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Original research

Roseburia intestinalis generated butyrate boosts anti-PD-1 efficacy in colorectal cancer by activating cytotoxic CD8⁺ T cells

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ABSTRACT

Objective *Roseburia intestinalis* is a probiotic species that can suppress intestinal inflammation by producing metabolites. We aimed to study the role of *R. intestinalis* in colorectal tumourigenesis and immunotherapy.

Design *R. intestinalis* abundance was evaluated in stools of patients with colorectal cancer (CRC) (n=444) and healthy controls (n=575). The effects of *R. intestinalis* were studied in *Apc*^{Min/+} or azoxymethane (AOM)-induced CRC mouse models, and in syngeneic mouse xenograft models of CT26 (microsatellite instability (MSI)-low) or MC38 (MSI-high). The change of immune landscape was evaluated by multicolour flow cytometry and immunohistochemistry staining. Metabolites were profiled by metabolomic profiling.

Results *R. intestinalis* was significantly depleted in stools of patients with CRC compared with healthy controls. *R. intestinalis* administration significantly inhibited tumour formation in *Apc*^{Min/+} mice, which was confirmed in mice with AOM-induced CRC. *R. intestinalis* restored gut barrier function as indicated by improved intestinal permeability and enhanced expression of tight junction proteins. Butyrate was identified as the functional metabolite generated by *R. intestinalis*. *R. intestinalis* or butyrate suppressed tumour growth by inducing cytotoxic granzyme B⁺, interferon (IFN)-γ⁺ and tumour necrosis factor (TNF)-α⁺ CD8⁺ T cells in orthotopic mouse models of MC38 or CT26. *R. intestinalis* or butyrate also significantly improved antiprogrammed cell death protein 1 (anti-PD-1) efficacy in mice bearing MSI-low CT26 tumours. Mechanistically, butyrate directly bound to toll-like receptor 5 (TLR5) receptor on CD8⁺ T cells to induce its activity through activating nuclear factor kappa B (NF-κB) signalling.

Conclusion *R. intestinalis* protects against colorectal tumourigenesis by producing butyrate, which could also improve anti-PD-1 efficacy by inducing functional CD8⁺ T cells. *R. intestinalis* is a potential adjuvant to augment anti-PD-1 efficacy against CRC.

INTRODUCTION

Colorectal cancer (CRC) is the third major cancer type and a major cause of cancer-related death worldwide.¹ Despite the high response rate to immune checkpoint blockade in patients with different cancers, the efficacy of immunotherapy

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Gut microbiome and metabolites are associated with colorectal cancer (CRC) tumourigenesis and immunotherapeutic response.
- ⇒ *Roseburia intestinalis* is a probiotic species that can suppress intestinal inflammation by producing metabolites.

WHAT THIS STUDY ADDS

- ⇒ *R. intestinalis* is depleted in patients with CRC compared with healthy controls by faecal metagenomic sequencing.
- ⇒ *R. intestinalis* administration significantly inhibits tumour formation and restores gut barrier function in multiple CRC mouse models.
- ⇒ Butyrate is the functional metabolite produced by *R. intestinalis* identified by non-target, target metabolomic profiling and biofunctional investigations.
- ⇒ Butyrate inhibits CRC tumourigenesis, and in particular boosts antiprogrammed cell death protein 1 (anti-PD-1) efficacy by enhancing the activity of cytotoxic CD8⁺ T cells in mice.
- ⇒ Mechanistically, butyrate directly binds to toll-like receptor 5 (TLR5) receptor on CD8⁺ T cells to induce its activity through activating nuclear factor kappa B (NF-κB) signalling.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ *R. intestinalis* protects against colorectal tumourigenesis by producing butyrate.
- ⇒ *R. intestinalis* and its generated butyrate improve anti-PD-1 efficacy by inducing functional CD8⁺ T cells.
- ⇒ *R. intestinalis* is a potential adjuvant therapy to augment anti-PD-1 efficacy against CRC.

against CRC is limited.² Clinical evidence demonstrated that only patients with CRC with microsatellite instability (MSI)-high subtype could respond to programmed cell death protein 1 (PD-1) inhibitor,³ which only accounts for 10%–15% of total CRC cases.⁴ In contrast, most CRC tumours are microsatellite stable (MSS), thus presenting a major challenge for immunotherapy against the

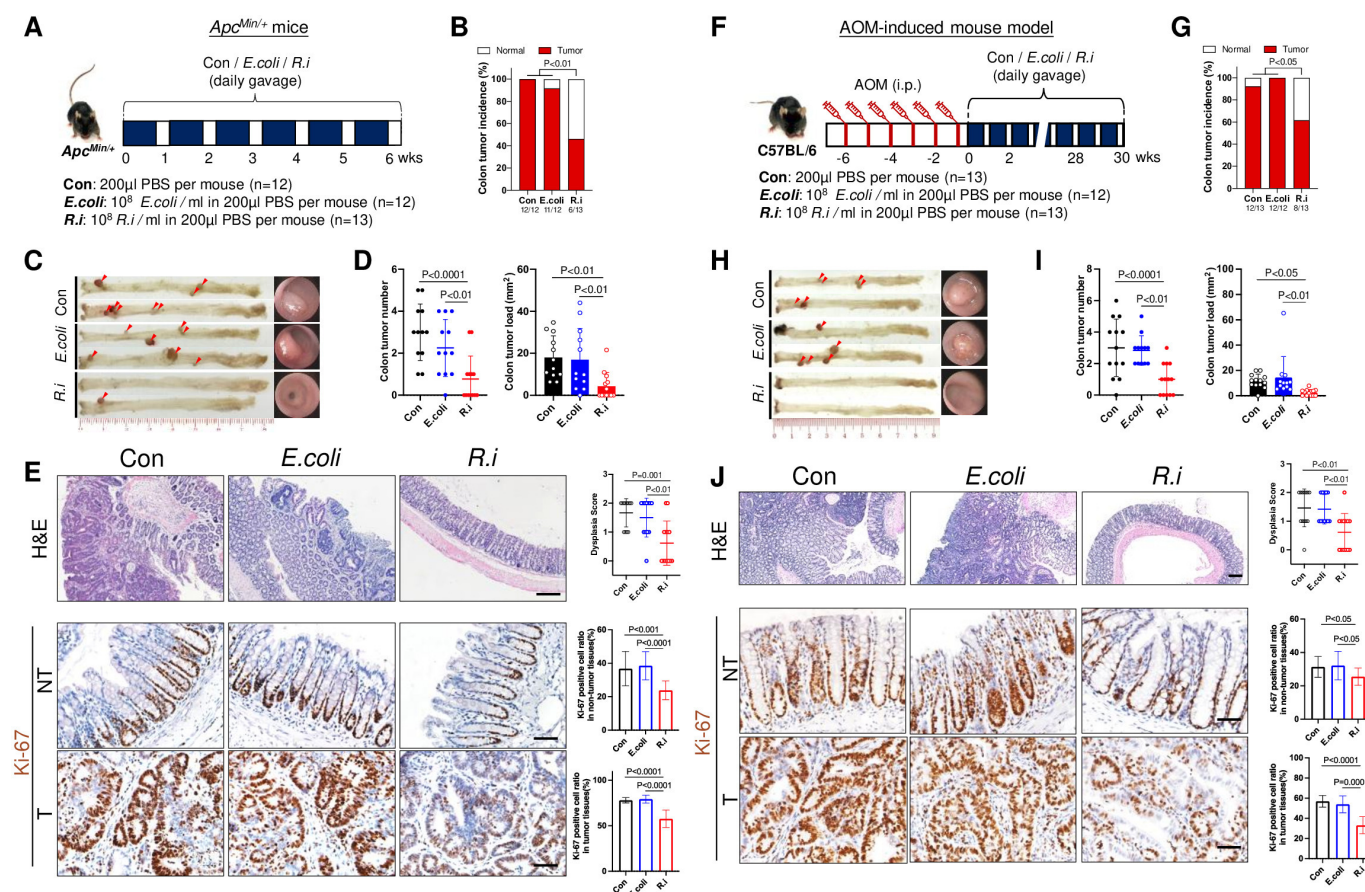


Figure 1 *Roseburia intestinalis* (*R.i*) reduces colorectal carcinogenesis in *Apc*^{Min/+} and azoxymethane (AOM)-induced mice. (A) Schematic diagram showing the experimental design and timeline of *Apc*^{Min/+} mice experiment. (B) Representative colonic morphologies (left panel) and colonoscopy video captures (right panel) of *Apc*^{Min/+} mice. Colon tumour incidence (C), colon tumour number and colon tumour load (total tumour area, mm²) (D) of *Apc*^{Min/+} mice. (E) Representative H&E staining images (scale bar=200 µm) and Ki-67 immunohistochemistry (IHC) staining (scale bar=50 µm) of *Apc*^{Min/+} mice. (F) Schematic diagram showing the experimental design and timeline of AOM-induced mice. (G) Representative colonic morphologies (left panel) and colonoscopy video captures and incidence (right panel) of AOM-induced mice. Colon tumour incidence (H), colon tumour number and colon tumour load (total tumour area, mm²) (I) of AOM-induced mice. (J) Representative H&E staining images (scale bar=200 µm) and Ki-67 IHC staining (scale bar=50 µm) of AOM-induced mice. Con, control; *E. coli*; *Escherichia coli*; PBS, phosphate-buffered saline.

majority of patients with CRC. Given that CD8⁺ T cells play an essential role in antitumour immunity, promoting functional CD8⁺ T cell infiltration to the tumour microenvironment may improve immunotherapy efficacy in patients with CRC, especially for those with MSS subtype. The gut microbiota is known to be associated with the efficacy of cancer immunotherapy by modulating host antitumour immune response.⁵ For instance, a depleted microbiota by antibiotics or in germ-free mice was irresponsive to immunotherapy, whereas oral supplementation of bacteria or faecal microbiota transplantation to these resistance mice restored immunotherapy response.^{6,7} Nevertheless, whether and how certain bacteria improve immunotherapy efficacy in patients with CRC remain largely unclear.

Metabolites produced by the gut microbiota have been shown to influence the development of CRC.⁸ Butyrate, as one of the essential bacterial metabolites,⁹ its concentration is negatively correlated with CRC incidence.¹⁰ Meanwhile, several butyrate-producing bacteria such as *Roseburia* were depleted in patients with CRC.¹⁰ In particular, *Roseburia intestinalis*, a Gram-positive, obligate anaerobic, butyrate-producing bacterium, was first isolated from human faeces in 2002,¹¹ and its metabolites have been shown to prevent intestinal inflammation.¹² These data prompted us to investigate whether *R. intestinalis* and its

metabolites could contribute to the suppression of colorectal tumorigenesis as well as improving immunotherapy efficacy against CRC. In this study, the protective role of *R. intestinalis* in CRC was investigated in multiple mouse models. We found that *R. intestinalis* produced butyrate which could directly boost cytotoxic CD8⁺ T cell function through toll-like receptor 5 (TLR5)-dependent nuclear factor kappa B (NF-κB) signalling. *R. intestinalis* also restricted both MSI-high and MSS orthotopic tumour growth by modulating antitumour CD8⁺ T cell response. Collectively, our findings suggested that *R. intestinalis* and its metabolite butyrate have potential clinical impact in improving immunotherapy efficacy in patients with CRC.

MATERIALS AND METHODS

Mouse models

Two CRC mouse models, transgenic *Apc*^{Min/+} and carcinogen azoxymethane (AOM)-induced, were established in this study. The intestinal adenoma spontaneous mouse strain C57BL/6J-*Apc*^{Min}/J (The Jackson Laboratory, Maine, USA) was orally treated with 1 × 10⁸ colony-forming unit of *R. intestinalis* daily at the age of 6 weeks, for consecutive 6 weeks. The same amount of *Escherichia coli* MG1665 or phosphate-buffered saline (PBS)

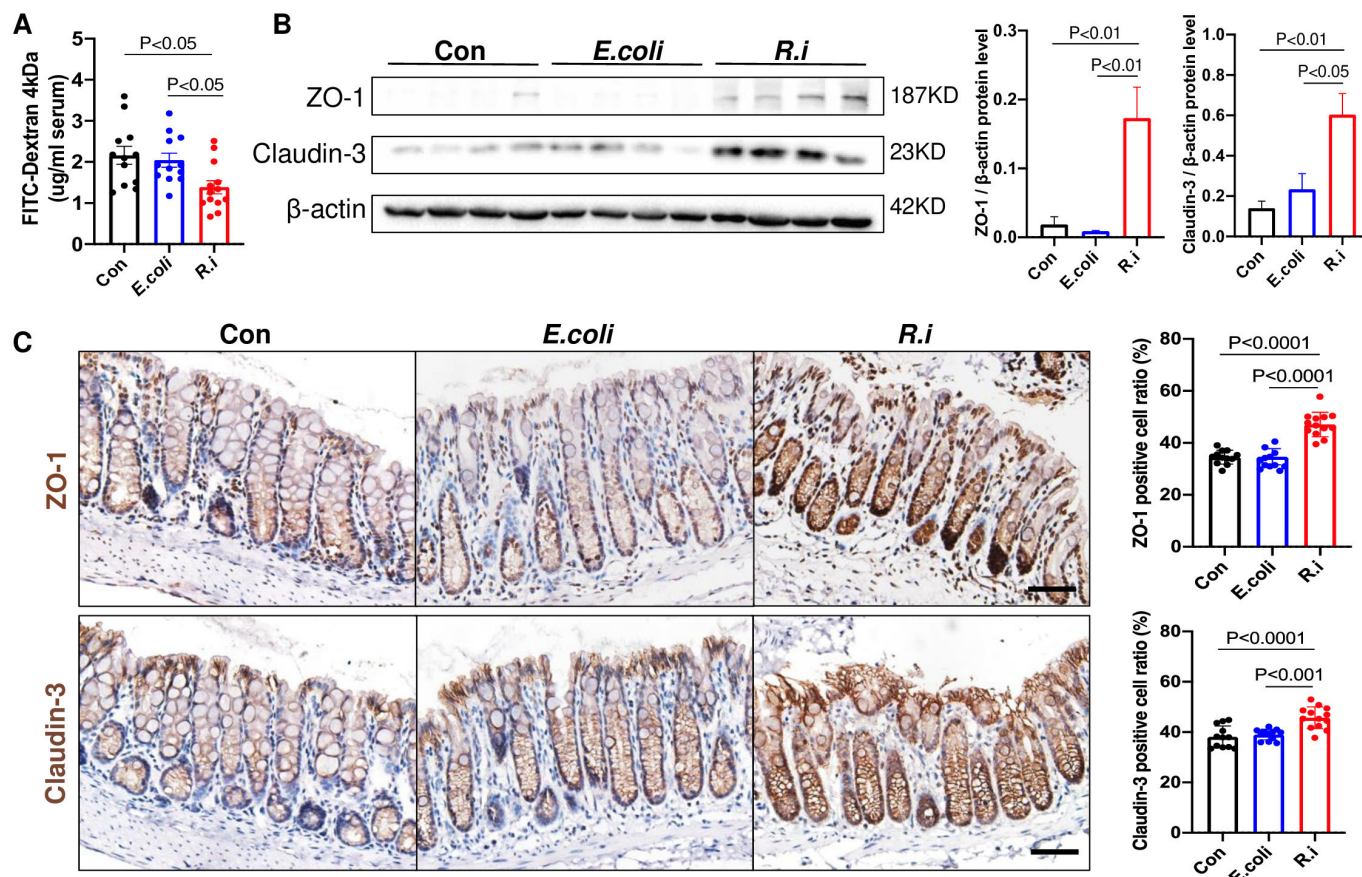


Figure 2 *Roseburia intestinalis* (*R.i*) enhances epithelial barrier functions in *Apc*^{Min/+} mice. (A) FITC-dextran (4 kDa) concentration (ng/mL) in *Apc*^{Min/+} mice serum. (B) Expression levels of tight junction proteins ZO-1 and claudin-3 determined by western blot analysis in colon tissues of *Apc*^{Min/+} mice. (C) Representative ZO-1 and claudin-3 immunohistochemistry staining images (scale bar=50 μm). Con, control; *E. coli*, *Escherichia coli*; FITC, fluorescein isothiocyanate; ZO-1, zonula occludens-1.

was used as bacterial control or negative control, respectively. For another tumorigenesis model, six doses of AOM (A5486, Sigma-Aldrich, Delaware, USA) were injected into C57BL/6 mice aged 6 weeks to induce sporadic tumour development. AOM-injected mice received the same treatment as in *Apc*^{Min/+} mouse model, which initiated 1 week after the last AOM injection and lasted for 30 weeks.

