Supplementary Materials and Methods

Metagenomic Cohorts of Colorectal Cancer Patients and Healthy Controls

Independent Chinese cohort named as "YuJ_2021" of 110 CRC patients and 112 healthy controls were recruited from the participants under colonoscopy screening at the Jockey Club Bowl Cancer Education Centre, The Chinese University of Hong Kong. The clinical study protocol was approved by Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. All participants signed an informed consent form. The mycobioa partial of the metagenomic data was published by our team and the details of sample collection was shown in that study. [1]

Four published metagenomic data cohorts from China, Germany, France, and Japan, including 334 CRC patients and 463 healthy individuals, were used to evaluate the abundance of *R. intestinalis*. Metagenomic data sets were downloaded from the European Nucleotide Archive (ENA) with the following ENA identifiers: PRJEB10878 for YuJ_2017, [2] PRJEB27928 for WirbelJ_2019, [3] ERP005534 for ZellerG_2014 [4] and DNA Data Bank of Japan for YachidaS_2019 [5] with accession number: DRA006684 and DRA008156.

Metagenomic Cohorts of Responders and Non-responders to Immune Checkpoint Inhibitors

Metagenomic sequences data to analyze the relative abundance of *R. intestinalis* in responders and non-responders to immune checkpoint inhibitors (ICI) were downloaded from SRA under the Bioproject accession PRJNA751792 and PRJNA782662. [6] This France cohort included 333 non-small-cell lung cancer patients (258 non-responders and 75 responders). Stool samples were collected before the ICI therapy start.

Bacterial culture

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291

R. intestinalis (DSM 14610) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and cultured at 37 ℃ in modified Yeast Casitone Fatty Acids (YCFA) Broth with Hungate bottles filled with nitrogen. *Escherichia coli* MG1665 was used as bacterial control and cultured at the same condition as *R. intestinalis.* When the absorbance of *R. intestinalis* reached optical density 600 of 1.0, the culture medium was centrifuged and filtered with 0.22 μm pore size filter to collect *R. intestinalis* culture medium (*R.i* CM) or *E. coli* culture medium (*E. coli* CM). Fraction larger or less than 3kDa was separated by 3,000 Dalton molecular weight cut-off filters (UFC9003, Merck KGaA, DE).

Targeted Metabolomics Cohort of Colorectal Cancer Patients and Negative Controls

A published metabolomics cohort including 118 CRC patients and 128 negative control individuals was performed gas chromatography coupled to time-of-flight mass spectrometer (GC-TOFMS) targeted a human gut microbiota-host co-metabolism panel by the authors and the significantly altered metabolites between CRC and NC were downloaded from Pubmed. [7] Metabolites with variable importance in projection (VIP) score >1 and P value <0.05 were considered as significant.

Histology evaluation

Formalin-fixed paraffin-embedded colon tissues from $Apc^{Min/+}$ and AOM-induced mouse were stained with hematoxylin and eosin (H & E) and scored by pathologists. Benign tissues were scored as “0”; tissues with low-grade dysplasia was scored as “1”; and tissues with high-grade dysplasia were scored as “2”.

Immunohistochemistry (IHC) staining

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291

Colon tissues were deparaffinized and rehydrated, followed by antigen unmasking and blocking. Slides were then incubated with primary antibodies (**S Table 1**) at 4°C overnight, followed by biotinylated secondary antibodies and streptavidin HRP at room temperature for 30 minutes each. Signals were developed with DAB chromogen. High-power fields were randomly chosen, and positive cells were calculated by ImageJ 1.53a with IHC Profiler plugin.

Intestinal permeability assay

Mouse was orally gavaged with 150 μL of 80 mg/mL 4 kDa FITC-dextran (68059, Sigma-Aldrich) after 6-hours fasting. 50 μL blood was collected 4 hours after FITC-dextran gavage. 100 μL of PBS-diluted (1:5) plasma and standards were transferred to a 96-well plate and the fluorescence was measured at 528 nm with 485 nm excitation. Permeability was expressed as relative fluorescence unit between groups.

Western blot

Total proteins from mouse colon tissues, CRC cell lines and human isolated T cells were extracted and separated by SDS-PAGE gel, then transferred onto 0.2 μm Nitrocellulose Membrane (1620112, Bio-Rad, WA). The membrane was blocked and incubated at 4°C overnight with primary antibodies, followed by one-hour secondary antibody incubation at room temperature. Primary antibodies used in this study were listed in **S Table 1**. β-actin was used as internal control.

