Gut microbiota regulates AML via alternation of intestinal barrier function mediated by butyrate

Ruiqing Wang

Qilu Hospital of Shandong University

Xinyu Yang

Qilu Hospital of Shandong University

Jinting Liu

Qilu Hospital, Shandong University

Fang Zhong

Shandong Provincial Hospital

Chen Zhang

Qilu Hospital of Shandong University

Yuhong Chen

Qilu Hospital of Shandong University

Tao Sun

School of Medicine, Shandong University

Chun-yan Ji

Qilu Hospital of Shandong University <https://orcid.org/0000-0003-4438-1768>

Daoxin Ma (✉ daoxinma@sdu.edu.cn)

Qilu Hospital of Shandong University

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1 **Gut microbiota regulates AML via alternation of intestinal barrier**
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4 Ruiqing Wang¹, Xinyu Yang¹, Jinting Liu¹, Fang Zhong², Chen Zhang¹, Yuhong Chen¹,
5 Tao Sun¹, Chunyan Ji¹, Daoxin Ma^{1*}

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8 ¹Department of Hematology, Qilu Hospital of Shandong University, Jinan, Shandong
9 250012, P.R. China; ²Department of Endocrinology and Metabolism, Shandong
10 Provincial Hospital affiliated to Shandong University, Jinan, Shandong 250012, P.R.
11 China

12

13

14

15 Corresponding author: Daoxin Ma, Professor

16 Address: Department of Hematology, Qilu Hospital of Shandong University, Jinan,
17 Shandong 250012, P.R. China

18 Phone: 86-0531-82169887 Fax: 86-0531-86927544

19 E-mail address: daoxinma@sdu.edu.cn

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21 Running head: Gut microbiota regulates AML progression

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24 **Key points**

- 25 • Patients with newly diagnosed AML have disordered gut microbiota together with
26 reduced butyrate and other SCFAs.
- 27 • AML causes intestinal barrier damage in mice, with increased plasma LPS that
28 accelerates AML progression.

Abstract

The gut microbiota has been linked to many cancers, yet the role of intestinal microbes in acute myeloid leukemia (AML) progression remains unclear. Here, we observed a significant shift in the gut microbiota in AML patients, characterized by reduced *Faecalibacterium* abundance. According to a murine AML model, we found that intestinal microbial diversity decreased as the disease progressed. On the other side, gut microbiota dysbiosis induced by antibiotic treatment accelerated AML progression with a higher leukemia cell burden and shorter overall survival (OS), while fecal microbiota transplantation altered this process. Metabolome analyses showed that microbiota-derived butyrate concentration obviously decreased in AML patient feces, and butyrate gavage postponed AML progression in a mouse model. Moreover, our study revealed that intestinal barrier function is decreased in AML mice which may be related to the microbiota disorder caused by AML. Lower intestinal barrier function increased the bacterial-associated lipopolysaccharide (LPS) concentration in the peripheral blood of AML patients or mice through enhancing intestinal permeability. Butyrate repaired the intestinal barrier damage and inhibited LPS absorption in AML mice. Collectively, these findings demonstrate that the gut microbiota promotes AML progression in a metabolite dependent manner, and targeting the gut microbiota might provide a novel therapeutic option for AML.

Key words: gut microbiota; butyrate; lipopolysaccharide; acute myeloid leukemia; progression

Introduction

Acute myeloid leukemia (AML) is a hematological malignancy characterized by excessive proliferation of immature myeloid cells. Although chemotherapy has been proven to be effective against this malignancy¹, long-term survival is modest². The

long-term survival and prognosis of AML are closely related to many factors in AML progression. Thus, improved outcomes may depend on novel treatment strategies derived from a better understanding of the mechanism of AML progression.

The progression of AML is governed by many factors including genetic and homeostatic environment changes. Accumulating evidence suggests that the human gut microbiota can affect the homeostatic environment and is linked to the development of inflammation, autoimmune diseases, and even cancers³⁻⁶. With further research, the relationship between the gut microbiota and hematological diseases has gradually been reported. In a typical and stable environment, the gut microbiota regulates and sustains normal steady-state hematopoiesis⁷. Recently, it was reported that structural changes in the gut microbiota can occur in acute lymphoblastic leukemia (ALL) patients, and the microbiota diversity was significantly reduced⁸. Shifts in the intestinal microbiota following allogeneic hematopoietic cell transplantation (HCT) were found to be associated with the development of gastrointestinal graft-versus-host disease (GVHD), suggesting that the intestinal microbiota may be an essential factor in GVHD⁹. However, the role that gut microbiota plays in AML progression has not been studied.