For orthotopic mouse models, MSI-high type CRC cell line MC38 (5×10^5 cells in 10 μL Matrigel (354248, Corning, Virginia) per mouse) or mouse MSS type CRC cell line CT26 was orthotopically implanted into the rectum of male C57BL/6 or BALB/c mice aged 5–6 weeks, respectively. When the tumour was established or 3 days after implantation, mice were started with *R. intestinalis*, *E. coli* or sodium butyrate (150 mM in drinking water, 303410, Sigma-Aldrich) daily treatment. One week after implantation, antimouse PD-1 monoclonal antibody (BP0146, clone: RMP1-14, Bio X Cell, New Hampshire, USA) or control IgG (BP0089, clone: 2A-3, Bio X Cell) was administered into mice by intraperitoneal injection every 3 days (100 μg per mouse, total of five injections).

T cell isolation and activation

T cells isolated from healthy human peripheral blood mononuclear cells (PBMC) using immunomagnetic negative selection kit (17951, STEMCELL Technologies, Canada) according to manufacturer's instructions. Isolated T cells were then cultured in RPMI-1640 medium supplied with human CD3/CD28 magnet beads (11 161D, Thermo Fisher Scientific) and 30 U/mL

recombinant human IL-2 (589104, BioLegend) for expansion and activation for 3 days. Mouse T cells were isolated from the spleen of naïve mice and purified with Mouse T cell isolation kit (19851, Thermo Fisher Scientific), which were then cultured in RPMI-1640 medium with mouse CD3/CD28 beads (11456D, Thermo Fisher Scientific) and 30 U/mL recombinant mouse IL-2 (575404, BioLegend).

Immune cell characterisation by flow cytometry

Mouse tumour tissues were minced and digested in PBS containing 0.5 mg/mL collagenase IV and 0.25 mg/mL DNase I at 37°C for 30 min. The cell suspension was then filtered through 70 μm cell strainers and resuspended in 1% bovine serum albumin for surface marker staining. For isolated human T cells after treatment (*R. intestinalis* cultured medium (*R.i* CM), sodium butyrate or TLR5 inhibitor TH1020 (HY-116961, MedChemExpress, New Jersey, USA)), they were stimulated with 30 ng/mL phorbol myristate acetate (PMA), 1 μg/mL ionomycin and 2.5 μg/mL monensin in RPMI-1640 medium at 37°C for 4 hours. After surface staining, cells were fixed by transcription factor fixation/permeabilisation buffer (00-5521-00, Invitrogen, UK), followed by intracellular cytokine staining in 1× permeabilisation buffer (00-8333-56, Invitrogen). Granzyme B, interferon (IFN)-γ, tumour necrosis factor (TNF)-α negative cells were gated in CD8⁺ T cells (CD45⁺ CD3⁺ CD8⁺) without intracellular staining. Flow cytometry was performed on FACSaria cell sorter (BD Biosciences). Data were analysed by FlowJo V.10.4. Antibodies used in this study are listed in online supplemental

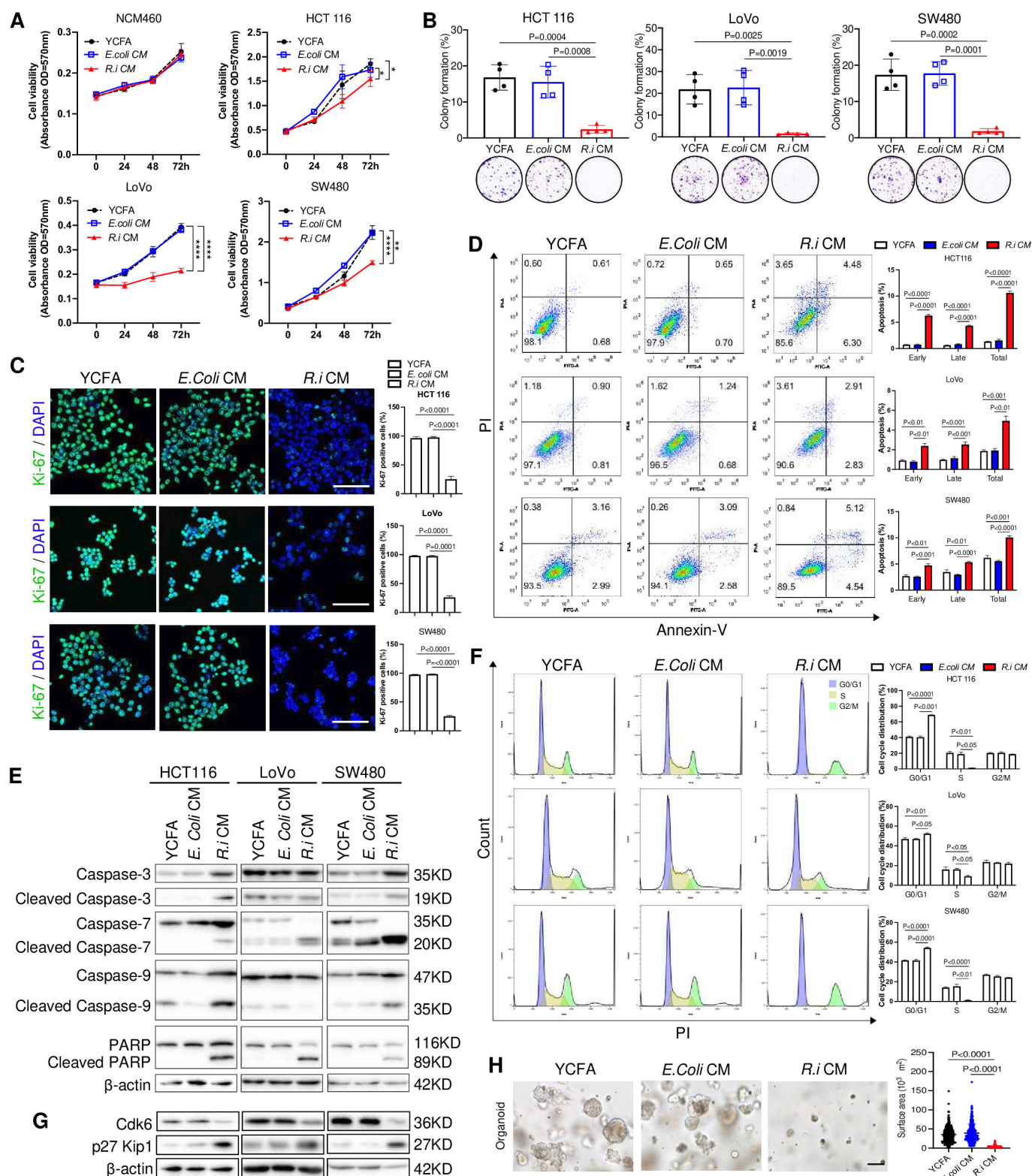


Figure 3 *Roseburia intestinalis* cultured medium (*R.i* CM) exhibits tumour-suppressive effects in vitro. (A) *R.i* CM (5%) reduced the colonic cell viability, except NCM460. *Escherichia coli* conditioned medium (*E. coli* CM) and yeast casitone fatty acids broth (YCFA) were used as control. (B) *R.i* CM (5%) suppressed the colony formation of colorectal cancer (CRC) cells. (C) *R.i* CM reduced CRC cell Ki-67 expression levels determined by immunocytochemistry. (D) *R.i* CM increased CRC cell apoptosis. (E) *R.i* CM induced CRC cell apoptosis of CRC cells by cleaved caspase-3, cleaved caspase-7, cleaved caspase-9 and cleaved poly (ADP-ribose) polymerase (PARP) expression. (F) *R.i* CM inhibited CRC cell cycle G1/S transition. (G) *R.i* CM induced G1 arrest by Cdk6 downregulation and p27 Kip1 upregulation. (H) *R.i* CM inhibited the growth of the organoid derived from a patient with CRC.

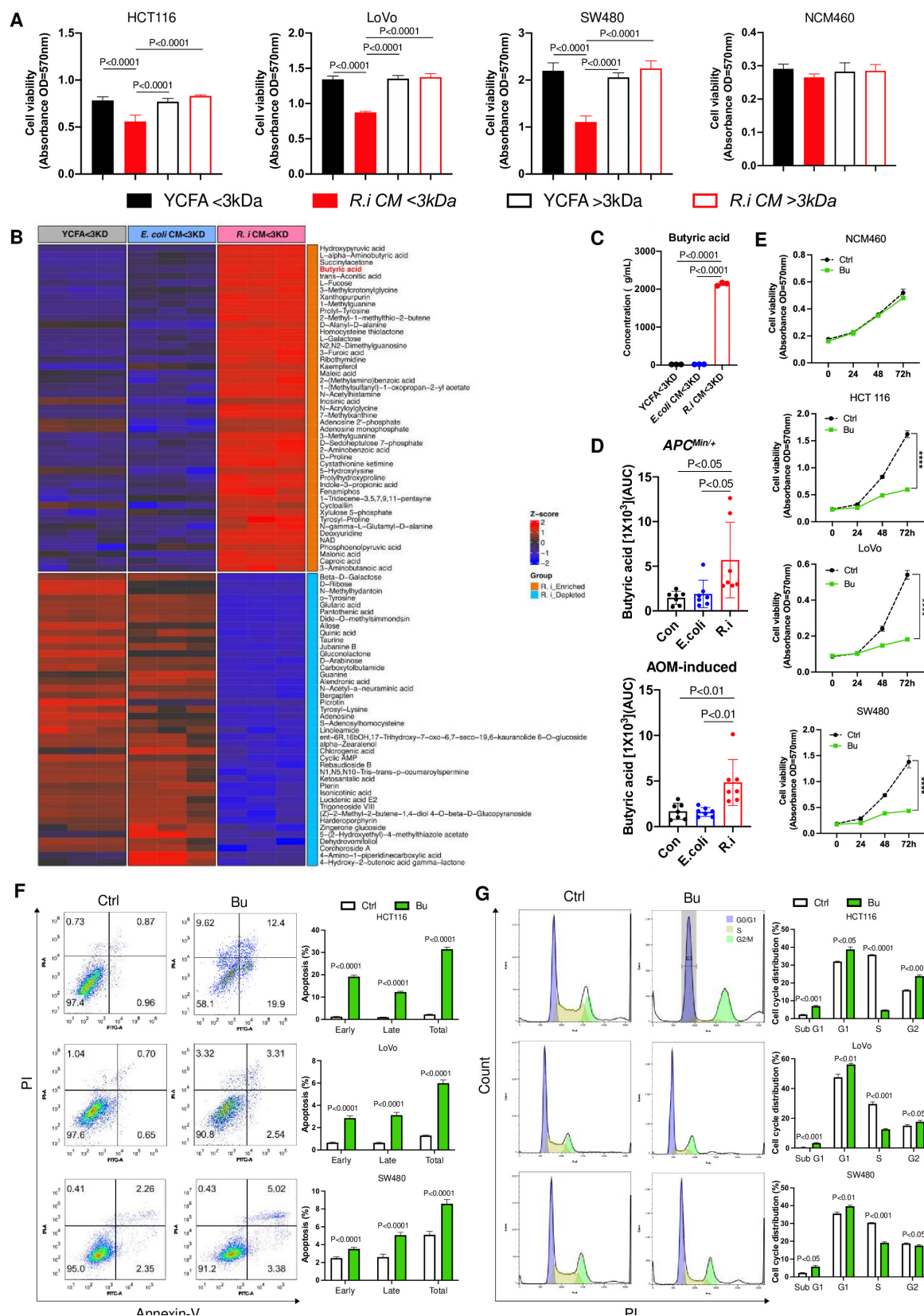


Figure 4 The tumour-suppressive component produced by *Roseburia intestinalis* is butyric acid. (A) The efficient components in *R. intestinalis* cultured medium (*R. i* CM) are <3 kDa. (B) The *R. i* CM <3kDa fraction was assessed by untargeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and butyric acid was identified as the candidate tumour-suppressive molecule. (C) Butyrate acid concentration (μg/mL) in *R. i* CM <3kDa was determined by targeted short chain fatty acid gas chromatography-mass spectrometry (GC-MS) analysis. (D) Relative concentration of butyrate acid (area under curve (AUC)) in *Apc*^{Min/+} and azoxymethane (AOM)-induced mice faeces was determined by GC-MS analysis. (E) Comparable dosage (1 mM) of butyrate in *R. i* CM reduced the colonic cell viability, except NCM460. (F) Butyrate-induced colorectal cancer (CRC) cell apoptosis. (G) Butyrate inhibited CRC cell cycle G1/S transition. Con, control; *E. coli*, *Escherichia coli*.