Cell culture

Normal human colon epithelial cell NCM460, human CRC cell lines HCT116, LoVo and SW480, and mouse CRC cell lines MC38 and CT26 were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Jurkat E6.1 (TIB-152, ATCC, VA) was cultured in RPMI-1640 supplied with 10% FBS and 1% P/S, and

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291

activated in 3 μg/ml anti-CD3 (300332, BioLgend, CA), 5 μg/ml anti-human CD28 (302934, BioLegend), and 100 IU/ml recombinant human IL-2 (589104, BioLgend) pre-coated wells for 3 days.

Cell viability and colony formation assay

1000 cells per well were seeded onto a 96-well plate and treated with live or pasteurized *E. coli* (CFU=10^6/ml) or *R. intestinalis* (CFU=10^6/ml), as well as 5% (vol/vol) YCFA, *E. coli* CM, or *R.i* CM in DMEM. Cell viability was determined by Thiazolyl Blue Tetrazolium Bromide (M5644, Merck KGaA) for 3 consecutive days. For cell colony formation assay, cells were treated for one to two weeks and the culture medium was changed every 3 days. After fixation by ice-cold methanol, cells were stained with 0.5% crystal violet solution. Cell colonies were measured by ImageJ 1.53a.

Immunofluorescent staining

Human CRC cells were seeded on poly-L-lysine coated coverslips in a 4-well plate and fixed with ice-cold methanol for 10 minutes. Then, cells were blocked with 1% bovine serum albumin (BSA) in PBST for 30 minutes and incubated with Ki-67 (8D5) Mouse mAb in a humidified chamber at 4°C overnight, followed by Alexa Fluor 488 goat anti-mouse IgG secondary antibody for one hour at room temperature in dark. Cell nucleus was stained with DAPI (P36935, Thermo Fisher Scientific) for one minute before mounting. Images were captured by a confocal laser scanning microscope (TCS SP8, Leica, IL). The proportion of Ki-67 positive cell in each randomly selected field was measured by ImageJ 1.53a.

Cell apoptosis and cell cycle

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291

Cell apoptosis was detected by FITC Annexin V apoptosis detection kit (556547, BD Biosciences, NJ) according to manufacturer's instructions. For cell cycle analysis, cell pellet was washed by PBS and incubated in 70% ethanol at -20°C for two hours. A concentration of 1×10^6 cells was used for staining in 0.5 ml PI/RNase staining buffer (550825, BD Biosciences) for 15 minutes at room temperature in dark. Stained cells were analyzed by flow cytometer for cell apoptosis and cell cycle analysis (FACSAria cell sorter, BD Biosciences). Data was analyzed by FlowJo 10.4.

Patient-derived CRC organoid culture

CRC patient-derived organoid was obtained from the Princess Margaret Living Biobank (CAN), originally from a 46-year-old female colorectal adenocarcinoma patient. The minced pathologic specimens were digested and embedded into Matrigel and maintained in Advanced DMEM/F12, supplied with 1% P/S, 10 mM HEPES, 1×GlutaMAX medium containing N2 and B27 supplements, 50% (vol/vol) Wnt3a conditional medium, 10% (vol/vol) R-spondin-1 conditional medium, 100 ng/ml Noggin conditional medium, 50 ng/ml EGF, 10nM Gastrin, 100 ng/ml FGF10, 1.25 mM N-acetylcysteine, 500 nM A8301, 1 μM SB202190, and 10 mM Nicotinamide. Treatment such as 5% (vol/vol) YCFA, *E. coli* CM or *R. i* CM was added into the culture medium directly, which was freshly changed every three days. Surface area of organoid was measured by Image J 1.53a.

Untargeted metabolomic profiling

400 μL extract solution containing isotopically labelled internal standard mixture was added into 100 μL of sample (YCFA<3kDa, *E. coli* CM<3kDa, or *R.i* CM<3kDa). The sample mixture was sonicated in iced water bath for 10 minutes and incubated at -40 °C for one hour to precipitate proteins. After centrifugation, the supernatant was transferred to a glass vial for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using UHPLC system (Vanquish, Thermo Fisher Scientific) with

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291

UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm), coupled to Orbitrap Exploris 120 mass spectrometer on information-dependent acquisition (IDA) mode which was controlled by the acquisition software (Xcalibur, Thermo Fisher Scientific).

