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Impact of 5 Fluorouracil Chemotherapy on Gut Inflammation, Functional Parameters, and Gut Microbiota

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Abstract

Emerging evidence suggests that gut microbiota may influence the response to chemotherapy. We sought to characterize the effects of 5 fluorouracil (5FU) chemotherapy on colon inflammation and functional measures in colorectal cancer (CRC) and to further determine whether gut microbiota can influence this response. 50 C57BL/6 were randomized into four groups; Control + Vehicle (n=10), Control + 5FU (n=10), AOM/DSS + Vehicle (n=15), and AOM/DSS + 5FU (n=15). CRC was induced chemically by a single 10 mg/kg injection of azoxymethane (AOM) followed by two cycles (2% and 1%) of dextran sodium sulfate (DSS). Mice were then treated with 3 cycles of vehicle or 5FU (cycle 1: 40 mg/kg, cycle 2+3: 20 mg/kg). Functional tests (grip strength and run-to-fatigue) were performed prior to 5FU treatment (baseline) and at the completion of the second cycle of 5FU. Following the third 5FU cycle, mice were euthanized and the colon was evaluated for expression of inflammatory genes using RT-qPCR and stool samples were profiled using 16S rRNA sequencing. A second experiment used fecal microbiota transplantation from 5FU treated mice to control mice (n=10– 15/group) to determine whether 5FU associated changes in the microbiota could influence functional measures and colon inflammation. 5FU reduced grip strength ($p<0.05$) and caused a trending decrease in run-to-fatigue performance in cancer mice ($p=0.06$). Select intestinal inflammatory genes were significantly elevated with 5FU treatment and this was further exacerbated with cancer ($p<0.05$). Microbiota analysis revealed increased

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dissimilarity and alterations in bacterial taxonomy in 5FU and AOM/DSS-treated mice ($p<0.05$). Fecal transplant from 5FU treated mice reduced functional performance ($p<0.05$) and altered select colon inflammatory markers ($p<0.05$). This study provides evidence of an effect of 5FU on inflammatory responses and functional measures in a mouse model of CRC and suggests that gut microbes may play a role in some, but not all, 5FU related perturbations.

Keywords

5-Fluorouracil; Chemotherapy; Toxicity; Colorectal Cancer; Inflammation; Microbiota; Fecal Transplant

1. Introduction

5 Fluorouracil (5FU) chemotherapy is widely used in the treatment of colorectal cancer (CRC), and has been the first-choice chemotherapy drug for CRC for many years[1,2]. However, its clinical utility remains hampered by acquired resistance and hematopoietic and gastrointestinal toxicities resulting from its non-selectivity[3,4]. 5FU functions as a thymidylate synthase inhibitor with a half-life of approximately 15 minutes when injected intravenously (I.V.)[2,5]. When used in the treatment of CRC, it is estimated that approximately 1% of the injected compound reaches the colon to induce a potential anti-tumor effect. This translates to a 10–15% success rate in the clinic[6]. Currently, the common regimen of 5FU therapy involves 4 consecutive days of 5FU treatment with daily monitoring of behavior and phenotypic changes in the patient. If the patient is described as well adapted and responsive to the treatment on day 5 another single injection will be delivered every other day for 6 days or until the patient starts to show symptoms of non- specific toxicity[7,8]. As such, many side effects are associated with 5FU treatment, the most common including fatigue, loss of appetite, and diarrhea, all of which can lead to a reduced quality of life[9]. Furthermore, 7.5% of cancer patients treated with chemotherapy die as a result of non-selective chemotherapy toxicity rather than the cancer itself[10]. Therefore, identifying methods to better evaluate the associated toxicity related to chemotherapy and the mechanisms involved is of great public health importance.

One of the most potent side effects of 5FU treatment is intestinal mucositis. Mucositis usually appears along the entire gastrointestinal tract from mouth to anus and causes general debility[3,4,7,11]. Mucositis of the intestine is characterized by increased crypt apoptosis and villus atrophy, leaving the mucosal tissue open to infection and ulceration[12,13]. Several factors or genes contributing to the 5FU-induced mucositis have been previously studied; the formation of reactive oxygen species (ROS) and the production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) have been implicated in this process[11,12,14,15]. Furthermore, histological evaluation of the small intestine indicates obvious villus destruction and inflammation of the crypts[12,14,16]. However, the majority of literature explaining 5FU-induced intestinal mucositis fails to utilize a disease model limiting a complete understanding of the side effects of 5FU specifically in the context of CRC.

Gut microbiota have been implicated in 5FU associated toxicity[17,18]. 5FU leads to gut dysbiosis (i.e. an imbalance of gut microbes) and the ensuing inflammation leads to exacerbated intestinal mucositis[17]. Several preclinical studies have reported a drastic shift from commensal bacteria (i.e., *Bifidobacterium* and *Lactobacillus* spp.) to *Escherichia*, *Clostridium*, and *Enterococcus* spp. following even a single intraperitoneal dose of 5-FU[17]. Mechanistic support for this relationship is provided by the reduced intestinal mucositis and decreased cytokine levels in 5FU treated mice after antibiotic induced depletion of microbes[17]. Understanding the influence of gut microbes on 5FU related toxicity may lead to the identification of targets (i.e. bacteria themselves or pathways that they mediate) to reduce side effects of chemotherapy.

Using the azoxymethane/dextran sodium sulfate (AOM/DSS) model of CRC, we sought to characterize the effects of 5FU on colon inflammation and functional measures in CRC and to further determine whether gut microbiota can influence this response. We detected a general anti-tumor effect of 5FU in AOM/DSS treated mice; however, side effects including mortality, adverse physiological outcomes, mucositis, and colon inflammation were evident. Further, we observed significant alterations in gut microbiota in the presence of 5FU. Fecal transplantation experiments also were conducted in control mice to investigate the potential causality between gut microbiota and 5FU toxicity. Our findings indicate that gut microbes may play a role in some, but not all, 5FU related perturbations.

2. Methods

2.1 Animals

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were cared for in the animal facility at the University of South Carolina. Mice, randomized upon arrival to the animal facility to prevent litter biases in microbiome data, were housed five per cage and maintained on a 12:12-h light-dark cycle in a low-stress environment (22^0C, 50% humidity, low noise). Mice were kept in a room isolated from all other ongoing animal experiments and were handled only by the primary investigators. All mice were habituated to the AIN-76A diet prior to any interventions and were given food and water *ad libitum* through the course of the study. All methods were in accordance with the American Association for Laboratory Animal Science, and the Institutional Animal Care and Usage Committee of the University of South Carolina approved all experiments.

2.2 AOM/DSS protocol and 5FU Treatment Experiment 1

In experiment 1 (Fig 1A), 50 mice were randomly divided into four groups: Control + Vehicle (n=10), Control + 5FU (n=10), AOM/DSS + Vehicle (n=15), and AOM/DSS +5FU (n=15). At 12 wk of age (*baseline week 0*) mice received either an intraperitoneal injection of the carcinogen[19], AOM (10 mg/kg) (Sigma, St. Louis, MO), diluted in PBS (AOM/DSS) or PBS alone (Control). Mice receiving the AOM injection were subjected to two cycles of DSS (36–50 kDa) (MP Biomedical, Solon, OH)-supplemented water at final concentrations of 2 and 1% at weeks 1 and 4, respectively. Each DSS cycle lasted for a 1-wk period. Body weights and symptom scores were determined semi-weekly along with food and water measurements. Calculation of symptom score was performed as previously described[20],

taking into account percent body weight loss, stool consistency, and rectal bleeding. Briefly, fresh colonic evacuates were smeared onto “Hemoccult” tape to assess severity of diarrhea and were tested with developer (Beckman Coulter, Brea, CA) to assess rectal bleeding. Bleeding was scored; no positive detection of blood (0), detection of blood but not grossly visible (2), and gross visibility of blood (4). Diarrhea was scored; solid cylinder (0), soft cylinder and easily spreadable (2), and non-cylindrical or runny (4). Body weight was calculated as percent body weight loss; 0–5% (0), 6–10% (1), 11–15% (2), 15–20% (3), 20–25% (4), and >25% (5). Scores of all three categories were summed to obtain an overall symptom score for each mouse.

5FU (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile phosphate buffered saline (PBS), pH7.4 and then sterile filtered through a 0.2μm syringe filter. 5FU was administered in 3 cycles; cycle 1: 40 mg/kg, cycle 2 and 3: 20 mg/kg via intraperitoneal injection. 5FU was prepared fresh at the beginning of each cycle. Sterile filtered PBS alone was used as the vehicle control. Each cycle consisted of 5 consecutive days of injections followed by 9 days of recovery. The treatment period lasted for 5 weeks and mice were sacrificed 24 hr after the final injection of the third cycle.