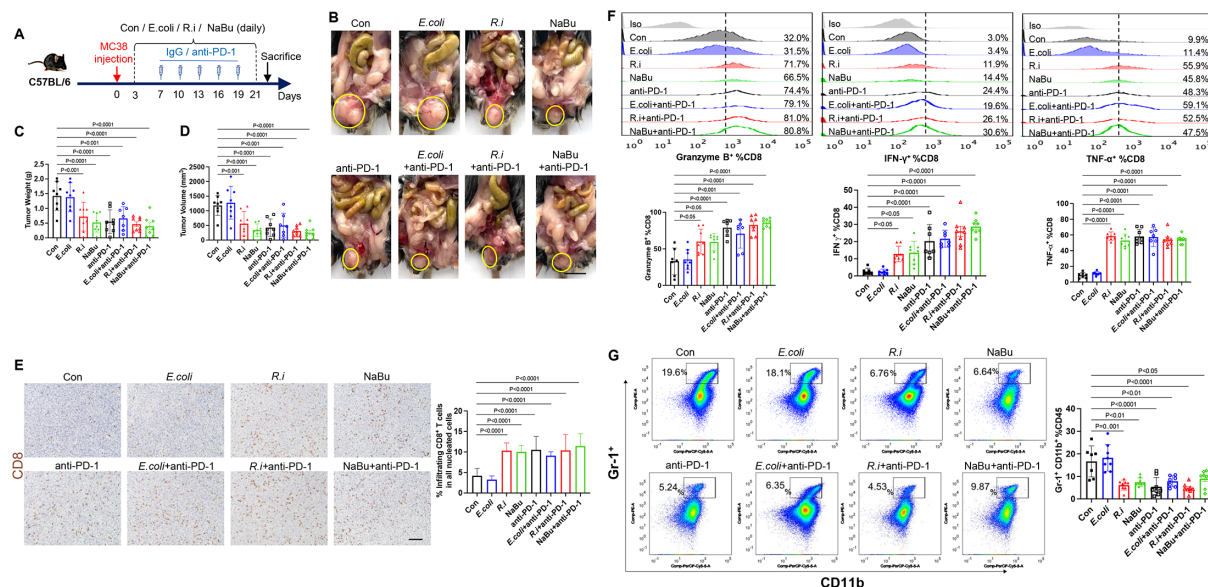


Figure 5 *Roseburia intestinalis* (*R.i*) and butyrate restrict MC38 orthotopic tumour growth by directly boosting CD8⁺ T cells. (A) Schematic diagram showing the experimental design and timeline of MC38 orthotopic model. (B) Representative MC38 rectum orthotopic tumour morphologies. (C) Tumour volume (mm³) and (D) tumour weight. (E) Proportion of tumour infiltrating CD8⁺ T cells in all nucleated cells of MC-38 orthotopic tumour tissues determined by immunohistochemistry. (F) Frequencies of granzyme B, interferon (IFN)-γ, tumour necrosis factor (TNF)-α within CD8⁺ populations of tumours from control, *Escherichia coli*-treated and *R.i*-treated C57BL/6J mice. (G) Frequencies of MDSC from tumour tissues in control, *E. coli*-treated and *R.i*-treated C57BL/6J mice measured by flow cytometry. Con, control; PD-1, programmed cell death protein 1.

table 1. Gating strategy is illustrated in online supplemental figure 1.

Surface plasmon resonance binding assay

To investigate the binding affinity between TLR5 and butyric acid, the Biacore T200 biosensor (GE Healthcare) was used to perform surface plasmon resonance. TLR5 human recombinant protein (TP315293, OriGene, Maryland, USA) was immobilised on a sensor chip CM5 (10328391, GE Healthcare) using an amine coupling kit (BR-1000-50, GE Healthcare) for 7 min, with a flow rate of 10 μL/min, resulting in a final ligand coupling of approximately 8500 response units (RU). Different concentrations of butyric acid (15.635, 31.25, 62.5, 125, 250, 500, 1000 μM) in pH 7.4 PBS were introduced using a microflow system. Association was allowed for 120 s at 30 μL/min, followed by dissociation for 200 s at 30 μL/min using a buffer solution wash, and regeneration for 30 s at 30 μL/min using glycine-HCl (10 mM). All binding experiments were conducted at 25°C, and the Biacore T200 evaluation software V3.1 was used to determine the binding affinity.

Receptor-ligand interaction analysis

To analyse the TLR5 receptor-butyrates interactions, a molecular docking analysis was conducted. The three-dimensional structure of butyrate was obtained from the PubChem database (Compound CID: 104775) in SDF format. The structure was minimised and constructed in ChemDraw Ultra V12.0¹³ and converted into PDB format using PyMOL visualisation software. The crystal structure of TLR5 (PDB code: 3J0A) was downloaded from the Protein Data Bank. MGLTools 1.5.6 from AutoDock Vina 1.1.2 programme was used for processing TLR5,¹⁴ while AutoDock was used for docking analysis. The docking runs were performed at a radius of 15 Å with specific coordinates x-axis: 52.981, y-axis: 63.073 and z-axis: 65.640. The top five results with the highest binding energy values were

selected. Additionally, two-dimensional ligand-protein interaction diagrams were used to analyse hydrophobic interactions with LigPlus software,¹⁵ calculating the number of van der Waals contacts of non-polar atoms.

Pull-down assay

Jurkat E6.1 cell was lysed with the pull-down buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl and 0.05% Tween-20) containing protease inhibitors. The lysate was then incubated with either the anti-TLR5 antibody (ab13876, abcam) or mouse IgG (sc-2027, Santa Cruz, California, USA) with or without 10 mM sodium butyrate at 4°C overnight while gently rotating in the pull-down buffer. The mixture was then incubated with Dynabeads Protein G beads (Invitrogen) for 2 hours at room temperature. Following this, samples were thoroughly washed with pull-down buffer 5 times and pull-down buffer without Tween-20 4 times before being eluted with 50 mM glycine (pH 2.8). A small portion of the eluate was used for western blot analysis to confirm successful pull-down of TLR5 while the remainder was used for liquid chromatography-mass spectrometry analysis to detect the level of butyrate binding to TLR5.

Statistical analyses

All measurements are shown as mean ± SD. Independent Student's t-test was used to compare the difference between two groups, and multiple t-tests were used to compare the difference at different timepoints or stages between two groups. Two-way analysis of variance (ANOVA) was used to compare the difference among three groups, and one-way ANOVA with Turkey's multiple comparison was used to compare the difference among three or more groups. The χ^2 test was used to evaluate the proportion of tumour incidence between groups. Two-tailed p value < 0.05 was considered statistically significant. Additional methods are provided in online supplemental file 1.

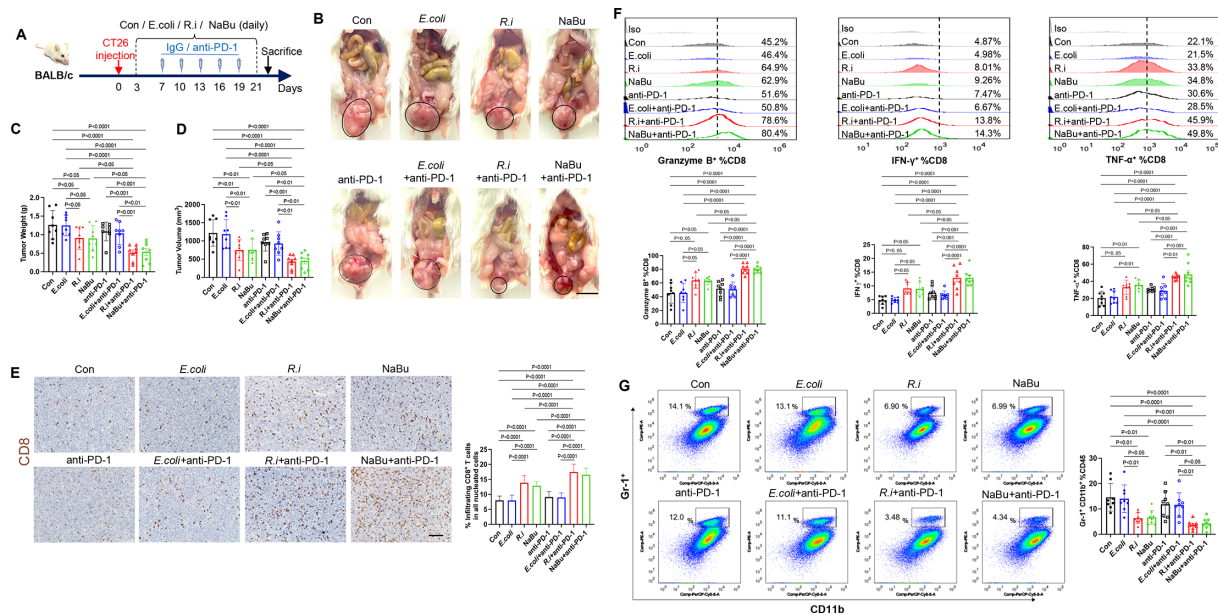


Figure 6 *Roseburia intestinalis* (*R.i*) and butyrate treatments are effective in CT26 orthotopic tumour via CD8⁺ T cells independently from anti-programmed cell death protein 1 (anti-PD-1) therapy. (A) Schematic diagram showing the experimental design and timeline of CT26 orthotopic model. (B) Representative CT26 rectum orthotopic tumour morphologies. (C) Tumour volume (mm³) and (D) tumour weight. (E) Proportion of tumour infiltrating CD8⁺ T cells of CT-26 orthotopic tumour in all nucleated cells determined by immunohistochemistry. (F) Frequencies of granzyme B, interferon (IFN)- γ , tumour necrosis factor (TNF)- α within CD8⁺ populations of tumours from control, *Escherichia coli*-treated and *R.i*-treated BALB/c mice. (G) Frequencies of MDSC from tumour tissues in control, *E. coli*-treated and *R.i*-treated BALB/c mice measured by flow cytometry. Con, control.

RESULTS

R. intestinalis is depleted in stools of patients with CRC

We first evaluated the abundance of *R. intestinalis* in our in-house cohort of 110 patients with CRC and 112 healthy controls (online supplemental table 2). *R. intestinalis* was significantly depleted in faecal samples of patients with CRC compared with healthy controls (online supplemental figure 2A). For verification, we retrieved four published metagenomic datasets of 334 patients with CRC and 463 healthy controls.^{16–19} The abundance of *R. intestinalis* was consistently decreased in all cohorts of patients with CRC (online supplemental figure 2B), thus confirming the depletion of *R. intestinalis* in CRC.

R. intestinalis inhibits CRC tumourigenesis in two different mouse models

The role of *R. intestinalis* in CRC tumourigenesis was first evaluated in *Apc*^{Min/+} mice, receiving orally gavage with *R. intestinalis*, *E. coli* strain MG1655 or PBS for 6 weeks (figure 1A). *R. intestinalis* significantly reduced colon tumour incidence (figure 1B,C), tumour number and load (figure 1D, online supplemental figure 3) compared with mice treated with *E. coli* or PBS, respectively. *R. intestinalis*-treated mice also had lower dysplasia score with significantly less Ki-67-positive cells in both tumour and non-tumour colon tissues (figure 1E). Meanwhile, mouse body weight (online supplemental figure 4A), liver function (serum alanine aminotransferase and aspartate aminotransferase) and renal function (serum blood urea nitrogen and creatinine) (online supplemental figure 4B) were not altered with *R. intestinalis* treatment.

For validation, another mouse model with carcinogen-induced CRC (AOM) was used with 30 weeks administration of *R. intestinalis* (figure 1F). Similar to the findings of *Apc*^{Min/+} mice, *R. intestinalis* significantly suppressed colorectal tumourigenesis with decreased tumour incidence (figure 1G,H), tumour number and load (figure 1I) as compared with controls. Lower

dysplasia score and less Ki-67-positive cells were also observed in *R. intestinalis*-treated AOM mice (figure 1J). Collectively, our consistent results from two CRC mouse models demonstrated the suppressive role of *R. intestinalis* against colorectal tumourigenesis.

R. intestinalis improves gut barrier function in mice

Gut barrier dysfunction is known to play a pivotal role in CRC. We therefore examined the gut barrier integrity in *Apc*^{Min/+} mice treated with *R. intestinalis*. *R. intestinalis* significantly reduced intestinal permeability as evidenced by decreased serum FITC-dextran level (figure 2). The protein expression of tight junction proteins ZO-1 and claudin-3 was also significantly increased in the colon tissues of *R. intestinalis*-treated mice, compared with mice treated with *E. coli* or PBS control (figure 2B). The upregulation of these genes in colon tissues was confirmed by IHC staining (figure 2C). Taking together, *R. intestinalis* could improve gut barrier function in CRC mice.

R. intestinalis culture medium suppresses the growth of CRC cells and patient-derived organoids

To investigate the tumour-suppressive mechanism of *R. intestinalis*, we first cocultured human CRC cell lines HCT116, LoVo and SW480, as well as a normal colon epithelial cell line NCM460, with live or pasteurised *R. intestinalis*, *E. coli* or broth control. However, neither live (online supplemental figure 5A) nor pasteurised (online supplemental figure 5B) *R. intestinalis* showed any difference in cell viability, indicating that *R. intestinalis* itself has no effect on CRC cell growth. We speculated that the tumour-suppressive role of *R. intestinalis* is attributed to its derivatives, and therefore treated CRC cells with *R.i* CM. *R.i* CM (5% vol/vol) significantly inhibited the cell viability of HCT116, LoVo and SW480, but not the normal colon cell line NCM460 (figure 3A). Similarly, *R.i* CM treatment reduced

