs 24% (NS) in the placebo group experienced symptomatic relapse at the study endpoint in this cohort of UC patients.[12] These data support the benefits of β-fructans while also demonstrating the potential negative impacts in select patients with IBD, even in remission. There are clear differences between CD and UC, and between patients with

IBD diagnosed as very early onset (<6 years), pediatric (6–18 years), and adult (>18 years), indicating potential differences between our pediatric population and adult RCT cohort in this study. Nevertheless, we were able to use this RCT cohort to validate that cytokines (IL-1β, IL-23, IL-5) associated with proinflammatory response to β-fructans in select patients with IBD (identified in Figure 1–2) were also increased in intestinal biopsy lysates from the RCT cohort, but only in patients with UC who flared following consumption of β-fructans, and not in the placebo arm (Figure 4*A*). These results further confirmed the proinflammatory response to β-fructans in select patients with IBD, supporting the clinical relevance of our ex vivo biopsy findings.

Furthermore, examining the microbial enzyme pathways identified in Figure 3*C*, we found that riboflavin, which was reduced in patients who displayed a proinflammatory response to β-fructans in our ex vivo patient biopsy model, was also significantly lower in baseline stool samples collected only from patients in the β-fructan RCT[12] who relapsed in response to β-fructan consumption (Figure 4*B*). Riboflavin negatively correlated with fecal calprotectin (gut inflammation marker) fold-change from baseline to month 6 (Figure 4*C*) in this RCT; riboflavin was not predictive of relapse in placebo RCT patients.

Fiber Avoidance Correlated With Inflammation

Given reported reduced consumption of dietary fibers in patients with IBD,[31] we used FFQs (reflecting previous diet) to calculate consumption of approximate daily dietary fiber intake (inulin, FOS, pectin, and β-D-glucan), using a fiber content database (Supplementary Table 4). Despite variability in consumption of dietary fibers, pediatric patients with IBD with active disease consumed significantly less β-D-glucan compared with patients in remission (Supplementary Figure 7A; $P < .05$). Supporting our hypothesis, we found significantly lower FOS consumption in pediatric patients with matching proinflammatory biopsy responses to FOS (IBD-R), compared with IBD-NR (Supplementary Figure 7B; $P < .01$); FOS consumption negatively correlated with IL-1β secretion in response to FOS in matching pediatric biopsies (Supplementary Figure 7C). Although these findings do not prove causality, they support a link between intestinal proinflammatory response to fiber and dietary fiber avoidance in pediatric IBD.

Discussion

Patients with IBD describe variable intolerance of fiber consumption,[8] which can lead to avoidance of generally beneficial fibers and worse patient outcomes.[9–11] We used IBD as a model to confirm our hypothesis that fibers that remain unfermented could drive inflammation. Supporting our findings, β-fructans have been shown to induce reactive oxygen species production and associated inflammation, possibly through NLRP3 signaling,[32] and clinical studies show that FOS consumption can worsen outcomes in select patients.[5,12] Nevertheless, the potentially negative effects of dietary fibers are poorly documented and usually overlooked.

Here, unfermented FOS induced proinflammatory cytokines in PBMCs, THP-1 macrophages, and IBD patient biopsies cultured ex vivo, via pathways previously associated with IBD including TLR2 and NLRP3, which are known to corporate in response to ligand stimuli such as lipopolysaccharide.[33,34] This was confirmed in select patients with IBD in an RCT inulin/FOS-treated cohort.[12] We propose that interactions between unfermented fibers in the luminal contents with leukocytes found in the mucosal lining or lamina propria exposed due to epithelial barrier breakdown, could drive these responses in a physiological setting. TLR2 is differentially expressed in cell types, with greater levels in monocytes and macrophages, supporting increased potential for interaction between unfermented β-fructans and TLR2 in patients with IBD where these cell populations are increased, particularly in patients with active disease. Response could be explained further by the increased presence of inflammatory macrophages in inflamed tissues of patients with IBD, although the presence of specific macrophage populations was not examined in this study.[35] The epithelial barrier, which typically prevents undesired immune interaction with luminal content, is commonly disrupted in IBD.[36] Here, β-fructans improved barrier formation in vitro, whereas β-D-glucan reduced barrier formation, possibly due to structural differences between β-

fructans and β-D-glucan.[2] Our data support future investigation of these pathways using organoid and animal models, which would provide more mechanistic findings in relation to the effects of these fibers on epithelial barrier integrity.

Sensitivity to fibers was further supported by our findings that patients with inflammatory response to β-fructans (IBD-R) consumed less dietary fiber than nonresponders (IBD-NR), and lower FOS consumption (measured by FFQ) correlated with higher proinflammatory response to FOS in matching patient biopsies cultured ex vivo. This suggests that patients with fiber sensitivities might unknowingly avoid consumption of select FOS-containing foods, possibly in attempts to ameliorate symptoms.