2.3 Fecal Transplantation Experiment 2

For the fecal transplantation experiment (Fig 1B), 35 mice were randomly divided into four groups: Donor Vehicle, Donor 5FU, Recipient Vehicle (Vehicle FMT), Recipient 5FU (5FU FMT) (n=5 per Donor group and n=10–15 per Recipient group). The Donor 5FU group was injected with 40 mg/kg 5FU for 5 consecutive days as described earlier. All Donor mice were sacrificed 9 days after initial injection and a mucosal scrape was performed of the distal enteric tract (cecum to anus) to harvest the microbiota for fecal transplantation. Fecal materials were suspended in sterile 10% glycerol/PBS, allowed to settle by gravity for 10 minutes, and aliquoted into daily requirements for Recipient groups. To perform the FMT study we adopted a protocol by Reikvam *et. al.* 2011[21]. Recipient mice were pretreated with the following antibiotic cocktail every 12 hrs for 10 days prior to FMT: Vancomycin (50 mg/kg), Neomycin (100 mg/kg), Metronidazol (100 mg/kg), and Amphotericin-B (1mg/kg) via oral gavage supplemented with Ampicillin (1mg/ml) in the drinking water given *ad libitum*. All antibiotics were purchased from ACROS Organics, New Jersey. FMT was administered via oral gavage for 14 days with 150 μl respective fecal suspension from PBS or 5FU injected mice.

2.4 Grip strength assessment

All experimental mice were utilized to evaluate the effects of 5FU treatment on grip strength. Grip strength was measured prior to 5FU administration (*week 8*) and during the recovery period of the second cycle (*week 11*) in experiment 1 and following antibiotic treatment *(week 0*) and fecal transplantation *(week 2*) in experiment 2. Briefly, holding the mice by the tail, the front and back feet were allowed to grip the grate. Mice were then pulled from the grate, generating a force that was measured by the force transducer (Aurora Scientific, Ontario, Canada). Five measurements were taken consecutively, with 2 min rest between sets until a total of 15 measurements were taken for each mouse. The averages of

the 15 measurements were used in the data analysis. Measurements were performed by the same investigator through the entirety of the study.

2.5 Run-to-fatigue assessment

To determine the effect of 5FU on fatigability, mice were subjected to a run-to-fatigue test prior to 5FU administration (*week 8*) and during the recovery period of the second cycle (*week 11*) in experiment 1 and following antibiotic treatment *(week 0*) and fecal transplantation *(week 2*) in experiment 2. After 3 consecutive days of habituation during the night cycle, mice were subjected to the following run-to fatigue protocol: 15-min warm-up, 20 m/min for 30 min, and 25 m/min thereafter until fatigue was reached. Fatigue was defined as the time at which mice were no longer able or willing to keep up with the treadmill despite hand prodding for at least 1 minute. All treadmill tests were performed during the night cycle by the same two investigators (ATS, BNV) through the entirety of the study.

2.6 Tissue collection

Mice were euthanized by isoflurane overdose 24 hr post final injection (cycle 3, day 6) in experiment 1 and on day 15 in experiment 2 (Fig 1A,B). Blood was collected from the inferior vena cava and tissues were removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at -80^0C or fixed in 10% formalin until further analysis. Briefly, the colon was carefully dissected distal to the cecum and proximal to the anus. Mesentery adipose tissue was removed with forceps. Colons were then flushed with PBS, opened longitudinally, and flattened with a cotton swab. In experiment 1, colonic polyps were counted under a dissecting microscope and were categorized according to size (>2, 2–1, and <1). For both experiment 1 and 2, a 5-mm piece of the distal colon was cut and fixed in 10% buffered formalin (Fisher Scientific, Pittsburg, PA) for 24 hr for immunohistochemical and morphological analysis. The remaining colon was cut in half and snap-frozen for RNA analysis. The distal portion of the ileum also was cut and fixed in 10% buffered formalin for 24 hr for morphological analysis. The liver was dissected and a small portion was fixed in 10% buffered formalin for 24 hr, for morphological analysis.

2.7 Blood Profile

In both experiment 1 and 2, a complete blood count was performed using the VetScan HMT (Abaxis, Union City, CA) for determination of white blood cells (WBCs), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), red blood cells (RBCs), Hemoglobin (HGB), Hematocrit (HCT), and platelets (PLT). Neutrophil/lymphocyte ratio (NLR) was calculated from obtained values. Briefly, whole blood removed from the inferior vena cava was placed in an EDTA microtube and analyzed on the VetScan HMT according to the manufacturer’s instructions.

2.8 Hematoxylin and eosin staining & histopathology

Hematoxylin and eosin staining of the colons, small intestines, and liver were performed as previously described[22] . Colon specimens were characterized according to the presence of epithelial neoplasia to: no adenomas/dysplasia (ND), adenomas with low-grade dysplasia

(LGD) adenomas with high-grade dysplasia (HGD) and adenocarcinomas. The presence of non-specific colitis also was evaluated in the small intestine and the colon (data not shown). Liver specimens were assessed and characterized according to the severity of hepatocellular injury: normal, early steatosis without inflammation, and acute hepatitis (AHI). All histological analyses were performed blindly by a certified pathologist (I.C.). All tissue morphological measurements were performed in triplicate and the coefficient of variance was determined to be less than 5%. Monocytes/macrophages were observed by positive staining of CD68+ (Abcam, ab125212) cells in the colon tissue.

2.9 Gene expression

Quantification of colonic expression of TNF-α, MCP-1, NOS2, IL-6, IL-10, IL-1β, IL-4, EMR-1, Ly6G, IFN-γ, and FOXP3, were performed as previously described[22]. Briefly, RNA was extracted using TRIzol reagent (Life Technologies, GIBCO-BRL, Carlsbad, CA) and chloroform procedures. Because DSS has been shown to inhibit polymerase and reverse transcriptase activity, lithium chloride was used to purify the RNA as described in detail by Viennois et al. 2013[23]. RNA sample quality and quantities were verified using an Agilent Bioanalyzer and determined to be of good quality based on A260/A280 values (>1. 8) prior to cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen 205313). Quantitative RT-PCR analysis was carried out as per the manufacturer's instructions (Applied Biosystems) using Taq-Man Gene Expression Assays. Data were normalized to vehicle treated controls and compared to two reference targets (TBP and H2AFV), which were evaluated for expression stability using GeNorm[24,25].

2.10 Microbiome Analysis

All Microbiome analysis and sequencing was performed by the University of North Carolina Microbiome Core. Fecal evacuates were collected from each mouse at the conclusion of the study and were chosen at random for 16S rRNA sequencing, at least one sample from each cage was used to prevent a cage effect in the data. A final n=5/group was obtained and used for 16S rRNA sequencing. DNA was isolated from fecal evacuates homogenized with bead-beating using the Qiagen Qiamp Fast DNA Stool Mini Kit (Qiagen #51604) and was used for 16S rRNA gene targeted sequencing using the V4 region on an Illumina MiSeq sequencer[26,27]. Amplification of the 16S rRNA V4 hypervariable region was carried out using the 16S V4 515F forward (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA 3') and V4 806R reverse primer (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAA T3') with added Illumina adapter overhang nucleotide sequences. The PCR conditions used were initial denaturing step at 95°C for 3 minutes, followed by a cycling of denaturing of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (25 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN). In the next step each sample was amplified using a limited cycle PCR program, adding Illumina sequencing adapters and dualindex barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30

seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (8 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter), quantified and normalized prior to pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer and heat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired–end sequencing with dual reads were performed according to the manufacturer's instructions. Multiplexed paired-end fastq files were produced from the sequencing results of the Illumina MiSeq using the Illumina software configure BclToFastq. The paired-end fastq files were joined into a single multiplexed, single-end fastq using the software tool fastq-join. Demultiplexing and quality filtering was performed on the joined results. Quality analysis reports were produced using the FastQC software. Bioinformatics analysis of bacterial 16S amplicon sequencing data was conducted using the Quantitative Insights Into Microbial Ecology (QIIME) software[28]. Operational taxonomic units (OTUs), an operational definition used to classify groups of closely related individuals, [29] were picked from the quality filtered results using pick_de_novo_otus.py. Chimeric sequences were detected and removed using ChimeraSlayer. Alpha diversity and beta diversity analysis were performed on the data set using the QIIME routines: alpha_rarefaction.py and beta_diversity_through_plots.py, respectively. Summary reports of taxonomic assignment by sample and all categories were produced using QIIME summarize_taxa_through_plots.py and summarize_otu_by_cat.py[29]. Samples were rarefied at 10,000 reads/sample. FastTree2 (Price MN, Dehal PS, Arkin AP FastTree 2- approximately maximum-likelihood tress for large alignments) to build the phylogenetic tree. Finally we used an open reference method for OTU picking. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the functional gene content in the fecal microbiota based on taxonomy obtained from the Greengenes reference database. PICRUSt and LefSe were performed online in the Galaxy workflow framework.