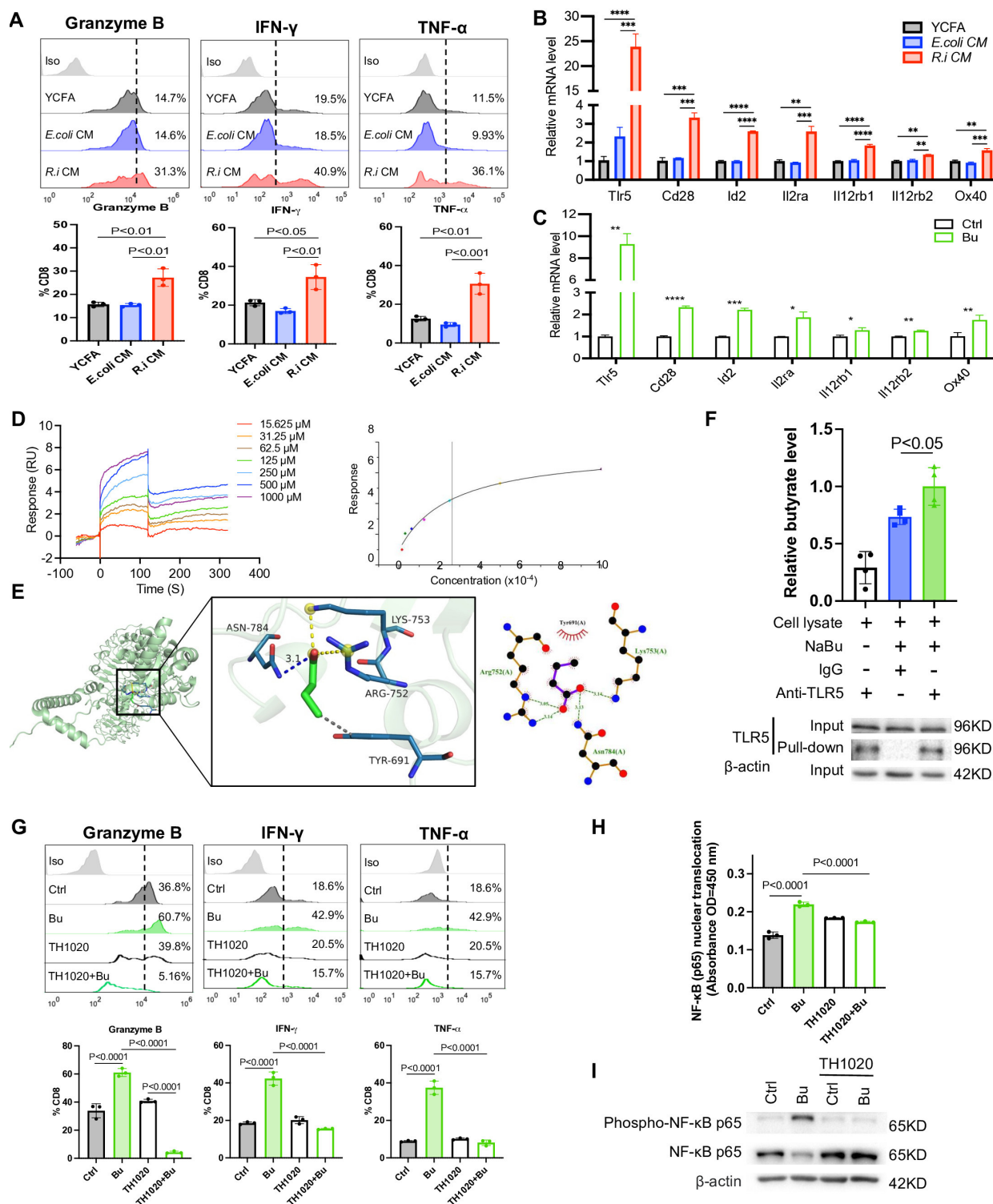


Figure 7 Comparable dosage of butyrate in *Roseburia intestinalis* cultured medium (*R. i* CM) suppresses colorectal cancer cells by direct CD8⁺ T cell boosting in vitro. (A) *R. i* CM directly boosted granzyme B⁺, interferon (IFN)- γ ⁺, tumour necrosis factor (TNF)- α ⁺ CD8⁺ T cells isolated from human peripheral blood mononuclear cells (PBMC). (B) The messenger RNA (mRNA) expression level of significant changed receptors on CD8⁺ T cells after treatment with *R. i* CM or (C) butyrate. (D) Binding affinity between toll-like receptor 5 (TLR5) and butyric acid was measured by surface plasmon resonance (left), affinity of TLR5 to butyric acid was 264 μ M (right). (E) Representative structure of butyrate and TLR5 after molecular docking. The structure of TLR5 is represented in green and the butyrate is shown as stick in the centre (left). Relative interactions between butyrate and four amino acids of TLR5 generated by LigPlot (right). (F) Jurkat E6.1 T cell lysate incubation with anti-TLR5 or mouse IgG, together with or without 10 mM NaBu in pull-down buffer. Elute was evaluated by liquid chromatography-mass spectrometry (upper) and western blot analysis (down). (G) granzyme B⁺, IFN- γ ⁺, TNF- α ⁺ CD8⁺ T cells isolated from human PBMC were directly boosted by comparable dosage of butyrate and abolished by TLR5 antagonist TH1020. (H) Nuclear factor kappa B (NF- κ B) (p65) nuclear translocation determined by NF- κ B (p65) transcription factor assay. (I) Phospho-NF- κ B p65 protein expression level of CD8⁺ T cells isolated from human PBMC after butyrate treatment with or without TH1020 inhibition. *E. coli*, *Escherichia coli*; YCFA, yeast casitone fatty acids broth. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

colony formation (figure 3B) and proliferation (figure 3C), but increased both early and late apoptosis in CRC cells (figure 3D), as well as the protein expression of apoptosis markers cleaved caspase-7 and cleaved poly (ADP-ribose) polymerase (figure 3E). *R.i* CM also inhibited cell cycle G1-S phase transition with an increased proportion at G0/G1 phase and decreased in S phase in CRC cells (figure 3F). In keeping with this, the protein expression of cell cycle markers CDK6 was decreased and p27 Kip1 was increased by *R.i* CM (figure 3G). Moreover, *R.i* CM significantly suppressed the growth of organoids derived from patients with CRC (figure 3H). Altogether, these results suggested that molecules produced by *R. intestinalis* inhibit the growth of CRC cell lines and patient-derived organoids.

Butyrate is the functional tumour-suppressive metabolite produced by *R. intestinalis*

To determine the functional antitumour molecules generated by *R. intestinalis*, we separated *R.i* CM into two different fractions with molecular weight <3 kDa or >3 kDa. We found that only *R.i* CM with <3 kDa fraction exhibited suppressive effects on all three CRC cell lines HCT116, LoVo and SW480, but not in normal epithelial cell line NCM460 (figure 4A). This was not observed in *R.i* CM with >3 kDa fraction. *R.i* CM with <3 kDa fraction was therefore subjected to untargeted metabolomics profiling by LC-MS/MS, and butyric acid emerged as one of the most differentially enriched metabolites, compared with broth control (figure 4B). Subsequent targeted metabolomics analysis using GC-MS identified short-chain fatty acids (SCFAs) (online supplemental figure 6), confirming the substantial increase of butyric acid concentration to approximately 2000 µg/mL in the <3 kDa fraction of *R.i* CM (figure 4C). The elevation of butyric acid was further confirmed in stools of *Apc^{Min/+}* and AOM-induced CRC mice receiving *R. intestinalis* supplementation (figure 4D).

The direct suppressive effect of butyrate was then assessed in vitro. The growth of CRC cells was significantly inhibited (figure 4E), together with increased apoptosis (figure 4F) and inhibited G1-S phase progression (figure 4G), by butyrate treatment with a comparable concentration (1 mM) in 5% *R.i* CM. Apart from butyric acid, inosine was also identified by untargeted metabolomics profiling as the top differentially enriched metabolites in *R.i* CM with <3 kDa fraction (online supplemental figure 7A). The concentration of inosine in *R.i* CM was around 8 µg/mL (online supplemental figure 7B). However, the comparable dosage of inosine in *R.i* CM had no significant suppressive effect on the growth of CRC cells (online supplemental figure 8A–C).

We further tested the association of *R. intestinalis* and butyric acid with colorectal tumorigenesis in human patients with CRC. Metabolomic profiling was performed on human faecal samples from our in-house cohort with 118 patients with CRC and 128 healthy controls,²⁰ which confirmed the significant depletion of butyric acid in patients with CRC (online supplemental figure 9A). *R. intestinalis* depletion was also observed in patients with CRC from this cohort (online supplemental figure 9B).²⁰ Collectively, these findings indicated that butyrate is the major metabolite responsible for the tumour-suppressive function of *R. intestinalis*.

R. intestinalis and butyrate improve anti-PD-1 efficacy in orthotopic CRC mouse models

From a published metagenomic dataset,²¹ we found that *R. intestinalis* was significantly enriched in stool samples of 75

immune checkpoint blockade responders compared with 258 non-responders (online supplemental figure 10). We therefore tested whether *R. intestinalis* and butyrate could enhance immunotherapy efficacy against CRC. Oral gavage of *R. intestinalis*, sodium butyrate or *E. coli* was started 3 days after orthotopic injection of a mouse CRC cell line MC38 (MSI-high type), and anti-PD-1 therapy was initiated after the tumour was established (figure 5A). Both *R. intestinalis* and sodium butyrate significantly inhibited MC38 orthotopic tumour growth (figure 5B–D). Tumour growth was also suppressed in mice receiving anti-PD-1 monotherapy, while combining anti-PD-1 with *R. intestinalis* or sodium butyrate could not further enhance anti-PD-1 efficacy. *R. intestinalis* or sodium butyrate treatment of MC38 tumours increased infiltrating CD8⁺ T cells (figure 5E) and granzyme B⁺, IFN-γ⁺ and TNF-α⁺ CD8⁺ T cells (figure 5F) while decreasing myeloid-derived suppressor cell (MDSCs) (figure 5G). MC38 syngeneic mice are known to be MSI-high with good immunotherapy response. Our findings suggested that *R. intestinalis* or butyrate has similar efficacy of tumour suppression as anti-PD-1 therapy in MSI-high type CRC allografts.

We then examined whether *R. intestinalis* or butyrate could also be effective in CT26 orthotopic mouse xenografts, which is a model with MSS and inherited immunotherapy resistance (figure 6A). As expected, CT26 orthotopic tumours were insensitive to anti-PD-1 therapy (figure 6B–D). Both tumour weight (figure 6C) and tumour size (figure 6D) were significantly reduced by the treatment of *R. intestinalis* or sodium butyrate compared with anti-PD-1 therapy and control groups. Moreover, the combination of *R. intestinalis* or sodium butyrate with anti-PD-1 further synergised the antitumour effects of *R. intestinalis* or sodium butyrate (figure 6B–D). Consistently, the proportion of infiltrating CD8⁺ T cells (figure 6E) and granzyme B⁺, IFN-γ and TNF-α⁺ CD8⁺ T cells was significantly increased in CT26 tumours with monotherapy of *R. intestinalis* or sodium butyrate (figure 6F), and their combination with anti-PD-1 showed additional effects on these immune cells (figure 6E–F). Treatment of *R. intestinalis* or sodium butyrate but not anti-PD-1 monotherapy also reduced the proportion of MDSCs in tumours (figure 6G). The increase of butyric acid was verified in the stools of both MC38 and CT26 orthotopic mice after administration of either *R. intestinalis* or sodium butyrate (online supplemental figure 11). These results collectively revealed that *R. intestinalis* and sodium butyrate are effective on facilitating anti-PD-1 therapeutic efficacy against CRC with MSS phenotype.

R. intestinalis-produced butyrate directly boosts cytotoxic CD8⁺ T cell function

We examined the mechanism of how *R.i* CM and butyrate interact with CD8⁺ T cells to modulate the antitumour immunity. T cells isolated from human PBMC or mouse spleen were treated with or without *R.i* CM or butyrate (1 mM). Both *R.i* CM and butyrate treatment promoted the production of granzyme B, IFN-γ and TNF-α by CD8⁺ T cells (figure 7A, online supplemental figure 12). To understand their mechanistic association with CD8⁺ T cells, the messenger RNA (mRNA) expression of receptors and transporters on CD8⁺ T cells including GPR43, SLCL6A1 and TLR1–6 was examined after *R.i* CM treatment.^{22–26} *R.i* CM did not change the expression of SCFAs receptors or transporters, but altered the expression of genes associated with effector T cells like *ID2*, *IL2RA* and *CD28* (figure 7B, online supplemental figure 13A). Particularly, the mRNA expression of pattern recognition receptor *TLR5* was over 10 times higher in *R.i* CM-treated CD8⁺ T cells compared with

those treated with *E. coli* CM or YCFA control. Consistently, the mRNA expression of *TLR5* was significantly higher in butyrate-treated CD8⁺ T cells compared with controls (figure 7C, online supplemental figure 13B). We confirmed that butyrate could bind to TLR5 receptor on CD8⁺ T cells with an affinity of 264 μ M by surface plasmon resonance (figure 7D). Butyrate was able to dock into the TLR5 catalytic site and the best docking conformation of complex had a binding energy of -3.5 kcal/mol (figure 7E), indicating that butyrate could spontaneously bind to the active pocket of TLR5. The butyrate-TLR5 physical interaction was confirmed by pull-down assay (figure 7F). To validate our findings, a selective TLR5 antagonist TH1020 was used to inhibit TLR5 signalling in CD8⁺ T cells. TH1020 abolished the enhanced T cell response induced by butyrate, as evidenced by decreased production of granzyme B, IFN- γ and TNF- α in CD8⁺ T cells (figure 7G). These results collectively suggested that *R. intestinalis*-produced butyrate promotes CD8⁺ T cell activation through direct binding to TLR5 receptor on CD8⁺ T cells.

NF- κ B signalling is well-established as the downstream pathway of TLR5. Butyrate promoted nuclear translocation of NF- κ B (p65) and its transcription factor DNA binding ability, as shown in CD8⁺ T cell nuclear extracts (figure 7H). Butyrate also enhanced the expression of phospho-NF- κ B (p65) in CD8⁺ T cells, whereas such increase was reversed by TH1020 (figure 7H,I). Altogether, *R. intestinalis* produced butyrate to boost cytotoxic CD8⁺ T cell function through activating the TLR5-NF- κ B pathway.

DISCUSSION

In this study, we elucidated the protective role of *R. intestinalis* against colorectal tumourigenesis. The abundance of *R. intestinalis* in the gut microbiota of patients with CRC was found to be depleted in previous studies.^{16–19} We also confirmed the depletion of *R. intestinalis* in our in-house CRC cohort. Of note, although its depletion has been frequently reported, the mechanism of how *R. intestinalis* protects against colorectal tumourigenesis remains completely unknown. Here, using two different mouse models (transgenic *Apc*^{Min/+} and carcinogen AOM-induced CRC), for the first time we illustrated the tumour-suppressive function of *R. intestinalis* in vivo (figure 1). Moreover, gut barrier dysfunction plays a crucial role in CRC, of which increased intestinal permeability allows more pathogenic microbes and their derivatives to have easier access to the bloodstream, thereby contributing to microbial dissemination and metastasis.²⁷ Consistently, our results revealed the impairment of gut barrier in CRC mice, whereas *R. intestinalis* supplementation could significantly improve gut barrier integrity (figure 2).

We identified that the tumour-suppressive function of *R. intestinalis* is attributed to its derived metabolites (figure 3). To date, various antitumour mechanisms of probiotics have been discovered. Some of them exhibit anti-CRC effects by secreting protein derivatives,²⁸ while the others including *R. intestinalis*, produce metabolites to abolish colorectal tumourigenesis.²⁹ Through metabolomic profiling, we found that butyrate is the major antitumour metabolite produced by *R. intestinalis* (figure 4). Butyrate is an SCFA known to be negatively correlated with CRC incidence,²⁰ and accumulated evidence has demonstrated its diverse protective mechanisms against colorectal tumourigenesis. For instance, butyrate could epigenetically act as a histone deacetylase inhibitor to inhibit tumour cell proliferation,³⁰ while *Clostridium butyrium*, the major butyrate producer in the human gut, dampens CRC development by downregulating the oncogenic WNT signalling pathway.³¹ In this study, our results clearly

revealed that *R. intestinalis* is a butyrate-producing bacteria, and its derived butyrate could facilitate the direct killing of tumour cells with minimal effects on normal colon epithelial cells.