Microbial function was predictive of response to FOS in patient samples. The enzyme UDP-N-acetylglucosamine was increased in IBD-R, suggesting that this pathway may be involved in the proinflammatory response to fibers, possibly via T-cell activation.[37] In contrast, IBD-R had significantly reduced riboflavin synthase which displays anti-inflammatory, antioxidant, and microbe-altering properties in patients with IBD.[38] Both riboflavin synthase and glucosylceramidase inhibit a variety of proinflammatory cytokines (IL-6, tumor necrosis factor, IL-1β).[39] Riboflavin (vitamin B2) was lower in stool of patients with UC in our RCT who flared following 6-month consumption of β-fructans and its absence correlated with increased fold-change in fecal calprotectin, suggesting further links between riboflavin and response to fiber consumption in patients with IBD. *Faecalibacterium prausnitzii* is thought to use riboflavin as a mediator of butyrate and SCFA production,[40] suggesting a key link between fiber-fermenting microbes, enzyme abundance, SCFA production, and inflammatory response to dietary fibers.

IBD-R mucosal microbiota washes produced increased acetate and decreased propionate and butyrate through fermentation of FOS. Acetate is known to increase reactive oxygen species production in macrophages, whereas butyrate and propionate inhibit inflammation through various pathways,[41] suggesting that even when fermentation is not reduced, altered production of SCFA may promote inflammation. Although no individual microbe species associated explicitly with proinflammatory response to FOS, there were altered patterns of microbial species abundances that may help identify microbiome changes associated with altered fermentation. Abundance of microbes known to ferment fibers[2] was significantly reduced in patients with moderate and severe CD; particularly, the dominant fiber-fermenting and butyrate-producing microbes *Roseburia hominis* and *F. prausnitzii*, as expected.[2,42] Although recent studies have indicated the importance of these mucosal microorganisms in the gut ecosystem and in relation to diet, there is only limited research on the interactions of diet with mucosal microbiota in IBD.

It is important to note some limitations of our study. Although we confirmed the purity of the fibers used with low lipopolysaccharide (within test limits of detection), other microbial contaminants may co-purify with the 3 β-fructans (FOS/inulin) from chicory roots used in this study, along with other fibers. Further, samples were collected

following colonoscopy preparation, which is known to alter microbiota composition; while our main focus was on mucosa-associated microbes, which are less affected by bowel preparation, luminal microbiota play an important role in fiber fermentation. Mucosal microbes typically include important fiber-fermenting microbes (*Bacteroidetes, Firmicutes* [Veillonellaceae, Ruminococcaceae]) compared with stool microbiota; however, our understanding of the precise community of microbes (luminal or mucosal) involved in fiber fermentation remains limited by our ability to culture and identify these microbes.[2,43]

We propose that when fiber-fermenting microbes are present in the gut, and normal barrier integrity prevents interactions between fibers and underlying immune cells, fermentation of select fibers enhances the barrier and reduces inflammatory response. In contrast, select disease state scenarios, such as active IBD, provide conditions leading to increased exposure and sensitivity to unfermented dietary fibers to develop in the diseased gut microenvironment. These conditions include (1) a reduced abundance and capacity of the gut microbiota to ferment fiber, (2) increased presence of immune cells at the mucosal surface, and (3) inflammatory damage to the gut barrier. Interaction of FOS and inulin with host cells could then result in gut inflammation through direct effects of intact fiber and/or altered SCFA production. Our work could have significant impacts on patient care. Further clinical studies are warranted to determine if FOS should be avoided by patients with IBD when experiencing specific alterations in gut microbiota composition and functions, specifically associated with lack of fermentation, especially with active disease. Because altered microbiota is more frequently found in patients with active IBD, it could be speculated that FOS (and potentially other fibers) should be administered as adjunct therapy only after medical therapy has induced remission (with barrier repair/mucosal healing and healthy microbiota functions) in these individuals, to ensure the other benefits of fibers and their products.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2022.09.034.

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Correspondence
Address correspondence to Heather Armstrong, PhD, Department of Internal Medicine, University of Manitoba, 808 JBRC, 715 McDermot Avenue, Winnipeg, Manitoba, R3E 0W2 Canada. e-mail: Heather.Armstrong@umanitoba.ca; or Eytan Wine, PhD, MD, Department of Pediatrics, University of Alberta, 7–124 Katz Building, 114st and 87 Avenue, Edmonton, Alberta, T6G 1C9, Canada. e-mail: wine@ualberta.ca.