2.11 Statistical analyses

All data were analyzed using commercial software (SigmaStat, SPSS, Chicago, IL). All outcomes were analyzed using a two-way ANOVA (disease x treatment), three-way ANOVA (disease x treatment x time) or by a Two-Tailed Student's T-Test where only two groups were compared. A Student-Newman-Keuls test was used for all post-hoc analyses. Survival curve analysis was conducted by Log-rank (Mantel-Cox) test. Pearson correlations were used to test the relationship between microbiota and colonic gene expression. Any data that were not normally distributed or did not display equal variance were logarithmically transformed so that those criteria were met. Statistical significance was set with an alpha value of $p<0.05$. Data are presented as mean ± SEM.

3. Results

3.1 Experiment 1: 5FU causes adverse physiological effects, increases colon inflammation, and alters gut microbiome profile.

3.1.1. 5FU reduces tumor burden but decreases survival in AOM/DSS mice.—

As expected, there was a main effect of AOM/DSS on body weight loss during the DSS

administration (*weeks 0–8*) ($p<0.05$) but not during the 5FU treatment period (*weeks 8–13*). Three cycles of 5FU (*weeks 8–13*) caused noticeable weight loss in both control and AOM/DSS mice but this did not reach statistical significance. However, there was a significant interaction detected beginning at *week 9*; within AOM/DSS, 5FU treated mice experienced greater weight loss than AOM/DSS alone (2A, B, $p<0.05$). Consistent with this, a Mentel-Cox test indicated significant survival risk in AOM/DSS + 5FU mice compared to all other groups (Fig 2C, $p<0.05$). As expected, a main effect of AOM/DSS on severity of symptoms was detected during DSS administration as well as during the duration of the 5FU treatment (Fig 2D, $p<0.05$). Similarly, at *weeks 9–13* (beginning 1 week after 5FU treatment) a main effect of 5FU was detected for symptom score (Fig 2D, $p<0.05$). Further, there was a significant interaction at weeks 10–11; AOM/DSS + 5FU exhibited greater symptom scores than AOM/DSS alone. At necropsy, there was no main effect of AOM/DSS or 5FU in liver weight; however, a significant interaction was detected within AOM/DSS mice where 5FU significantly increased liver weight compared to AOM/DSS alone (Fig 2E, $p<0.05$). As expected a main effect of AOM/DSS was detected for spleen weight with AOM/DSS groups having significantly greater weight (Fig 2F, $p<0.05$) but there was no effect of 5FU and no interaction. Both AOM/DSS and 5FU reduced the total weight of the epididymal fat pad (Fig 2G, $p<0.05$) but there was no significant interaction. Similarly, a main effect of 5FU was detected in mesenteric fat pad mass with 5FU resulting in overall lower weight (Fig 2H, $p<0.05$). A trending decrease in total polyp number (Fig 2I, $p=0.18$) and a significant decrease in large polyp number (Fig 2J) ($p<0.05$) was recorded in AOM/DSS + 5FU mice compared to AOM/DSS alone. Consistent with this, histological analysis of the colonic polyps in AOM/DSS mice treated with 5FU, revealed less adenomas with adenocarcinoma and more low-grade dysplasia (Fig 2K, L) compared to AOM/DSS alone.

3.1.2 Nonspecific toxicity of 5FU destroys intestinal tissue morphology and induces hepatotoxicity.—To observe some of the most commonly associated side effects of chemotherapy, we analyzed the small intestine and liver tissue. Histological analysis revealed a main effect of 5FU in shortening of the length/atrophy of the villi in small intestine (Fig 3A, B, $p<0.05$). Histology of the livers revealed acute hepatitis in 80% of the AOM/DSS mice treated with 5FU and in 90% of the cases the presence of necrosis accompanied by the formation of abscesses (Fig 3C, D); findings compatible with liver toxicity.

3.1.3 5FU alters blood profile differently in the presence of tumor burden—A main effect of both 5FU and AOM/DSS was detected in LYM, RBC, HGB, HCT, and PLT counts (Table 1, $p<0.05$); 5FU treatment decreased LYM, RBC, HGB, HCT but increased PLT whereas AOM/DSS treated mice exhibited increased LYM but decreased RBC, HGB, HCT and PLT (Table 1, $p<0.05$). Further, there was a significant interaction detected within AOM/DSS; 5FU treated mice presented with greater WBC, MON, NEU counts, and NLR (Table 1, $p<0.05$) compared to AOM/DSS alone.

3.1.4 5FU alters the immune profile of the colon—There was a significant main effect of both 5FU and AOM/DSS in mRNA expression of inflammatory cytokines TNFα

and NOS2 (Fig 4, $p<0.05$). Further, a main effect of AOM/DSS was detected in mRNA expression of inflammatory cytokine IFNγ and macrophage marker EMR1. Interestingly, a significant interaction was detected within AOM/DSS where AOM/DSS mice treated with 5FU showed increased mRNA expression of inflammatory cytokines and chemokines MCP-1, IL6, IL1β, IL10 and the regulatory T-cell marker FOXP3 (Fig 4, $p<0.05$) compared to AOM/DSS alone. There was no significant difference in IL4 expression between any groups (Fig 4).

3.1.5 5FU affects functional measures of fatigue.—To evaluate the effects of 5FU on peripheral muscle fatigue we utilized grip strength testing (measured as absolute strength in N and relative N/Kg body weight). No significant differences between groups were detected in relative grip strength (N/Kg); however, analysis of absolute strength indicates a significant interaction within AOM/DSS treatment in which AOM/DSS mice treated with 5FU exhibited a significant decrease in strength (Fig 5A,B, $p<0.05$) compared to AOM/DSS alone. To measure the effect of 5FU on fatigability, mice were challenged with a run-to-fatigue test. Given that there were no differences in absolute values at baseline between any of the groups, the data is presented as percent performance change (Fig 5C). No significant main effects were detected; however, AOM/DSS mice treated with 5FU showed a strong trend for a decreased overall performance compared to AOM/DSS alone with a majority (7/9) of mice recording negative changes in performance (Fig 5C, $p=0.06$).

3.1.6 5FU treatment alters gut microbiota and correlates with colonic gene expression.—Recent investigations have indicated that 5FU significantly alters the gut microbiota in both the colon and small intestine. Further, alterations in gut microbiota composition may have detrimental effects on immune function in the gut. To focus our investigation on the colon microenvironment we performed 16S rRNA sequencing on fecal pellets collected during euthanasia (the full analysis will be available upon request to the corresponding author). A Shannon plot and total sequences per sample figures were generated to demonstrate differences in community richness and evenness to estimate within-community diversity (alpha-diversity) (Fig 6A, B). Results of the alpha-diversity plots indicate that three cycles of 5FU did not necessarily alter microbiota diversity in control mice but did alter the richness and evenness in AOM/DSS mice (Fig 6A, B). Unweighted UniFrac PCoA analysis demonstrated that there is a difference in beta-diversity across groups at the OTU level (Fig 6C). A two-way ANOVA was performed to determine main effects and interactions among phyla and class. AOM/DSS increased the relative abundance of OTUs in *Proteobacteria* and *Verrucomicrobia* (Fig 6D, $p<0.05$) but decreased it in *Actinobacteria, Bacteroidetes, and Tenericutes* (Fig 6D, $p<0.05$) whereas 5FU increased the relative abundance of OTUs in the *Actinobacteria* and *Verrucomicrobia* phyla (Fig 6D, $p<0.05$). Surprisingly, there were no main effects in the relative abundance of OTUs of the *Firmicutes* phylum. Interactions were evident within AOM/DSS and 5FU; within AOM/DSS, 5FU decreased the relative abundance of OTUs in the *Firmicutes* phylum compared to AOM/DSS alone (Fig 6D, $p<0.05$) and within 5FU, AOM/DSS had significantly less relative abundance of OTUs of *Actinobacteria* compared to 5FU alone (Fig 6D, $p<0.05$). Interestingly, the aforementioned differences were seen in a similar fashion at the class level. A main effect of AOM/DSS was detected for decreased relative abundance of

Actinobacteria, Bacteroidia, Erysipelotrichi, Deltaproteobacteria, and *Mollicutes* and increased abundance of *Betaproteobacteria* and *Verrucomicrobia* classes (Fig 6E, $p<0.05$). Further, a main effect of 5FU was detected in which the relative abundance of OTUs were decreased in the classes *Coriobacteria* and *Deltaproteobacteria* and increased in the *Actinobacteria, Bacilli, Betaproteobacteria,* and *Verrucomicrobia* classes (Fig 6E, $p<0.05$). There was no significance differences detected in the class *Clostridia*. A significant interaction was found within 5FU in which AOM/DSS mice had significantly greater abundance of OTUs of *Actinobacteria* compared to 5FU alone whereas 5FU resulted in a decrease in *Bacteroidia* in the absence of AOM/DSS (Fig 6E, $p<0.05$).