Apart from tumour cells, we illustrated a link between *R. intestinalis* and antitumour immunity. The gut microbiota is well-recognised for its influence on immunotherapy efficacy. Indeed, we confirmed the enrichment of *R. intestinalis* in immune checkpoint blockade responders from a published metagenomic dataset,²¹ while faecal butyrate concentration was reported to be associated with anti-PD-1 efficacy in solid cancer.³² We therefore tested the correlation between *R. intestinalis* or butyrate and anti-PD-1 therapy in two orthotopic mouse models bearing tumours with different CRC subtypes (MSI-high or MSS). Clinically, anti-PD-1 therapy is approved for patients with CRC with MSI-high subtype with a response rate of 40%.³³ For patients with MSS subtype, although they account for the majority of CRC cases (~85%), the immunological 'cold' tumour microenvironment in these patients has restricted the application of immunotherapy and how to improve their responsiveness to immune checkpoint blockade remains elusive.³⁴ Here, as expected, only mice bearing MSI-high but not MSS tumours responded to anti-PD-1 therapy (figures 5,6). Meanwhile, the combination of anti-PD-1 therapy with *R. intestinalis* or butyrate was effective to suppress the growth of MSS tumours with increased cytotoxic function of CD8⁺ T cells. These results collectively indicated that *R. intestinalis* and its derived butyrate may be a promising adjuvant to patients with CRC who are resistant to anti-PD-1 therapy. Moreover, we observed that *R. intestinalis* or butyrate alone is sufficient to induce shrinkage of MSI-high and MSS tumours, thus suggesting the feasibility to leverage probiotics as a direct treatment of CRC.

We examined the mechanism of how *R. intestinalis*-derived butyrate interacts with antitumour CD8⁺ T cells. G protein-coupled receptors (GPR)41, GPR43 and GPR109A are the major receptors on colon epithelial cells responsible for sensing butyrate and other SCFAs.³⁵ However, we observed that their expressions on CD8⁺ T cells are not altered by butyrate. Similarly, a previous study reported that GPR blockade does not impair the effects of butyrate on functional CD8⁺ T cells.²⁶ These findings therefore indicated that GPRs are dispensable for butyrate-mediated effects on CD8⁺ T cells. Instead, we identified the significant upregulation of TLR5 on CD8⁺ T cells and confirmed that TLR5 is a butyrate receptor by surface plasmon resonance and pull-down assay (figure 7). TLR5 is a receptor expressed on epithelial and immune cells specifically recognising flagellin on bacteria,³⁶ whereas currently there is a lack of studies reporting its interaction with microbial metabolites. Shen *et al* discovered that *R. intestinalis* stimulates TLR5 on intestinal epithelial cells to inhibit chronic inflammation.³⁷ In comparison, for the first time we revealed that *R. intestinalis*-derived butyrate but not *R. intestinalis* itself could directly bind to TLR5 on CD8⁺ T cells to enhance their cytotoxic functions. Altogether, our results suggested a novel link between butyrate and a well-studied innate immune receptor, which may provide the basis for new therapeutic strategies to boost the antitumour immunity.

In summary, we observed significant depletion of gut *R. intestinalis* and butyrate in patients with CRC. By using multiple mouse models, we demonstrated the tumour-suppressive roles of *R. intestinalis* against two different subtypes of CRC including MSI-high and MSS tumours. *R. intestinalis* exhibits its antitumour function by producing butyrate. Both *R. intestinalis* and butyrate enhance the efficacy of anti-PD-1 therapy in CRC. Mechanistically, we revealed the direct binding of *R. intestinalis*-derived butyrate to TLR5 on CD8⁺ T cells, thereby boosting

the antitumour immunity. Thus, *R. intestinalis* is a promising probiotic supplement, which potentially open a new avenue to improve treatment efficacy in patients with CRC, particularly for those with anti-PD-1 therapy resistance.

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Contributors XK was involved in the study design, conducted experiments and drafted the manuscript; CL and YD performed bioinformatics analysis; YN performed histological analysis; FJ operated LC-MS/MS; HCHL and LJ performed animal experiments; JJYS commented on the study; SW and JY conceived, supervised the study and revised the manuscript. JY acts as the guarantor of the study.

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Competing interests None declared.

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1 **Supplementary Materials and Methods**

2 **Metagenomic Cohorts of Colorectal Cancer Patients and Healthy Controls**

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

10 Four published metagenomic data cohorts from China, Germany, France, and Japan,
11 including 334 CRC patients and 463 healthy individuals, were used to evaluate the
12 abundance of *R. intestinalis*. Metagenomic data sets were downloaded from the
13 European Nucleotide Archive (ENA) with the following ENA identifiers: PRJEB10878
14 for YuJ_2017, [2] PRJEB27928 for WirbelJ_2019, [3] ERP005534 for ZellerG_2014
15 [4] and DNA Data Bank of Japan for YachidaS_2019 [5] with accession number:
16 DRA006684 and DRA008156.

17 **Metagenomic Cohorts of Responders and Non-responders to Immune Checkpoint** 18 **Inhibitors**

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

25 **Bacterial culture**

R. intestinalis (DSM 14610) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and cultured at 37 °C 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

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

7 **Intestinal permeability assay**

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

13 **Western blot**

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

20 **Cell culture**

21 Normal human colon epithelial cell NCM460, human CRC cell lines HCT116, LoVo
22 and SW480, and mouse CRC cell lines MC38 and CT26 were cultured in DMEM with
23 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Jurkat E6.1 (TIB-
24 152, ATCC, VA) was cultured in RPMI-1640 supplied with 10% FBS and 1% P/S, and

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=10⁶/ml) or *R. intestinalis* (CFU=10⁶/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

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 (FACS Aria 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

1 UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm), coupled to Orbitrap Exploris
2 120 mass spectrometer on information-dependent acquisition (IDA) mode which was
3 controlled by the acquisition software (Xcalibur, Thermo Fisher Scientific).

4 **Targeted gas chromatography-mass spectrometry**

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

21 **RNA isolation and quantitative PCR**

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

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

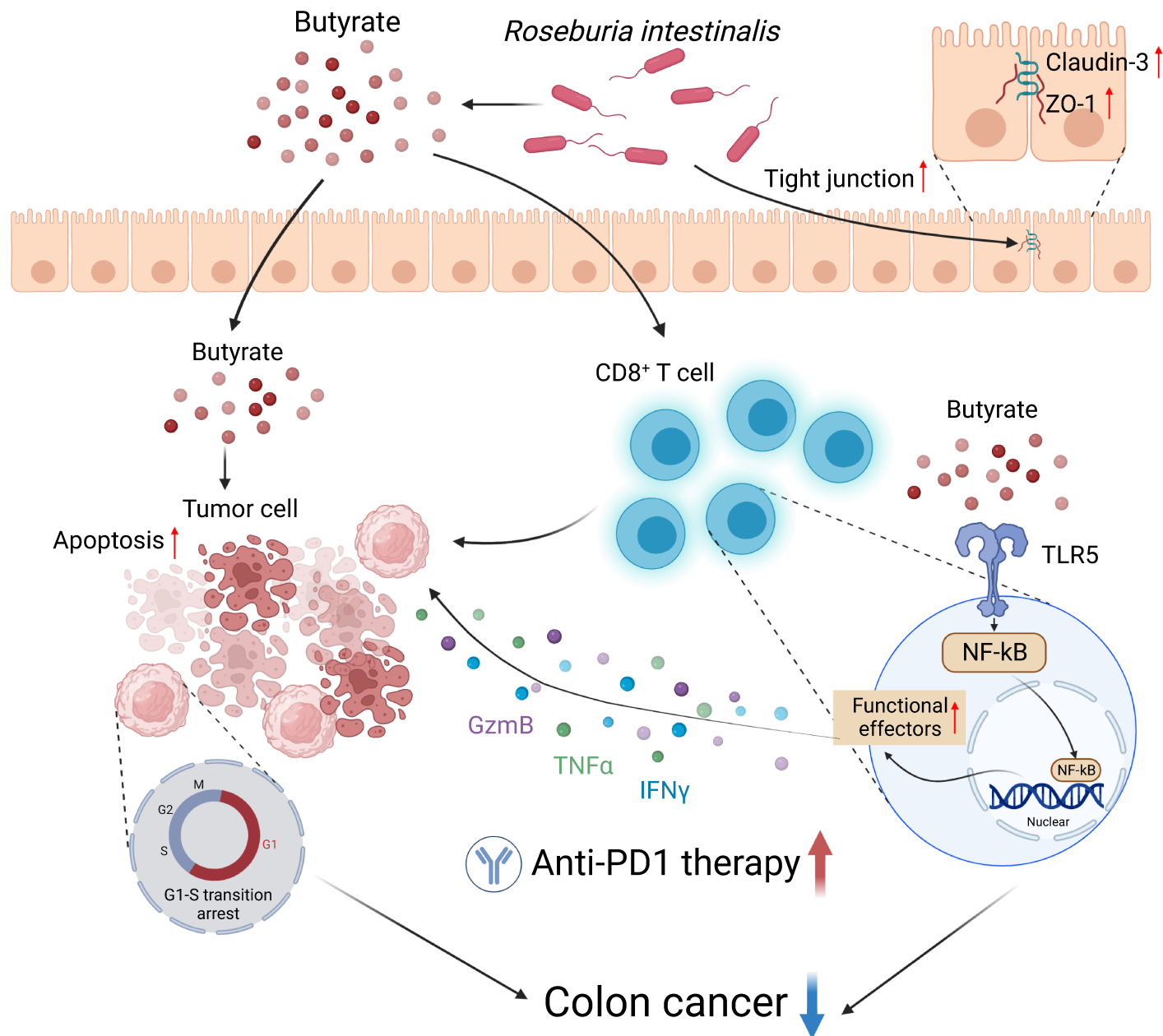
NF-κB (p65) transcription factor assay

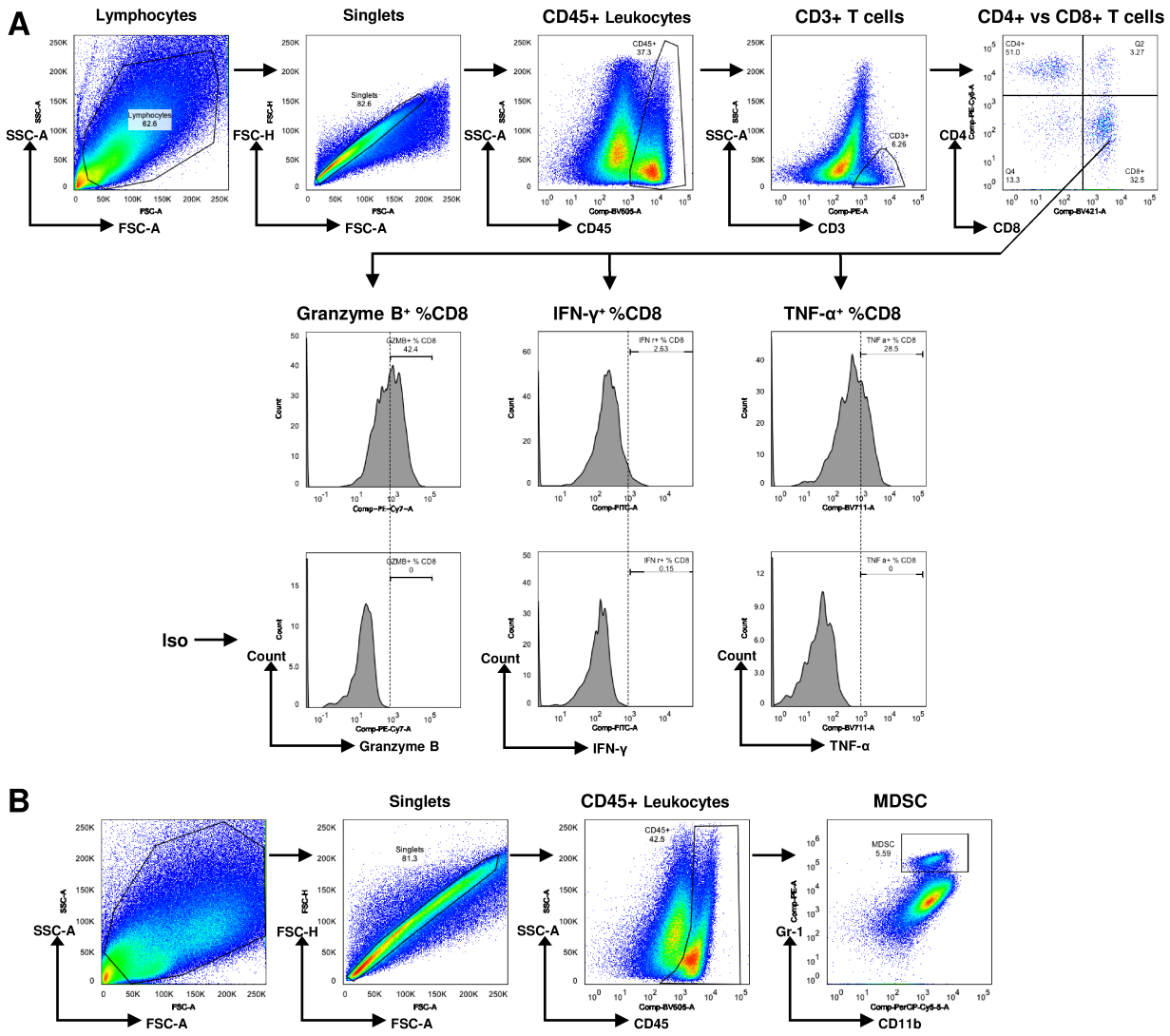
Human T cell was isolated and activated as described before. After treating with butyrate (1mM) with or without TH1020 for 3 days, nuclear protein of activated T cells was extracted with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Fisher Scientific). Nuclear protein concentration 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

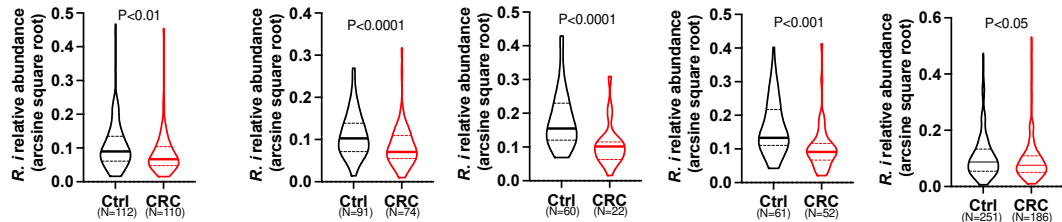
- 1 Lin Y, Lau HC, Liu Y, Kang X, Wang Y, Ting NL, *et al.* Altered Mycobiota Signatures and Enriched Pathogenic *Aspergillus rambellii* Are Associated With Colorectal Cancer Based on Multicohort Fecal Metagenomic Analyses. *Gastroenterology* 2022;**163**:908–21.
- 2 Yu J, Feng Q, Wong SH, Zhang D, Liang 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.