Targeted gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS, Shimadzu, JP) equipped with HP-FFAP capillary column (30m×250μm×0.25μm, Agilent, US) was applied to detect short-chain fatty acids (SCFAs) in mouse stool samples and cultured medium (YCFA<3kDa, *E. coli* CM<3kDa, or *R.i* CM<3kDa). For sample preparation, 10-20mg of stool sample was weighed and homogenized in 100ul DDI water. Then 100ul of the stool sample or bacterial cultured medium were individually mixed with 10ul of 1.0M HCl and internal standard 2-methylpentanoic acid. Further, a liquid-liquid extraction was performed by adding 200ul of cold methyl tert-butyl ether (MTBE). The mixture was subjected to 30 seconds of vortex, 10 minutes of oscillation and 10 minutes of sonication in ice water. After a 15-minute centrifugation at 10000 rpm at 4 °C, 100ul of upper MTBE layer was transferred into a glass vial for GC-MS analysis. The initial GC oven temperature was 80 °C, held for 1 min, raised to 200 °C at a rate of 10 °C/min, and held for 5 min, then raised to 240 °C at a rate of 40 °C/min and held for 1 min. The mass spectrometry data were acquired in Scan/SIM mode with the m/z range of 33-150. The concentration was canulated according to the standard curve established with individual SCFA standards purchased from Dr. Ehrenstorfer GmbH (DE).

RNA isolation and quantitative PCR

Total RNA was extracted with Trizol (15596018, Invitrogen) from T cells isolated from naïve mouse spleen according to manufacturer's instructions. The extracted RNA was then reversely transcribed into complementary DNA with RT reagent kit with gDNA eraser (RR047A, Takara, JP). Quantitative PCR was performed using TB Green Premix

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291

(RR420A, Takara) in QuantStudio 7 Flex System (Thermo Fisher Scientific). The relative RNA expression was normalized to *β-actin* as the denominator.

NF-κB (p65) transcription factor assay

Human T cell was isolated and activated as described before. After treating with butyrate (1mM) with or without TH1020 for 3 days, nuclear protein of activated T cells was extracted with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Fisher Scientific). Nuclear protein concentration was determined by BCA assay and normalized to 0.2 *μg/μl*. NF-κB (p65) Transcription Factor Assay Kit (10007889, Cayman, MN) was used to detect specific transcription factor DNA binding activity in T cell nuclear extracts accordingly with 450 nm absorbance.

Reference

1 Lin Y, Lau HC, Liu Y, Kang X, Wang Y, Ting NL, *et al.* Altered Mycobiota Signatures and Enriched Pathogenic Aspergillus rambellii Are Associated With Colorectal Cancer Based on Multicohort Fecal Metagenomic Analyses. Gastroenterology 2022;**163**:908-21.

2 Yu J, Feng Q, Wong SH, Zhang D, Liang QY, Qin Y, *et al.* Metagenomic analysis of faecal microbiome as a tool towards targeted non-invasive biomarkers for colorectal cancer. Gut 2017;**66**:70-8.

3 Wirbel J, Pyl PT, Kartal E, Zych K, Kashani A, Milanese A, *et al.* Meta-analysis of fecal metagenomes reveals global microbial signatures that are specific for colorectal cancer. Nat Med 2019;**25**:679-89.

4 Zeller G, Tap J, Voigt AY, Sunagawa S, Kultima JR, Costea PI, *et al.* Potential of fecal microbiota for early-stage detection of colorectal cancer. Mol Syst Biol 2014;**10**:766.

5 Yachida S, Mizutani S, Shiroma H, Shiba S, Nakajima T, Sakamoto T, *et al.* Metagenomic and metabolomic analyses reveal distinct stage-specific phenotypes of the gut microbiota in colorectal cancer. Nat Med 2019;**25**:968-76.

6 Derosa L, Routy B, Thomas AM, Iebba V, Zalcman G, Friard S, *et al.* Intestinal Akkermansia muciniphila predicts clinical response to PD-1 blockade in patients with advanced non-small-cell lung cancer. Nat Med 2022;**28**:315-24.

7 Coker OO, Liu C, Wu WKK, Wong SH, Jia W, Sung JJY, *et al.* Altered gut metabolites and microbiota interactions are implicated in colorectal carcinogenesis and can be non-invasive diagnostic biomarkers. Microbiome 2022;**10**:35.

Kang X, *et al. Gut* 2023; 72:2112–2122. doi: 10.1136/gutjnl-2023-330291