To observe the potential relationship between gut microbiota changes and colon gene expression we performed Pearson correlations on three of the most influenced microbial phyla that relate to inflammation and cancer outcome; *Bacteriodetes, Proteobacteria,* and *Verrucomicrobia*. Six genes; TNFa ($r=0.788$, $p<0.01$), MCP-1 ($r=0.588$, $p<0.01$), NOS2 ($r=0.679$, $p<0.001$), IL6 ($r=0.671$, $p<0.01$), IL1b ($r=0.516$ $p<0.05$), and FOXP3 ($r=0.535$, $p<0.05$) were positively correlated with *Verrucomicrobia* abundance (Fig S1A-F). Similarly, eight genes; TNFα ($r=0.528$, $p<0.05$), MCP-1 ($r=0.717$, $p<0.01$), IL6 ($r=0.685$, $p<0.01$), IFNγ ($r=0.582$, $p<0.01$), IL1β ($r=0.742$, $p<0.01$), IL10 ($r=0.647$, $p<0.01$), FOXP3 ($r=0.717$, $p<0.01$), and NOS2 ($r=0.624$, $p<0.01$) were positively correlated with *Protoeobacteria* abundance (Fig S1G-N). Lastly, three genes; IL10 ($r=-0.616$ $p<0.01$), IL6 ($r=-0.476$, $p<0.05$), and FOXP3 ($r=-0.680$, $p<0.01$) were negatively correlated with *Bacteriodetes* abundance (FigS1O-Q).

3.1.7 Metagenomic PICRUSt analysis indicates functional consequences of altered microbiota.—

To investigate the functional consequences of the taxonomic changes in gut microbial composition, we performed PICRUSt metagenomic analysis to generate a functional profile of the microbial communities investigated. The full analysis will be available upon request to the corresponding author. We present here the most interesting of the findings (Fig S2A-D). We detected a main effect of 5FU and AOM/DSS in predicted expression of nitrate reductase subunits (alpha, beta, gamma, and delta), [citrate (pro-3S)-lyase] ligase and [protein-PII] uridylyltransferase (Fig S2A, $p<0.05$), and acylphosphatase (Fig S2C, $p<0.05$). Similarly, there is a main effect of AOM/DSS in predicted expression of 5'nucleotidase (Fig S2A, $p<0.05$) and a main effect of 5FU on fumarate hydratase expression (Fig S2D, $p<0.05$). There were no significant interactions for the aforementioned outcomes. However, a significant interaction was detected for beta-mannosidase within 5FU; 5FU alone increased beta-mannosidase but it was significantly decreased in AOM/DSS mice treated with 5FU (Fig S2B, $p<0.05$).

3.2. Experiment 2: Fecal transplantation of 5FU-disturbed microbiota alters host physiology.

3.2.1. Fecal transplantation affects functional measures of fatigue.—

To elucidate the role of the gut microbiota on the adverse side-effects associated with 5FU treatment we performed fecal transplantation studies in control mice (Fig 1B). No body weight or organ weight difference was detected between the two groups (Vehicle FMT and 5FU FMT) (Fig 7A-F). All mice were challenged to the same fatigue tests used in

experiment 1. A significant decrease in absolute grip strength (Fig 7G, $p<0.05$) and a trending decrease in relative grip strength (Fig 7H, $p=0.06$) was detected in mice treated with 5FU FMT compared to vehicle control. In the run-to-fatigue test, a trending decrease (Fig 7 I, $p=0.07$) in performance was recorded in 5FU mice compared to control. Similar to experiment 1, we noticed a large number (6/15) of mice with negative performance changes following 5FU FMT.

3.2.2 Fecal transplantation manifests a unique blood profile—Upon analysis of the blood profile, we noticed a significant increase in WBC, MON, and LYM counts in 5FU FMT mice vs Vehicle FMT controls (Table 2, $p<0.05$). However, no differences in NEU and RBC counts were detected (Table 2). The increase in lymphocyte count contributed to a trending decrease in NLR in 5FU FMT mice compared to Vehicle FMT controls (Table 2, $p=0.07$).

3.2.3. 5FU fecal microbial transplantation reduces macrophage populations in the colon.—To further understand the potential contribution of gut microbiota on inflammatory responses following 5FU treatment, we performed gene expression analysis on the colon tissue. Contrary to our hypothesis, we found a decreased expression of MCP-1, IL10, and EMR1 in 5FU FMT mice compared to Vehicle FMT controls (Fig 8A, $p<0.05$). A trending decrease in IL1β ($p=0.08$) also was detected with no differences in TNFα, NOS2, IL4, and Ly6G expression (Fig 8A). Investigating the morphology of the colon tissue, we did not notice any histological differences between Vehicle FMT and 5FU FMT mice (Fig 8B). The gene expression data explained above indicate a decreased macrophage population in 5FU FMT mice relative to Vehicle FMT controls. To confirm this finding, we performed immunohistochemical analysis of CD68+ cells present in the colons (Fig 8C). Although we did not directly quantify, observation suggests fewer CD68+ positive cells in the lamina propria and epithelial layers in 5FU FMT mice.

4. Discussion

5FU chemotherapy is widely used in the treatment of CRC. However, side effects including fatigue, loss of appetite, and diarrhea, all of which can lead to a reduced quality of life, are common. Emerging evidence suggests that the gut microbiota may play a role in this response. In the current study, we sought to characterize the effects of 5FU on colon inflammation and functional measures in CRC and to further determine whether gut microbiota can influence this response. Despite a general anti-tumor effect of 5FU, we document findings of non-selective toxicity in intestinal and liver tissue that which are associated with reduced functional performance and decreased survival. Select intestinal inflammatory genes were significantly elevated with 5FU treatment and this was further exacerbated with cancer. Both 5FU and AOM/DSS treatment result in significant alterations in gut microbiota and predicted functional parameters. Finally, using fecal transplantation we demonstrate that a 5FU altered microbiome influences the systemic and colonic immune environment and can impact functional parameters.

Our findings indicate that a clinically relevant chemotherapy regimen has general anti-tumor effects in the AOM/DSS model of CRC. This is consistent with previous reports of a

beneficial effect of 5FU in intestinal tumorigenesis in the $Apc^{min/+}$ mice model where a 60–80% reduction in polyps was reported.[30] Despite these benefits however, our data indicates that 5FU reduces body weight, exacerbates symptom severity score, increases liver weight, and decreases epididymal fat mass, consistent with a decrease in survival. These results are not surprising given the well documented toxicities resulting from 5FU non-selectivity[3,4]. The manifestations of 5FU induced toxicity have been mainly attributed to 'mucositis' or the disruption of the mucosal barrier. [14,31]. We confirm this damaging mechanism as we report a main effect of 5FU treatment on reducing villus length in the distal ilium. Further complications of chemotherapy in CRC include progressive liver failure leading to premature death [16,31,32]. Our results are consistent with this as the majority of AOM/DSS mice treated with 5FU presented with acute necrotizing inflammation with abscesses in the liver. These histological findings are indicative of an immune response against a bacterial infection[33,34], evidence that can be associated with the presence of leaky gut syndrome; however, further investigation would be needed to confirm this finding. Other studies have reported similar results using short, high-dosage treatment regimens 12,18 but have utilized non-diseased mouse models. Our findings suggest that the chemotherapy regimen administered in this experiment, using a mouse model of CRC, is effective in replicating the clinical outcome of 5FU treatment.

Given 5FU's well documented effects on blood parameters, we used a diverse blood panel analysis to specifically investigate its effects on circulating immune cells and factors contributing to chronic anemia in the context of CRC. Consistent with clinical and experimental literature, we observe decreased circulating immune cells and indications of severe anemia with 5FU treatment and in the presence of cancer [14,35]. As expected, the combined treatment of 5FU and AOM/DSS had worsening effects on anemia including RBCs, HCT and HGB. Interestingly, however, where 5FU seemingly causes significant immune cell death in a healthy physiological state, it appears that the already altered physiology caused by the AOM/DSS model of tumor burden increased the systemic immune response when combined with 5FU. This specifically caused an increase in neutrophils and monocytes, but not lymphocytes. The mechanism for this increase in circulating innate immune cells is unclear and warrants further investigation.