1 7 Coker OO, Liu C, Wu WKK, Wong SH, Jia W, Sung JJY, *et al.* Altered
2 gut metabolites and microbiota interactions are implicated in
3 colorectal carcinogenesis and can be non-invasive diagnostic
4 biomarkers. *Microbiome* 2022;**10**:35.
5

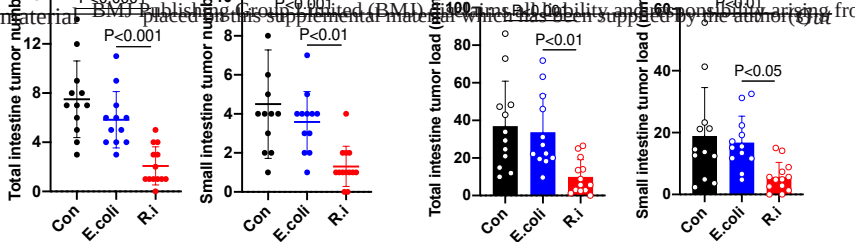




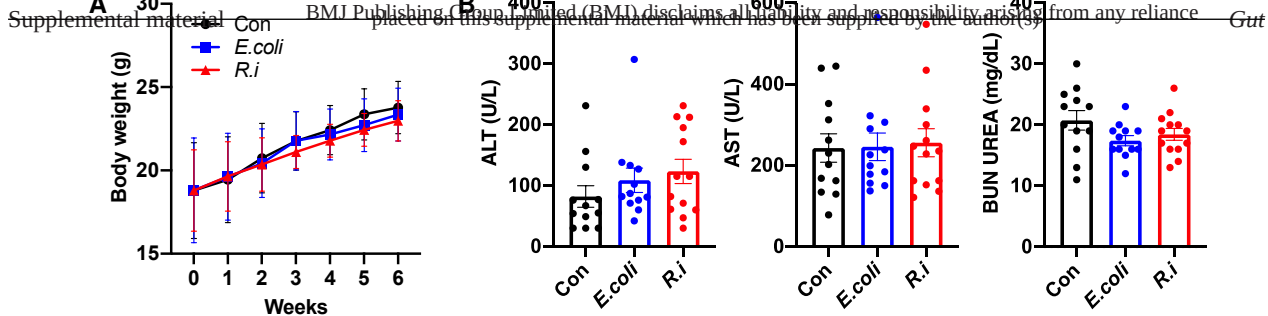
Supplementary Figure 1. Gating strategy of cytotoxic T cells (A) and MDSC (B).



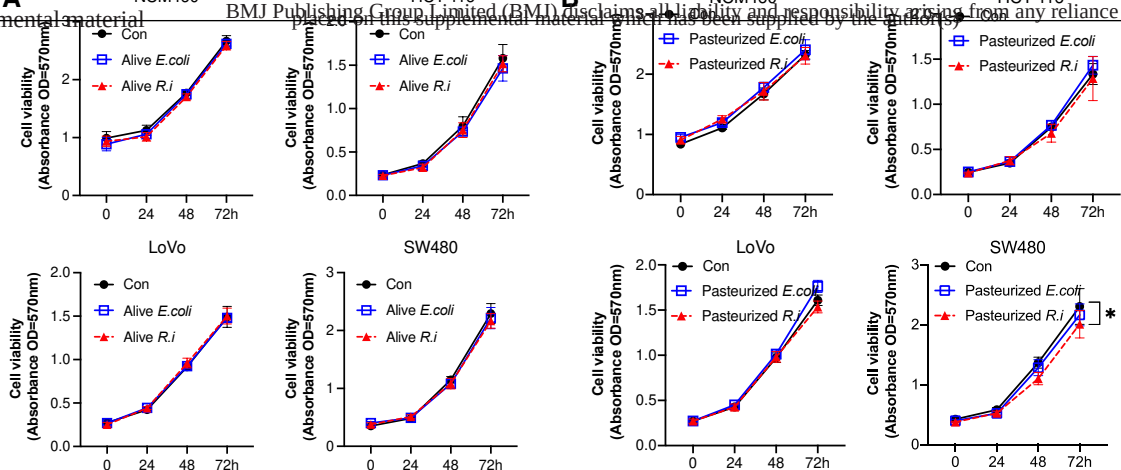
Supplementary Figure 2. *R. intestinalis* is depleted in fecal samples of CRC patients. Arcsine square root transferred relative abundance of *R. intestinalis* in in-house metagenomic cohort (A) and published metagenomic cohorts (B) of colorectal cancer patients and healthy controls. CHN, China; DE, Germany; FR, France; JP, Japan.



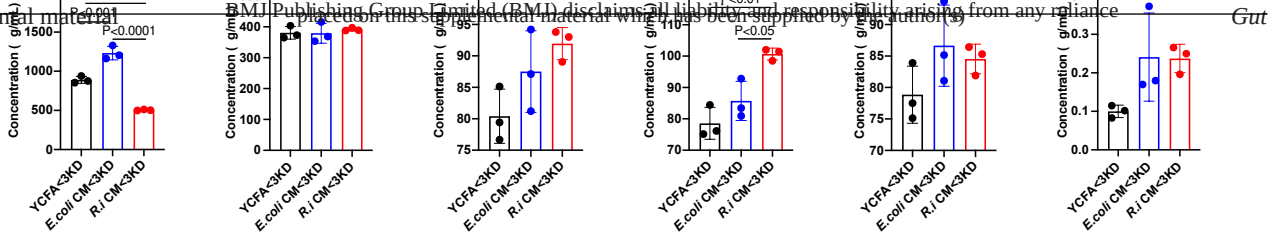
Supplementary Figure 3. Total and small intestine tumor number (A) and tumor load (total tumor area, mm²) (B) of *Apc^{Min/+}* mice.



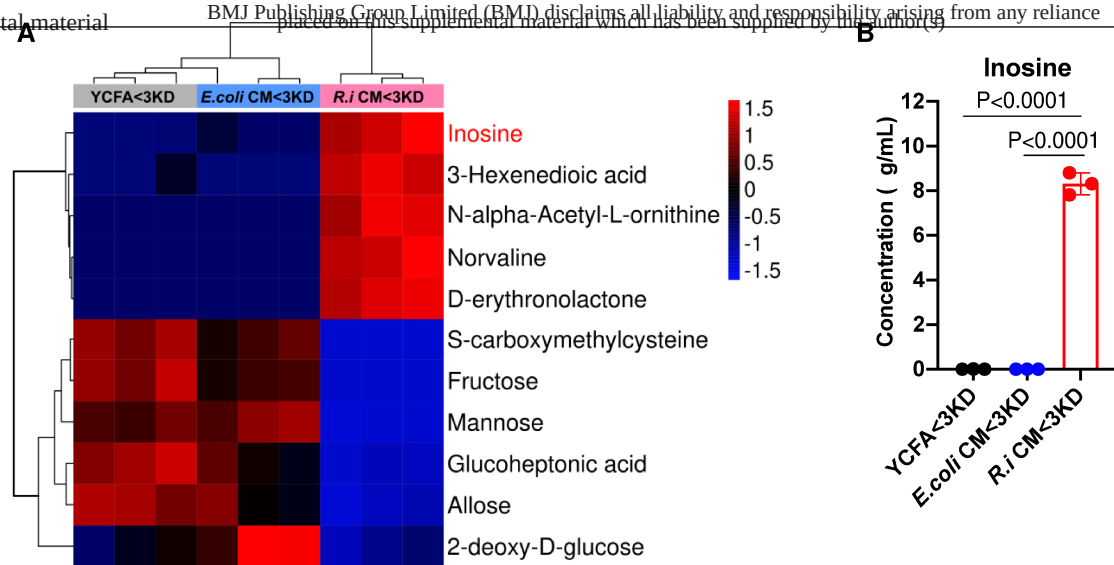
Supplementary Figure 4. Safety assessment of *R. intestinalis* on *APC^{Min/+}* mice. The dosage and frequency of *R. intestinalis* treatment on mouse body weight (A), liver (ALT, AST) and renal function (BUN) (B). ALT, Alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CREA, creatinine, all the samples were under 0.3mg/dL and the results were not shown.



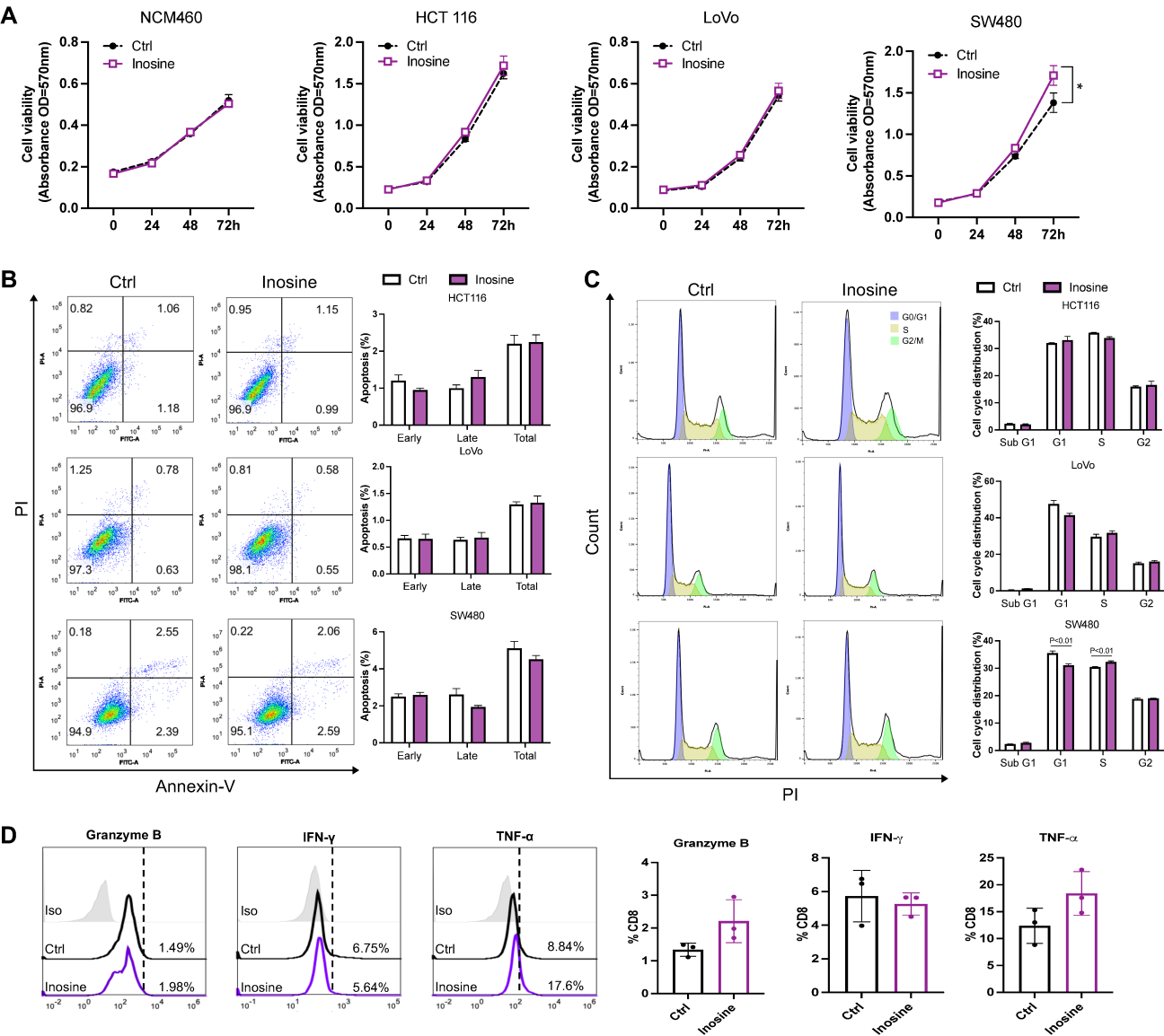
Supplementary Figure 5. Alive and pasteurized *R. intestinalis* have no tumor-suppressive effects in vitro. (A) Both alive *R. i* (CFU=10⁶/ml) and (B) pasteurized (70 ° C, 30min) *R. i* had no effects on colonic cell viability.



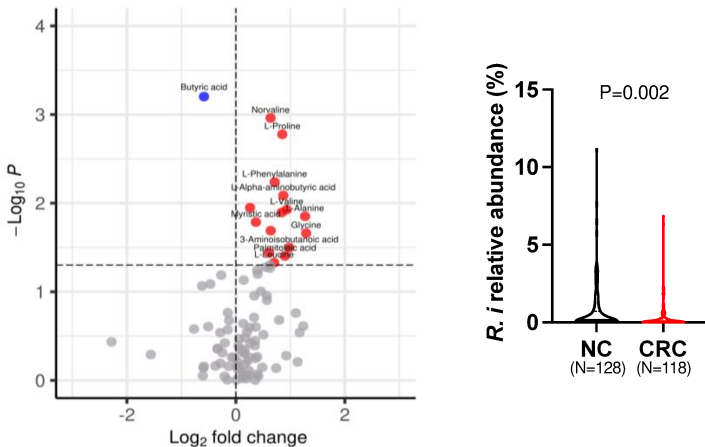
Supplementary Figure 6. The concentration ($\mu\text{g/ml}$) of other short-chain fatty acids besides butyric acid (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and hexanoic acid) in *R.i* CM <3KD was determined by targeted short-chain fatty acid GC-MS analysis.



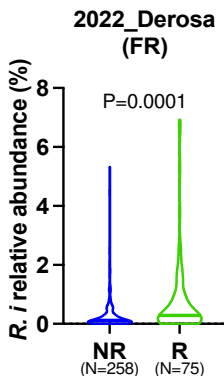
Supplementary Figure 7. Inosine might be another tumor suppressive component produced by *R.i*. (A) The *R.i* CM <3KD fraction was assessed by untargeted GC-MS/MS analysis and inosine was identified as the candidate tumor suppressive molecule. (B) Inosine concentration ($\mu\text{g/ml}$) in *R.i* CM <3KD was determined by targeted LC-MS analysis.