Some microbial metabolites are exclusively derived from the gut microbiota and are not host derived, which mediate the biological effects of the gut microbiota. Microbial metabolites such as short chain fatty acids (SCFAs) have been implicated in hematological diseases. Payen et al. revealed a dramatic decrease in the levels of the main SCFAs, acetate, propionate, and butyrate, in severe GVHD patients¹⁰. In addition, butyrate, as an important SCFA¹¹⁻¹³, has been reported as a chief energy source for intestinal epithelial cells, and administration of butyrate could effectively alleviate GVHD¹⁴⁻¹⁶. This finding suggests that the gut microbiota may play an important role in diseases through metabolic pathways, especially in GVHD. However, related research

on SCFAs in AML is still lacking.

The existence of the gut microbiota, along with its nutritious SCFA metabolites, can make the growth of intestinal epithelial cells more active and regulate their differentiation and repair¹⁷⁻¹⁹. The intestinal barrier is the sole gate where the entry of the gut microbiota or metabolites into the blood can be prevented. Its integrity and whether it can function normally are directly linked to the fluctuation of the amounts of bacteria and their metabolites in the blood. Cui et al. found that the physical, chemical, immunological, and microbiological barriers in the intestinal tract constitute the complete intestinal barrier, which plays an important defensive role against the invasion of harmful substances from the intestines²⁰. Another study confirmed that in hematological diseases, GVHD patients showed significant incapacitation of the gut barrier functionality, compared to that of healthy volunteers²¹. The extent of integrity and permeability of the intestinal barrier often determine how the gut flora and their metabolites affects human systemic immunity²². Intestinal barrier tends to be disregarded in AML and focusing on intestinal barrier may provide a novel insight for improving the therapeutic effect of AML.

In this study, we found that the gut microbes of AML patients and mice are significantly disorder and the gut microbiota dysbiosis further aggravate the progression of the AML. SCFA metabolome analyses showed that the concentration of butyrate was significantly decreased in the intestinal contents of AML patients, which resulted in lower intestinal barrier function and higher absorption of LPS into the blood. Moreover, the increased LPS content promoted AML progression, while the addition of intestinal butyrate protected intestinal epithelial cells and decreased AML severity. Our study on the relationship between AML and intestinal barrier function has confirmed that intestinal health is also worthy of attention in the process of AML

progression, which will provide a new understanding for the diagnosis and treatment of AML.

Methods

Patient recruitment and specimens

Sixty-one adults were enrolled from 2018 to 2019. Stool, peripheral blood and bone marrow (BM) included in this study were obtained from 31 newly diagnosed (ND) AML patients and 30 healthy controls. These specimens were collected at Qilu Hospital of Shandong University. The exclusion criteria were as follows: age <16 years and > 70 years, diarrhea, receiving antibiotics or hormone therapy within the last 10 weeks, and blood pressure anomalies. The patients were divided into an AML patient group (n = 31) and a healthy control (Con) group (n = 30). Their characteristics are summarized in supplementary table 1 and the risk stratification standard are shown in supplementary table 2. Forty-four blood samples were used for metabolic profiling analysis (n = 22 per group), and another 28 samples were utilized for cell culture and functional studies. Ten BM samples were randomly chosen for metabolic profiling analysis (n = 5 per group). Among the 61 individuals, all were chosen for stool 16S ribosomal RNA sequencing. The study was performed with the patients' written informed consent at the beginning of the trial and was approved by the Medical Ethical Committee of Qilu Hospital of Shandong University.