ROS produced following initiation of 5FU-related mucositis induces tissue injury and triggers a cascade of inflammatory pathways including nuclear factor kappa-B (NF-κB) activation. [36] Once activated by 5FU, NF-κB induces gene expression and production of pro- inflammatory cytokines, which, in turn, lead to tissue injury and apoptosis[36]. In addition, CRC itself leads to significant colon inflammation as we have previously reported [37]. Therefore, it is possible that colon-specific inflammation may be exacerbated following treatment of 5FU in CRC leading to worsening symptoms and poor prognosis. Thus, we next examined the effects of 5FU on colon inflammation in the context of CRC. As hypothesized, both 5FU and AOM/DSS lead to an increase in select inflammatory mediators with a greater degree of inflammation noted in AOM/DSS mice treated with 5FU. As such, it is possible that this 'inflammatory cytokine storm' may explain, at least in part, the increased mortality in AOM/DSS mice treated with 5FU.

To establish the functional deficits associated with chronic 5FU treatment, we performed a grip strength and run-to-fatigue test. AOM/DSS mice treated with three cycles of 5FU significantly reduced absolute grip strength and there was a strong trend towards a decreased run-to-fatigue time. This was consistent with findings of a previous study by our group that characterized the chronic fatigue of 5FU treatment[38]. The mechanisms responsible for 5FU-related fatigue in cancer patients have not yet been fully elucidated[39,40]. However, several hypotheses have been postulated and include: central serotonin dysregulation, hypothalamus- pituitary-adrenal axis dysfunction, circadian disruption, depression, anemia, and central and peripheral inflammation [39,40]. Arguably, the strongest of these are inflammation and anemia. Our data supports these hypotheses given the increase in anemia related outcomes and inflammation in AOM/DSS mice treated with 5FU – mice with the greatest performance deficits.

Recent investigations have implicated a direct role of gut microbiota in the development and treatment of CRC. Therefore, we next examined the effects of 5FU on the gut microbiota profile. Others have reported differences in gut microbiota following 5FU treatment in non-cancer mice[18]. However, to our knowledge this is the first report of a change in microbiota profile when 5FU is administered in the presence of CRC in an experimental mouse model; specifically, we note a decrease in alpha diversity. We focus our discussion on the most important and novel findings of the post-hoc analysis of bacterial phyla. We saw a decrease in abundance of *Firmicutes* phylum in AOM/DSS mice treated with 5FU but not in AOM/DSS alone. The *Firmicutes* represent one of the most abundant phyla of the gut microbiota and are important for short-chain fatty acid production and maintenance of gut homeostasis[18,41]. In-fact, decreases in *Firmicutes* have previously been associated with inflammatory bowel disease and CRC[41,42]. This suggests a possible role of chemotherapeutics in altering the gut microbiota in the presence of CRC in a manner that exacerbates gut dysbiosis and may contribute to a worse prognosis following chemotherapy treatments. Further, both 5FU treatment and AOM/DSS treatment increased relative abundance of *Verrucomicrobia.* This is not surprising given that the *Verrucomicrobia* phylum is suggested to be directly related to inflammation in the colon[43–45]. The increase in *Proteobacteria* phylum with AOM/DSS appeared to result from increased *Betaproteobacteria*. This is consistent with findings in human fecal samples in which *Betaproteobacteria* were found to be increased in patients with CRC[46]. Our findings of a decrease in *Bacteroidetes* in AOM/DSS treated mice are consistent with some reports of lower levels of *Bacteroidetes* being linked to increased cancer risk[42]. The strong correlations detected between the above-mentioned phyla and colon inflammatory mediators suggest a potential link between gut microbes and the exacerbated inflammation in AOM/DSS mice treated with 5FU. If *Verrucomicrobia* and *Proteobacteria* are indeed playing a role in the creation of an ‘inflammatory cytokine storm,’ selected targeting of these phyla may help lower the adverse toxicities common to 5FU treatment. Further, PICRUSt analysis of OTUs indicates significant changes in predicted genes involved in environmental processing and cellular processes. From these predicted changes it can be suggested that alterations in the gut microbiota may affect key processes outside the gut that may be contributing to the exacerbated disease state in AOM/DSS mice treated with 5FU.

While our gut microbiota findings are consistent with some of the previously reported literature for both 5FU and CRC, it is important to note that inconsistencies also exist. For example, a recent study by Hong-Li *et. al.* (2017), reported decreased *Firmicutes* and *Proteobacteria*, increased *Bacteroidetes*, and no significant change in *Verrucomicrobia* following 5FU treatment in a cancer-free model[18], whereas we saw increases in *Verrucomicrobia* and *Actinobacteria* with no changes in the other phyla. However, only 3 consecutive injections of 5FU (50mg/kg) were used by Hong-Li *et.al.* (2017), whereas we employed a cycled regime with the aim of investigating chronic effects of 5FU treatment. These differences in treatment regime likely contributed to the disparities in findings across studies. Further, some of these differences may stem from our utilization of the AIN-76A purified diet instead of standard chow and randomization of the mice upon arrival to the facility to prevent maternal bias in fecal analysis [43]. Similarly, while we report here a decrease in *Bacteroidetes* with AOM/DSS, it should be noted that reports of increases also have been reported in CRC [47] indicating inconsistencies in the literature.

Culminating from the differences in the gut microenvironment of 5FU treated mice, we next sought to determine whether gut microbes may be responsible for some of the side effects associated with 5FU. Thus, we designed an experiment utilizing gut microbiota depletion followed by subsequent engraftment by either Vehicle FMT or 5FU FMT (Fig 1B)[21]. An absence of body weight and organ weight changes from fecal transplantation was contrary to our hypothesis and the findings of Hong-Li *et. al.* (2017), which showed a significant decrease in body weight following transplantation of feces from 5FU treated mice[18]. As noted above, differences in the study design may have contributed to the disparities among findings. Interestingly, however, we did find a decrease in muscle strength and run-to-fatigue times following engraftment of 5FU FMT that is consistent with the findings in experiment 1. To our knowledge this is the first report of a direct relationship between 5FU, gut microbes, and performance deficits and probes the determination of the exact microbes that may be contributing to this response.

We next examined the effects of engraftment by either Vehicle FMT or 5FU FMT on circulating and colonic immune responses. Performing the same diverse blood panel as Experiment 1, we observe an increased number of circulating immune cells (WBC, LYM, MON) in 5FU FMT mice engrafted with 5FU treated feces, a finding contrary to the results of direct 5FU treatment in Experiment 1. Others have reported effects of gut bacteria on circulating immune cells. [48] However, to our knowledge this is the first report of a 5FU influenced gut microbiome on circulating immune cells. Fecal Microbial Transplantation of microbes from mice treated with 5FU (5FU FMT) to non-diseased (Control) mice specifically reduced the MCP-1 and EMR1 gene expression in the colon but Ly6G was unaltered. This indicates that the engrafted microbiome may be causing a reduction specifically in the macrophage population in the colon but not in neutrophils. This was consistent with a notable reduction in CD68+ cells in the colon. This finding was further supported by a decreased expression of IL-10 and a trending decrease in IL-1β, cytokines that are known to be released from macrophages[38]. This suggests that the altered microbial composition resulting from 5FU treatment may, in-itself, be eliciting an anti-inflammatory response independent of 5FU, where cellular damage from 5FU is most likely contributing to the pro-inflammatory response reported in Experiment 1. Although requiring further

investigation, this would explain the correlations of certain bacteria to both pro- and anti-inflammatory markers in the colon tissue in Experiment 1. Taken together, these data suggest that transfer of 5FU treated feces leads to an increased production of circulating immune cells including monocytes; yet appears to reduce macrophages in the colon. However, the exact microbes that are driving this response cannot be determined from the current study.

The novel findings from this study implicate that 1) the immune response to cancer therapy may be regulated by gut microbiota and 2) gut microbiota may influence functional outcomes following cancer therapy. This suggests that gut microbiota may serve as a potential therapeutic target for manipulating immune responses and improving quality of life outcomes during cancer therapy. It is certainly possible, and even likely, that some microbes may confer beneficial effects in this model whereas others may have detrimental effects. Thus, future pre- clinical studies should determine the exact microbes that are responsible for the aforementioned effects so that therapeutics can be developed. Administration of antibiotics and FMT are viable options for manipulating gut microbiota during cancer therapy and could be implemented in the clinic.