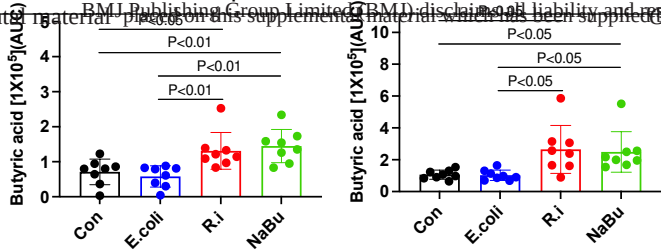
Supplementary Figure 8. Comparable dosage of inosine in *R.i* CM has no anti-tumor effects in vitro. Comparable dosage of inosine in *R.i* CM (0.4μg/ml) had no suppressive effects on colonic cell viability (A), cell apoptosis (B) and cell cycle (C) Comparable dosage of inosine had no effects on Granzyme B⁺, IFN- γ ⁺, TNF- α ⁺ CD8⁺ T cells isolated from C57BL/6 mouse spleen.



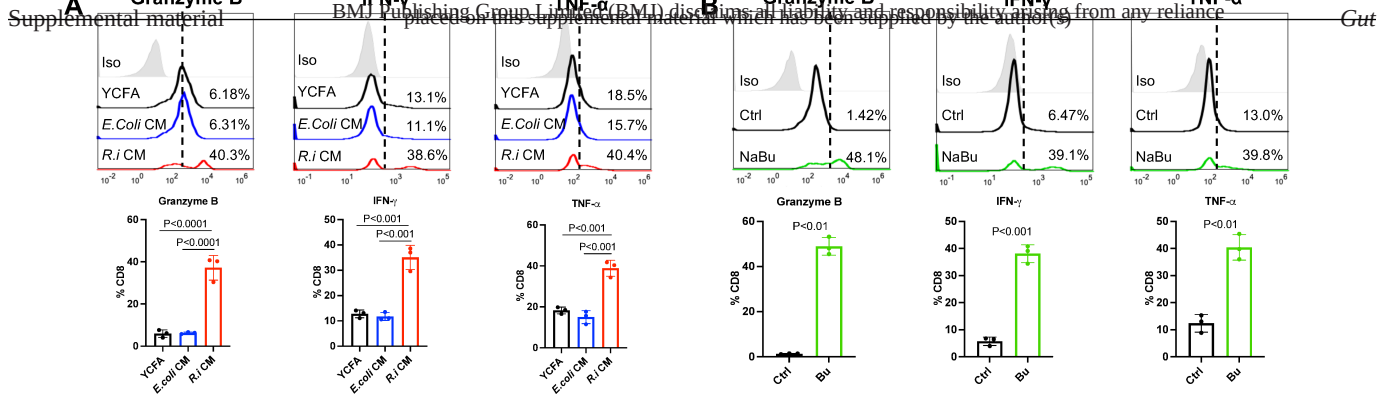
Supplementary Figure 9. *R. intestinalis* and butyric acid are depleted in fecal samples of CRC patients. Volcano plots of significantly altered human fecal metabolites between CRC and NC (A). Enriched metabolites are labelled as red dots and depleted ones are labelled as blue. $-\text{Log}_{10} P$ (0.05) was indicated with the horizontal dash line. CRC, colorectal cancer; NC, normal control. (B) Relative abundance of *R. intestinalis* (%) in fecal samples used for metabolomic sequencing in (A).



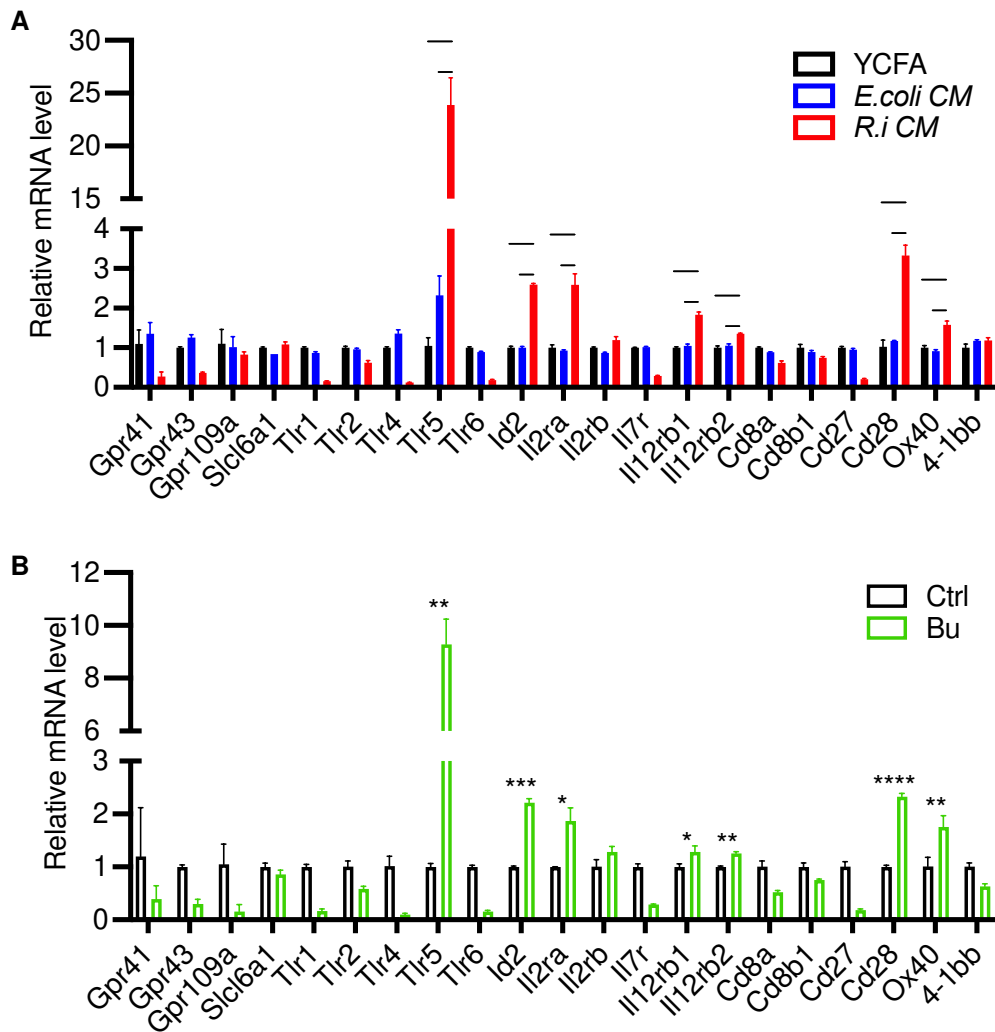
Supplementary Figure 10. Relative abundance of *R. intestinalis* in a France cohort of responders and non-responders to immune checkpoint inhibitors. NR, non-responder; R, responder.



Supplementary Figure 11. The relative concentration of butyrate acid (area under curve, AUC) in MC38 and CT26 orthotopic mice feces.



Supplementary Figure 12. Comparable dosage of butyrate in *R.i* CM suppresses colorectal cancer cells by CD8⁺ T cell boosting in vitro. *R.i* CM (A) and comparable dosage (1mM) of butyrate (B) directly boosted Granzyme B⁺, IFN- γ ⁺, TNF- α ⁺ CD8⁺ T cells isolated from C57BL/6 mouse spleen.



Supplementary Figure 13. The mRNA expression level of associated receptors on CD8⁺ T cells after treatment with *R.i* CM (A) and Butyrate (B).

S Table 1. Antibodies used in this study

ANTIBODIES	SOURCE	IDENTIFIER	APPLICATION	DILUTION / CONCERNTRATION
Ki-67 (D3B5) Rabbit mAb (IHC Formulated)	Cell Signalling Technology	Cat# 12202	IHC	1/200
Anti-ZO-1 tight junction protein antibody	abcam	Cat# ab96587	IHC, WB	1/100, 1/500
Claudin-3 polyclonal antibody	Thermo Fisher Scientific	Cat# 34-1700	IHC, WB	1/100, 1/1000
Recombinant Anti-CD8 alpha antibody [EPR21769]	abcam	Cat# ab217344	IHC	1/2000
Caspase-3 (8G10) Rabbit mAb	Cell Signalling Technology	Cat# 9665	WB	1/1000
Cleaved Caspase-3 (Asp175) antibody	Cell Signalling Technology	Cat# 9661	WB	1/1000
Caspase-7 antibody	Cell Signalling Technology	Cat# 9492	WB	1/1000
Cleaved Caspase-7 (Asp198) antibody	Cell Signalling Technology	Cat# 9491	WB	1/1000
Caspase-9 (C9) Mouse mAb	Cell Signalling Technology	Cat# 9508	WB	1/1000
PARP (46D11) Rabbit mAb	Cell Signalling Technology	Cat# 9532	WB	1/1000
Cleaved PARP (Asp214) Rabbit mAb	Cell Signalling Technology	Cat# 5625	WB	1/1000
Cdk6 (D4S8S) Rabbit mAb	Cell Signalling Technology	Cat# 13331	WB	1/1000
p27 Kip1 (D69C12) XP® Rabbit mAb	Cell Signalling Technology	Cat# 3686	WB	1/1000
Phospho-NF-κB p65 (Ser536) Rabbit mAb	Cell Signalling Technology	Cat# 3033	WB	1/1000
NF-κB p65 (D14E12) Rabbit mAb	Cell Signalling Technology	Cat# 8242	WB	1/1000
β-actin (13E5) Rabbit mAb	Cell Signalling Technology	Cat# 4970	WB	1/1000
Anti-rabbit IgG, HRP-linked Antibody	Cell Signalling Technology	Cat# 7074	WB	1/10000
Anti-mouse IgG, HRP-linked Antibody	Cell Signalling Technology	Cat# 7076	WB	1/10000
Ki-67 (8D5) Mouse mAb	Cell Signalling Technology	Cat# 9449	IF	1/6400
Alexa Fluor 488 goat anti-mouse IgG secondary antibody	Thermo Fisher Scientific	Cat# A-11001	IF	1 µg/mL
Brilliant Violet 605 anti-human CD45 antibody (2D1)	BioLegend	Cat# 368524	FCM	1/100
PE anti-human CD3 antibody (OKT3)	BioLegend	Cat# 317308	FCM	1/100
PE/Cyanine5 anti-human CD4 antibody (RPA-T4)	BioLegend	Cat# 300510	FCM	1/100
Brilliant Violet 421 anti-human CD8a antibody (SK1)	BioLegend	Cat# 344748	FCM	1/100
PE/Cyanine7 anti-human/mouse Granzyme B recombinant antibody (QA16A02)	BioLegend	Cat# 372214	FCM	1/100
Brilliant Violet 711 anti-human IFN-γ antibody (4S.B3)	BioLegend	Cat# 502540	FCM	1/100
FITC anti-human TNF-α antibody (Mab11)	BioLegend	Cat# 502906	FCM	1/100
Brilliant Violet 605 anti-mouse CD45 antibody (30-F11)	BioLegend	Cat# 103140	FCM	1/100
PerCP/Cyanine5.5 anti-mouse/human CD11b antibody (M1/70)	BioLegend	Cat# 101228	FCM	1/100
PE anti-mouse Ly-6G/Ly-6C (Gr-1) antibody (RB6-8C5)	BioLegend	Cat# 108408	FCM	1/100
Brilliant Violet 605 anti-mouse CD45 antibody (30-F11)	BioLegend	Cat# 103140	FCM	1/100
PE anti-mouse CD3 antibody (17A2)	BioLegend	Cat# 100206	FCM	1/100
Brilliant Violet anti-mouse CD8a antibody (53-6.7)	BioLegend	Cat# 100738	FCM	1/100
PE/Cyanine5 anti-mouse CD4 antibody (GK1.5)	BioLegend	Cat# 100410	FCM	1/100
FITC anti-mouse IFN-γ antibody (XMG1.2)	BioLegend	Cat# 505806	FCM	1/100
Brilliant Violet 711 anti-mouse TNF-α (MP6-XT22)	BioLegend	Cat# 506349	FCM	1/100

S Table 2. Patient information retrieved from our in-house cohort.