In summary, this study examined the functional and physiological effects of 5FU in the context of CRC. Despite a general anti-tumor effect of 5FU, we report findings of non-selective toxicity in intestinal and liver tissue that are associated with reduced functional performance and decreased survival. The side effects of 5FU in CRC were associated with alterations in gut bacteria and functional pathways. Finally, using fecal transplantation we demonstrate that a 5FU altered microbiome can influence the systemic and colonic immune environment and can impact functional parameters. However, further studies are necessary to determine the exact bacterial populations that are responsible for these effects and whether gut bacteria can be targeted for reducing side effects associated with chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 5FU increases mortality and symptom severity despite anti-tumor effects
- Colon inflammation is elevated with 5FU and this is further exacerbated with cancer
- 5FU and cancer lead to dissimilarity and alterations in gut bacterial taxonomy
- 5FU reduces grip strength and run-to-fatigue performance in cancer mice
- A 5FU altered microbiome influences the immune milieu and functional parameters

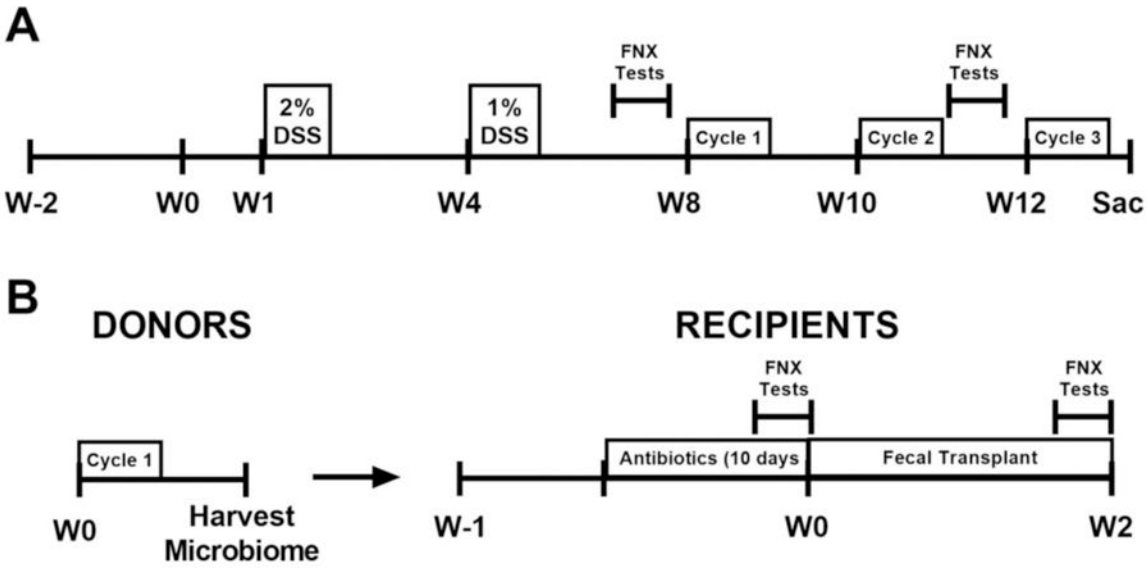

Figure 1. Study Design for experiment 1 and experiment 2.

A. Experiment 1 design and 5FU treatment regimen. B. Experiment 2 design, antibiotics treatment regimen and fecal transplantation.

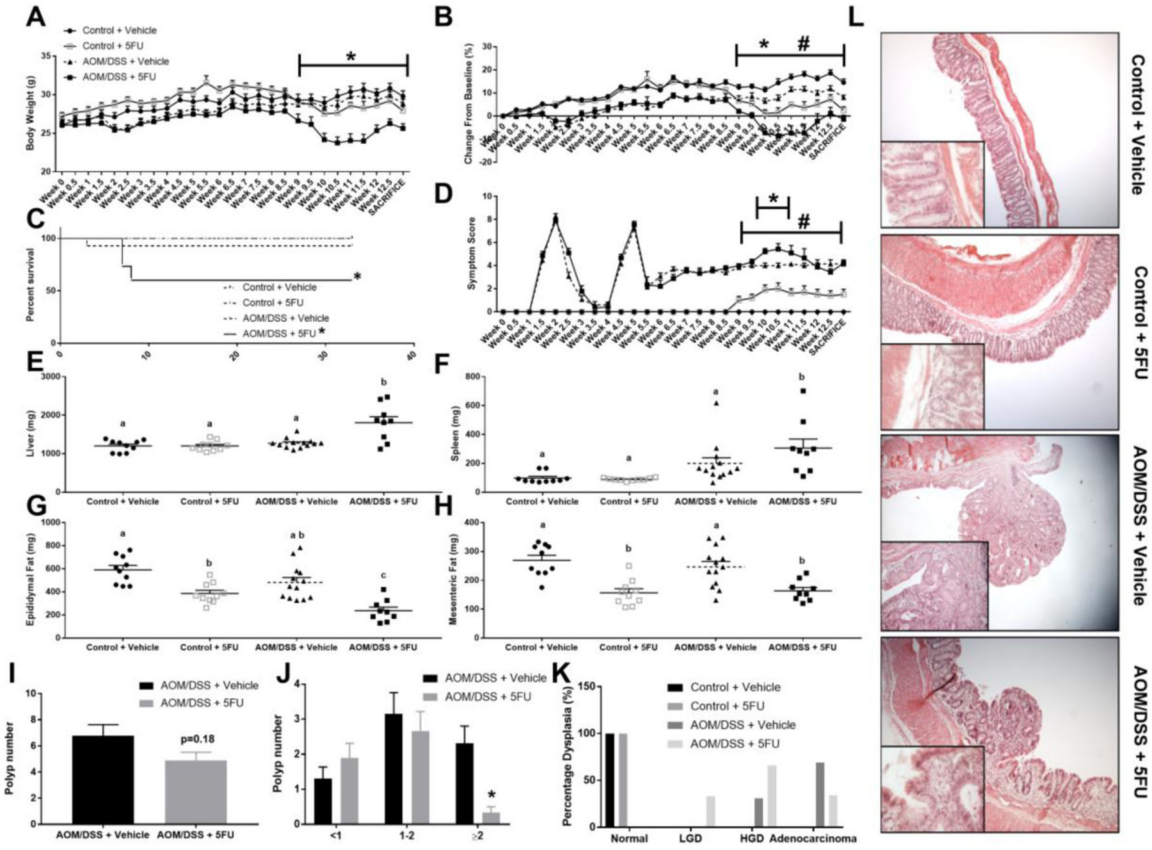

Figure 2. 5FU reduces tumor burden but decreases survival in AOM/DSS mice.

A. Gross body weight. B. Percent body weight change. C. Survival curve. D. Symptom score. * indicates statistical significance ($p<0.05$) for AOM/DSS + 5FU group vs. all groups, # indicates statistical signficiance ($p<0.05$) for Control + 5FU vs. Control + Vehicle from 3-way ANOVA. E-H. Liver, spleen, epididymal fat, and mesenteric fat, weights at euthanization. Groups not containing the same letters (a,b,ab) indicate statistical significance between groups ($p<0.05$) from two-way ANOVA, n=9–13/group. I. Total Polyp count. J. Polyp size. K. Dysplasia characterization. L. Representative H&E staining of distal colon specimens at 4X, insets are 20X. * indicates statistical significance ($p<0.05$) from students t-test, n = 9–13/group.