Sample	Cohort	Stage	Country	Continent	Age	Gender	AJCC	AJCC_simple	Labeling
S3_043	YuJ_2021	CRC	CHN	Asian	72	F	I	I	Downstream
S5_002	YuJ_2021	CRC	CHN	Asian	50	M	I	I	Downstream
S5_003	YuJ_2021	CRC	CHN	Asian	83	M	II	II	Downstream
S5_006	YuJ_2021	CRC	CHN	Asian	75	M	I	I	Downstream
S5_007	YuJ_2021	CRC	CHN	Asian	58	F	III	III	Downstream
S5_008	YuJ_2021	CRC	CHN	Asian	67	M	IV	IV	Downstream
S5_009	YuJ_2021	CRC	CHN	Asian	89	F	II	II	Downstream
S5_010	YuJ_2021	CRC	CHN	Asian	82	F	II	II	Discard
S5_011	YuJ_2021	CRC	CHN	Asian	71	M	II	II	Downstream
S5_012_merged	YuJ_2021	CRC	CHN	Asian	84	M	III	III	Downstream
S5_013	YuJ_2021	CRC	CHN	Asian	72	F	II	II	Downstream
S5_014	YuJ_2021	CRC	CHN	Asian	61	M	I	I	Discard
S5_015	YuJ_2021	CRC	CHN	Asian	65	M	IV	IV	Downstream
S5_016	YuJ_2021	CRC	CHN	Asian	83	M	II	II	Downstream
S5_017	YuJ_2021	CRC	CHN	Asian	81	M	III	III	Downstream
S5_018	YuJ_2021	CRC	CHN	Asian	74	M	III	III	Downstream
S5_019	YuJ_2021	CRC	CHN	Asian	65	F	IV	IV	Downstream
S5_020	YuJ_2021	CRC	CHN	Asian	72	F	I	I	Downstream
S5_021	YuJ_2021	CRC	CHN	Asian	64	F	I	I	Downstream
S5_022	YuJ_2021	CRC	CHN	Asian	73	M	I	I	Downstream
S5_023	YuJ_2021	CRC	CHN	Asian	68	F	III	III	Downstream
S5_025	YuJ_2021	CRC	CHN	Asian	60	F	III	III	Downstream
S5_026	YuJ_2021	CRC	CHN	Asian	80	F	I	I	Downstream
S5_027	YuJ_2021	CRC	CHN	Asian	84	F	III	III	Discard
S5_028	YuJ_2021	CRC	CHN	Asian	67	M	I	I	Downstream
S5_029	YuJ_2021	CRC	CHN	Asian	79	M	III	III	Downstream
S5_030	YuJ_2021	CRC	CHN	Asian	82	M	IV	IV	Downstream
S5_031	YuJ_2021	CRC	CHN	Asian	84	M	III	III	Downstream
S5_032	YuJ_2021	CRC	CHN	Asian	85	M	II	II	Downstream
S5_034	YuJ_2021	CRC	CHN	Asian	74	F	IV	IV	Downstream
S5_035	YuJ_2021	CRC	CHN	Asian	77	M	II	II	Downstream
S5_037	YuJ_2021	CRC	CHN	Asian	73	F	III	III	Downstream
S5_038	YuJ_2021	CRC	CHN	Asian	67	M	II	II	Downstream
S5_039	YuJ_2021	CRC	CHN	Asian	70	F	II	II	Downstream
S5_040	YuJ_2021	CRC	CHN	Asian	91	M	II	II	Downstream
S5_041	YuJ_2021	CRC	CHN	Asian	64	F	III	III	Discard
S5_042	YuJ_2021	CRC	CHN	Asian	77	M	II	II	Downstream
S5_043	YuJ_2021	CRC	CHN	Asian	63	F	II	II	Discard
S5_045	YuJ_2021	CRC	CHN	Asian	80	M	III	III	Downstream
S5_047	YuJ_2021	CRC	CHN	Asian	43	F	IV	IV	Downstream
S5_052	YuJ_2021	CRC	CHN	Asian	84	F	II	II	Downstream
S5_055	YuJ_2021	CRC	CHN	Asian	81	F	I	I	Downstream
S5_055-2	YuJ_2021	CRC	CHN	Asian	81	F	I	I	Discard
S5_056	YuJ_2021	CRC	CHN	Asian	79	M	IV	IV	Downstream
S5_057	YuJ_2021	CRC	CHN	Asian	92	M	III	III	Downstream
S5_060	YuJ_2021	CRC	CHN	Asian	52	F	I	I	Downstream
S5_061	YuJ_2021	CRC	CHN	Asian	84	M	III	III	Downstream
S5_062	YuJ_2021	CRC	CHN	Asian	96	F	II	II	Downstream
S5_064	YuJ_2021	CRC	CHN	Asian	78	M	I	I	Downstream
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S5_068	YuJ_2021	CRC	CHN	Asian	87	F	III	III	Downstream
S5_070	YuJ_2021	CRC	CHN	Asian	59	M	III	III	Downstream
S5_071	YuJ_2021	CRC	CHN	Asian	83	M	II	II	Downstream
S5_072	YuJ_2021	CRC	CHN	Asian	71	M	III	III	Downstream
S5_073	YuJ_2021	CRC	CHN	Asian	85	M	II	II	Downstream
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S5_076	YuJ_2021	CRC	CHN	Asian	82	F	II	II	Discard
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S5_078	YuJ_2021	CRC	CHN	Asian	74	F	II	II	Downstream
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S5_084_merged	YuJ_2021	CRC	CHN	Asian	68	F	III	III	Downstream
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S5_086	YuJ_2021	CRC	CHN	Asian	67	M	I	I	Downstream

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	S5_088	YuJ_2021	CRC	CHN	Asian	56	M	I	I	Downstream
	S5_089	YuJ_2021	CRC	CHN	Asian	75	M	I	I	Downstream
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	S6_007	YuJ_2021	CRC	CHN	Asian	82	F	II	II	Downstream
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	S6_013	YuJ_2021	CRC	CHN	Asian	64	F	I	I	Discard
	S6_014	YuJ_2021	CRC	CHN	Asian	78	M	IV	IV	Downstream
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	S6_024	YuJ_2021	CRC	CHN	Asian	89	M	II	II	Downstream
	S6_025	YuJ_2021	CRC	CHN	Asian	81	F	I	I	Downstream
	S6_026	YuJ_2021	CRC	CHN	Asian	67	M	I	I	Discard
	S6_027	YuJ_2021	CRC	CHN	Asian	62	M	II	II	Downstream
	S6_028	YuJ_2021	CRC	CHN	Asian	69	F	II	II	Downstream
	S6_029	YuJ_2021	CRC	CHN	Asian	68	F	III	III	Downstream
	S6_030	YuJ_2021	CRC	CHN	Asian	57	F	IV	IV	Downstream
	S6_031	YuJ_2021	CRC	CHN	Asian	72	F	I	I	Downstream
	S6_032	YuJ_2021	CRC	CHN	Asian	68	F	III	III	Downstream
	S6_035	YuJ_2021	CRC	CHN	Asian	64	F	III	III	Downstream
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	S1_002	YuJ_2021	Ctrl	CHN	Asian	57	F	NA	NA	Downstream
	S1_003	YuJ_2021	Ctrl	CHN	Asian	63	M	NA	NA	Downstream
	S1_004	YuJ_2021	Ctrl	CHN	Asian	59	F	NA	NA	Downstream
	S1_005	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Discard
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	S1_008	YuJ_2021	Ctrl	CHN	Asian	73	F	NA	NA	Downstream
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	S1_011	YuJ_2021	Ctrl	CHN	Asian	61	F	NA	NA	Downstream
S1_012_merged	YuJ_2021	Ctrl	CHN	Asian	59	F	NA	NA	Downstream	
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	S1_016	YuJ_2021	Ctrl	CHN	Asian	65	F	NA	NA	Downstream
	S1_017	YuJ_2021	Ctrl	CHN	Asian	71	F	NA	NA	Downstream
	S1_018	YuJ_2021	Ctrl	CHN	Asian	65	M	NA	NA	Discard
	S1_019	YuJ_2021	Ctrl	CHN	Asian	66	M	NA	NA	Downstream
	S1_020	YuJ_2021	Ctrl	CHN	Asian	79	F	NA	NA	Downstream
	S1_021	YuJ_2021	Ctrl	CHN	Asian	71	M	NA	NA	Downstream
	S1_022	YuJ_2021	Ctrl	CHN	Asian	57	M	NA	NA	Downstream

S1_023	YuJ_2021	Ctrl	CHN	Asian	64	M	NA	NA	Downstream	
	YuJ_2021	Ctrl	CHN	Asian	65	M	NA	NA	Downstream	
	S1_025	YuJ_2021	Ctrl	CHN	Asian	58	M	NA	NA	Downstream
S1_026	YuJ_2021	Ctrl	CHN	Asian	59	M	NA	NA	Downstream	
S1_027	YuJ_2021	Ctrl	CHN	Asian	67	M	NA	NA	Downstream	
S1_028	YuJ_2021	Ctrl	CHN	Asian	50	M	NA	NA	Discard	
S1_029	YuJ_2021	Ctrl	CHN	Asian	61	F	NA	NA	Downstream	
S1_030	YuJ_2021	Ctrl	CHN	Asian	47	F	NA	NA	Downstream	
S1_031	YuJ_2021	Ctrl	CHN	Asian	52	F	NA	NA	Downstream	
S1_032	YuJ_2021	Ctrl	CHN	Asian	51	M	NA	NA	Downstream	
S1_033	YuJ_2021	Ctrl	CHN	Asian	63	F	NA	NA	Downstream	
S1_034	YuJ_2021	Ctrl	CHN	Asian	68	F	NA	NA	Downstream	
S1_035	YuJ_2021	Ctrl	CHN	Asian	55	M	NA	NA	Downstream	
S1_036	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_037	YuJ_2021	Ctrl	CHN	Asian	64	M	NA	NA	Downstream	
S1_038	YuJ_2021	Ctrl	CHN	Asian	59	M	NA	NA	Downstream	
S1_039	YuJ_2021	Ctrl	CHN	Asian	54	F	NA	NA	Downstream	
S1_040	YuJ_2021	Ctrl	CHN	Asian	60	F	NA	NA	Discard	
S1_041	YuJ_2021	Ctrl	CHN	Asian	56	M	NA	NA	Downstream	
S1_042	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_043	YuJ_2021	Ctrl	CHN	Asian	75	M	NA	NA	Discard	
S1_045	YuJ_2021	Ctrl	CHN	Asian	67	F	NA	NA	Discard	
S1_047	YuJ_2021	Ctrl	CHN	Asian	66	M	NA	NA	Downstream	
S1_048	YuJ_2021	Ctrl	CHN	Asian	57	F	NA	NA	Downstream	
S1_049	YuJ_2021	Ctrl	CHN	Asian	61	F	NA	NA	Downstream	
S1_050	YuJ_2021	Ctrl	CHN	Asian	70	F	NA	NA	Downstream	
S1_051	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_052	YuJ_2021	Ctrl	CHN	Asian	61	F	NA	NA	Downstream	
S1_053	YuJ_2021	Ctrl	CHN	Asian	64	F	NA	NA	Downstream	
S1_054	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_057	YuJ_2021	Ctrl	CHN	Asian	57	F	NA	NA	Downstream	
S1_058	YuJ_2021	Ctrl	CHN	Asian	60	M	NA	NA	Downstream	
S1_060_merged	YuJ_2021	Ctrl	CHN	Asian	57	F	NA	NA	Downstream	
	S1_061	YuJ_2021	Ctrl	CHN	Asian	59	F	NA	NA	Downstream
S1_062_merged	YuJ_2021	Ctrl	CHN	Asian	61	F	NA	NA	Downstream	
	S1_064	YuJ_2021	Ctrl	CHN	Asian	72	M	NA	NA	Downstream
S1_065	YuJ_2021	Ctrl	CHN	Asian	64	F	NA	NA	Downstream	
S1_066	YuJ_2021	Ctrl	CHN	Asian	64	M	NA	NA	Downstream	
S1_067	YuJ_2021	Ctrl	CHN	Asian	57	F	NA	NA	Discard	
S1_068	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_069	YuJ_2021	Ctrl	CHN	Asian	65	F	NA	NA	Downstream	
S1_070	YuJ_2021	Ctrl	CHN	Asian	58	M	NA	NA	Downstream	
S1_071	YuJ_2021	Ctrl	CHN	Asian	65	F	NA	NA	Downstream	
S1_072	YuJ_2021	Ctrl	CHN	Asian	76	F	NA	NA	Downstream	
S1_073	YuJ_2021	Ctrl	CHN	Asian	65	F	NA	NA	Downstream	
S1_074	YuJ_2021	Ctrl	CHN	Asian	63	F	NA	NA	Downstream	
S1_077	YuJ_2021	Ctrl	CHN	Asian	67	F	NA	NA	Downstream	
S1_078	YuJ_2021	Ctrl	CHN	Asian	68	F	NA	NA	Discard	
S1_079	YuJ_2021	Ctrl	CHN	Asian	65	M	NA	NA	Downstream	
S1_081	YuJ_2021	Ctrl	CHN	Asian	67	F	NA	NA	Downstream	
S1_083	YuJ_2021	Ctrl	CHN	Asian	72	F	NA	NA	Downstream	
S1_084	YuJ_2021	Ctrl	CHN	Asian	59	M	NA	NA	Downstream	
S1_086	YuJ_2021	Ctrl	CHN	Asian	57	M	NA	NA	Downstream	
S1_087	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Discard	
S1_088	YuJ_2021	Ctrl	CHN	Asian	65	F	NA	NA	Downstream	
S1_089	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_089-2	YuJ_2021	Ctrl	CHN	Asian	62	F	NA	NA	Downstream	
S1_091	YuJ_2021	Ctrl	CHN	Asian	57	M	NA	NA	Downstream	
S1_092	YuJ_2021	Ctrl	CHN	Asian	62	M	NA	NA	Downstream	
S1_093	YuJ_2021	Ctrl	CHN	Asian	67	M	NA	NA	Downstream	
S1_094	YuJ_2021	Ctrl	CHN	Asian	60	F	NA	NA	Downstream	
S1_095	YuJ_2021	Ctrl	CHN	Asian	59	F	NA	NA	Downstream	
S1_100_merged	YuJ_2021	Ctrl	CHN	Asian	53	F	NA	NA	Downstream	
	S1_102	YuJ_2021	Ctrl	CHN	Asian	52	F	NA	NA	Downstream
	S1_105	YuJ_2021	Ctrl	CHN	Asian	61	F	NA	NA	Downstream
	S1_106	YuJ_2021	Ctrl	CHN	Asian	59	M	NA	NA	Downstream
	S1_107	YuJ_2021	Ctrl	CHN	Asian	72	F	NA	NA	Downstream

S1_108	YuJ_2021	Ctrl	CHN	Asian	63	M	NA	NA	Downstream
S1_109	YuJ_2021	Ctrl	CHN	Asian	51	M	NA	NA	Downstream
S1_110	YuJ_2021	Ctrl	CHN	Asian	53	F	NA	NA	Downstream
S1_111	YuJ_2021	Ctrl	CHN	Asian	72	M	NA	NA	Downstream
S1_112	YuJ_2021	Ctrl	CHN	Asian	57	M	NA	NA	Downstream
S1_113	YuJ_2021	Ctrl	CHN	Asian	56	F	NA	NA	Downstream
S1_114	YuJ_2021	Ctrl	CHN	Asian	59	M	NA	NA	Downstream
S1_115	YuJ_2021	Ctrl	CHN	Asian	57	F	NA	NA	Downstream
S1_116	YuJ_2021	Ctrl	CHN	Asian	54	F	NA	NA	Downstream
S1_118	YuJ_2021	Ctrl	CHN	Asian	48	F	NA	NA	Downstream
S1_120	YuJ_2021	Ctrl	CHN	Asian	58	F	NA	NA	Downstream
S1_121	YuJ_2021	Ctrl	CHN	Asian	76	M	NA	NA	Downstream
S1_122	YuJ_2021	Ctrl	CHN	Asian	78	F	NA	NA	Discard
S1_123	YuJ_2021	Ctrl	CHN	Asian	72	M	NA	NA	Downstream
S1_124	YuJ_2021	Ctrl	CHN	Asian	76	F	NA	NA	Downstream
S1_125	YuJ_2021	Ctrl	CHN	Asian	58	M	NA	NA	Downstream
S1_126	YuJ_2021	Ctrl	CHN	Asian	58	M	NA	NA	Downstream
S1_127	YuJ_2021	Ctrl	CHN	Asian	63	F	NA	NA	Downstream
S1_128	YuJ_2021	Ctrl	CHN	Asian	69	F	NA	NA	Downstream
S1_130	YuJ_2021	Ctrl	CHN	Asian	67	M	NA	NA	Downstream
S1_132	YuJ_2021	Ctrl	CHN	Asian	65	F	NA	NA	Downstream
S2_005	YuJ_2021	Ctrl	CHN	Asian	60	F	NA	NA	Downstream
S3_096	YuJ_2021	Ctrl	CHN	Asian	66	M	NA	NA	Downstream