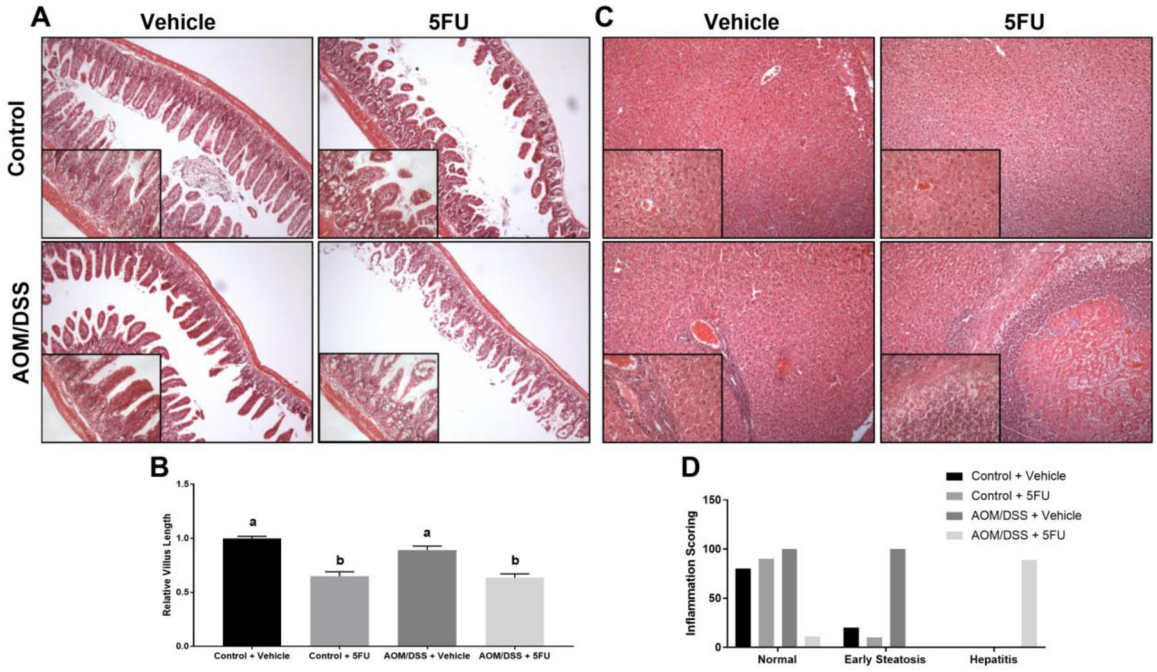

Figure 3. Nonspecific toxicity of 5FU in distal ileum and liver tissue

A. Representative H&E staining of distal ileum specimens at 4X, insets are 20X. B. Measurement of villus length in small intestine. Groups not containing the same letters (a,b) indicate statistical significance between groups ($p<0.05$) from two-way ANOVA. C. Representative H&E staining of liver tissue specimens at 4X, insets are 20X. D. Histopathological analysis of liver inflammation. n = 9– 13/group.

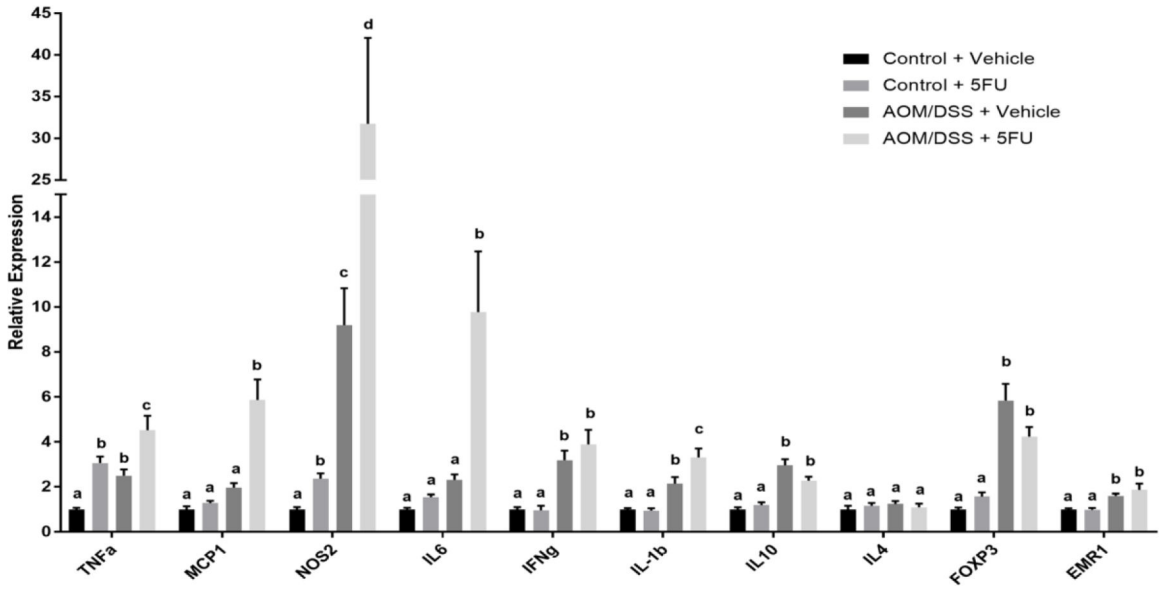

Figure 4. 5FU alters the immune profile of the colon.

A. qPCR analysis of TNFa, MCP-1, NOS2, IL6, IFNg, IL1b, IL10, IL4, FOXP3 genes. Data were normalized to vehicle treated controls and compared to two reference targets (TBP and H2AFV), which were evaluated for expression stability using GeNorm. Groups not containing the same letters (a,b,c,d) indicates statistical significance between groups ($p<0.05$) from two-way ANOVA, n = 9–13/group.

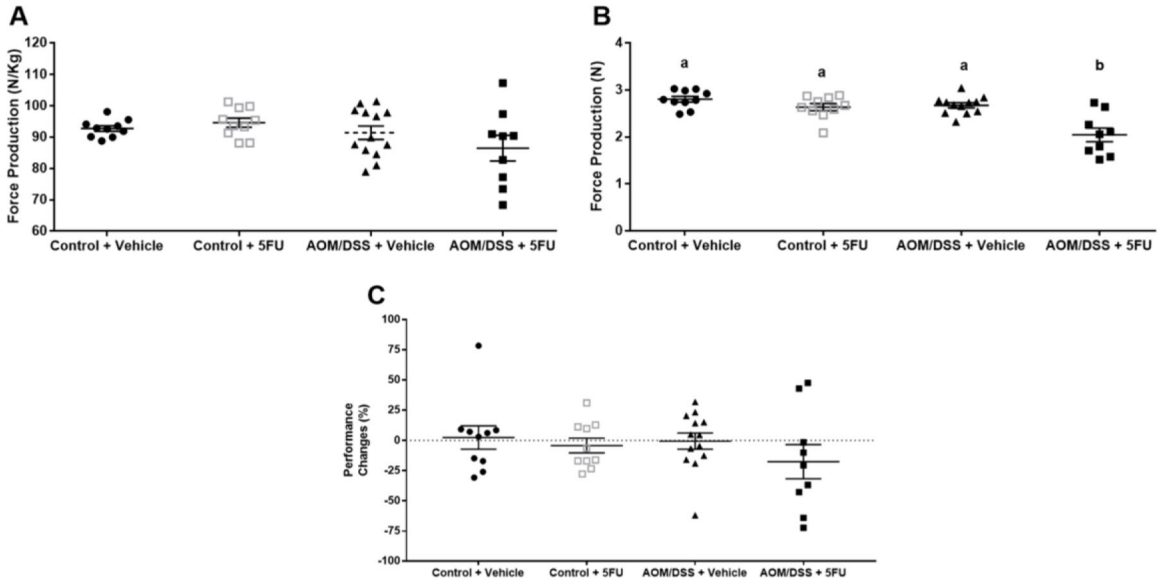

Figure 5. 5FU affects functional measures of fatigue.

A. Relative grip strength measured in newtons/kilogram (N/Kg). B. Absolute grip strength measured in newtons (N). C. Percent performance change in run-to-fatigue test from pre-5FU treatment to post-5FU treatment. Groups not containing the same letters (a,b) indicates statistical significance between groups ($p<0.05$) from two-way ANOVA, n = 9–13/group.

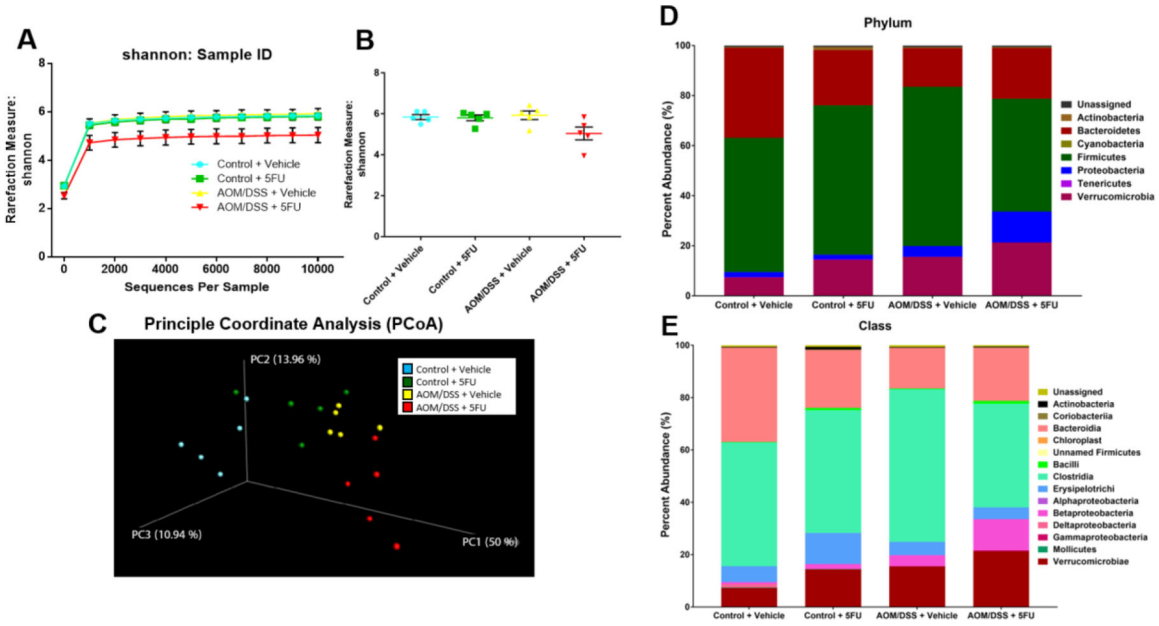

Figure 6. 5FU alters gut microbial profile.

A. Shannon plot of alpha-diversity in microbial communities. B. Total sequences per sample by Shannon rarefaction. C. Unweighted UniFrac Principle Coordinate Analysis (PCoA) of beta-diversity in microbial communities. D. Percent abundance of microbial phyla. E. Percent abundance of microbial class, n=5/group.

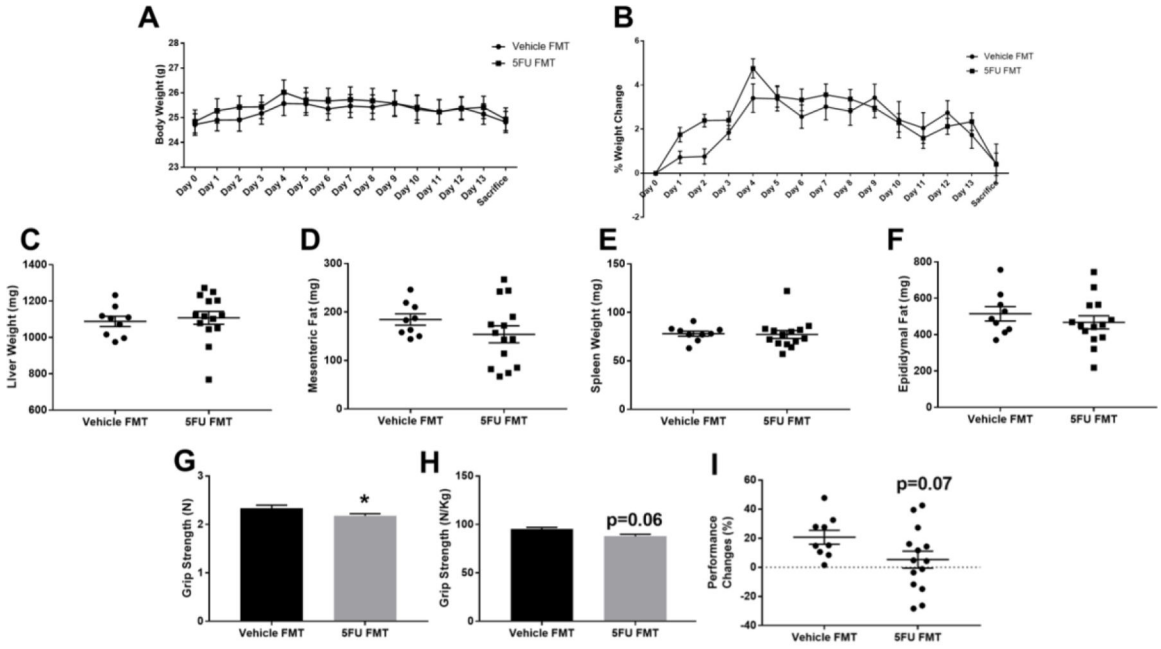

Figure 7. Fecal Transplantation of 5FU altered microbiome does not alter body weight but does affect functional measures of fatigue.

A. Gross body weight. B. Percent body weight change. C-F. Liver, spleen, epididymal fat, and mesenteric fat, weights at euthanization. G. Relative grip strength measured in newtons/kilogram (N/Kg). H. Absolute grip strength measured in newtons (N). I. Characterization of performance changes in run-to-fatigue test. J. Percent performance change in run-to-fatigue test from pre-5FU treatment to post-5FU treatment. * indicates statistical significance between groups ($p<0.05$) from students t-test n=10– 15/group.

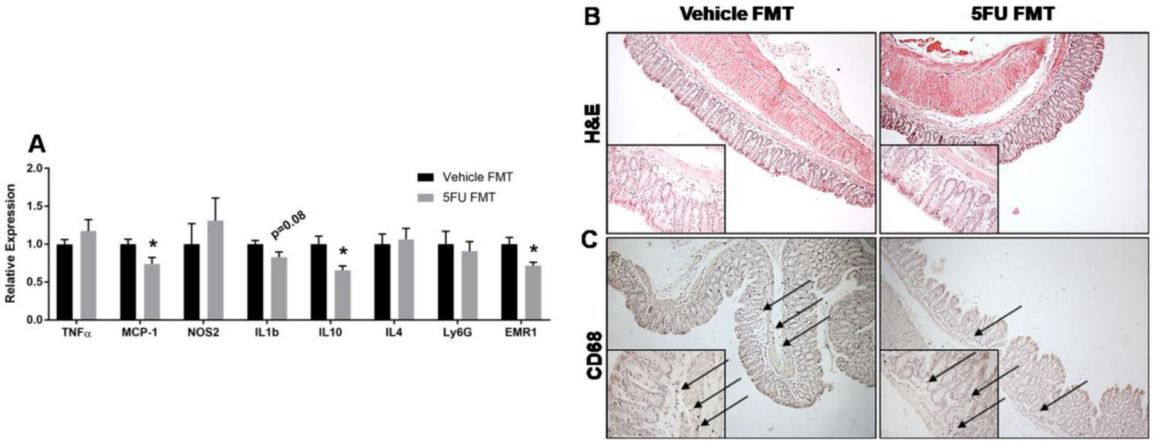

Figure 8. 5FU alters macrophage population in the colon lamina propria and is replicated with fecal transplantation.

A. qPCR analysis of TNFa, MCP-1, NOS2, IL1b, IL10, IL4, Ly6G, and EMR1 genes in fecal transplantation mice. B,C. Representative H&E staining and CD68+ staining, respectively, in distal colons of fecal transplantation mice. Arrows indicate examples of positive staining. * indicates statistical significance between groups ($p<0.05$) from students t-test, n=10–15/group.

Table 1.

5FU alters blood profile in AOM/DSS mice different from control.

White blood cells (WBC), Lymphocytes (LYM), Monocytes (MON), Neutrophils (NEU), Neutrophil:Lymphocyte Ratio (NLR), Red Blood Cells (RBC), Hemoglobin (HGB), and Hematocrit (HCT). Groups not containing the same letters (a,b,c,d) indicate statistical significance between groups ($p<0.05$) from two-way ANOVA, n=9–13/group.

Group	WBC (10^9/l)	LYM (10^9/l)	MON (10^9/l)	NEU (10^9/l)	NLR	RBC(10^12/l)	HGB
Control + Vehicle	4.19 ± 0.39[a]	2.58 ± 0.25[a]	0.20 ± 0.06[a]	1.40 ± 0.29[a]	0.61 ±0.12[a]	9.62 ± 0.48[a]	14.80 0.22 a
Control + 5FU	2.16 ± 0.12[b]	1.52 ± 0.11[b]	0.06 ± 0.01[b]	0.58 ± 0.05[b]	0.40 ± 0.04[b]	7.45 ± 0.25[b]	11.28 0.13 [b]
AOM/DSS + Vehicle	3.65 ± 0.3 [a]	2.70 ± 0.27[a]	0.17 ± 0.04[a]	0.78 ± 0.08[a]	0.33 ± 0.05 [b]	7.63 ± 0.48 [b]	11.38 0.62 [b]
AOM/DSS + 5FU	7.21 ± 1.50[c]	2.43 ± 0.26[a]	0.53 ± 0.17[c]	4.25 ± 1.33[c]	2.04 ± 0.67 [c]	6.85 ± 0.21[c]	8.80 ± [c]

Table 2.

5FU FMT manifests a unique blood profile.

White blood cells (WBC), Lymphocytes (LYM), Monocytes (MON), Neutrophils (NEU), Neutrophil:Lymphocyte Ratio (NLR), Red Blood Cells (RBC), Hemoglobin (HGB), and Hematocrit (HCT).

Group	WBC (10^9/l)	LYM (10^9/l)	MON (10^9/l)	NEU (10^9/l)	NLR	RBC (10^12/l)	HGB
Vehicle FMT	4.84 ± 0.40	3.76 ± 0.20	0.12 ± 0.00	0.97 ± 0.20	0.25 ± 0.00	9.79 ± 0.20	14.61 0.30
5FU FMT	6.34 ± 0.39 *	5.12 ± 0.31 *	0.34 ± 0.06 *	0.89 ± 0.10	0.18 ± 0.02	10.28 ± 0.24	14.27 0.25

* indicates statistical significance between groups ($p<0.05$) from students t-test, n=10–15/group.