Cells culture and reagents

MLL-AF9, murine AML cell labeled with a GFP reporter, was obtained from the spleen of the successfully established MLL-AF9 murine AML model. MV411 or primary leukemia cells were cultured in IMDM culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). MLL-AF9 cells were cultured in DMEM containing 10% FBS,

cytokines (IL-3, IL-6, G-msf and scf) and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. LPS, dimethyl sulfoxide (DMSO) and FITC-dextran were purchased from Sigma-Aldrich (USA). Adriamycin (ADR), daunorubicin (DNR) and cytosine arabinoside (Ara-C) were dissolved in normal saline and diluted in RPMI 1640 medium immediately before use. Primary antibodies against claudin-1 (#15101), claudin-2 (#8242), ZO-1 (#3033) and Bcl-2 (#3498) used for Western blot were obtained from Cell Signaling Technology (Beverly, MA, USA), and antibodies against cleaved caspase-3 (ab13847), BAX (ab199677), β -tubulin (ab0039) and β -actin (ab227387) were purchased from Abcam (Cambridge, UK). Secondary antibodies were obtained from Millipore (USA). All reagents were dissolved and preserved following the manual's instructions.

Murine AML models

Specific pathogen free (SPF) mice on a C57BL/6J background were purchased from the Laboratory Animal Center of Shandong University. A retrovirus vector containing the intracellular domain of MLL-AF9 (MSCV-MLL-AF9-IRES-GFP) was kindly provided by Dr. Hui Cheng (Institute of Hematology, Chinese Academy of Medical Sciences). To establish a murine AML model, C-kit⁺ cells from the bone marrow of wild-type (WT) mice transfected with MSCV-MLL-AF9-IRES-GFP (10⁷ cells/host), together with bone marrow mononuclear cells (BM-MNCs) from C57BL/6J mice (10⁷ cells/host), were transplanted into lethally irradiated C57BL/6J recipients. On day 30 after transplantation, AML mice were verified by detecting GFP frequency in peripheral blood from the lateral tail vein with a FACS Aria II sorter (BD Biosciences). Then, we collected the spleen cells from the successfully established MLL-AF9 AML mice and froze the cells as MLL-AF9 murine AML cells at -80 °C for subsequent experiments. All mice were six to eight weeks-old and were maintained in an SPF environment.

Animal protocols were approved by the Animal Ethics Committee of Qilu Hospital, Shandong University.

Preparation of fecal hydration liquid

Fresh feces were weighed and diluted with normal saline to adjust the volume of normal saline so that the fecal suspension concentration was approximately 150 mg/mL. Then, the fecal suspension was filtered to remove large particles, the filtrate was collected, glycerol was added to a concentration 20%, and the samples were stored at - 80 °C. Fecal hydration liquid was prepared from human samples for rodent gavage.

Fecal microbiota transplantation (FMT) and antibiotics

For fecal microbiota transplantation (FMT), mice were randomized into the following groups (n = 5 per group): AML-FMT (antibiotic-treated AML mice followed by FMT with fecal hydration liquid from AML patients) and Con-FMT (antibiotic-treated AML mice followed by FMT with fecal hydration liquid from healthy people). An antibiotic mix (ABX) was administered by oral gavage. Briefly, the antibiotic was used as follows: on days 21-7 before MLL-AF9 cell injection, mice received a daily gavage of metronidazole (100 mg/kg), while the antibiotic mix (ampicillin (1 g/L), vancomycin (0.5 g/L) and neomycin (1 g/L)) was added to the drinking water. Then, FMT was carried out via oral gavage with a fecal suspension (150 mg/mL) in a final volume of 250 μ L. FMT was performed daily from 14 to 35 days after the start of the antibiotic regimen.

Butyrate treatment

Mice received sodium butyrate (15 mg/kg) or vehicle (sterile PBS) through a 20G - 1.5-inch flexible intragastric gavage needle (Braintree Scientific; Braintree, MA) every day for 2 weeks after MLL-AF9 cell injection.

Dual-luciferase activity assay

MLL-AF9 cells were transfected with a Luciferase (MCS-Luciferase-IRES-Puro)

retrovirus vector. Firefly and Renilla luciferase activities were measured consecutively 24 h following transfection using a Dual-Luciferase Reporter Assay (Promega, USA) according to the manufacturer's instructions. Moreover, to monitor tumor progression and metastasis in vivo, bioluminescent imaging was performed with an IVIS Lumina Series III (PerkinElmer, USA) on days 10 and 14 after injection and was used to confirm the dynamic process of tumor cell load in vivo.

Cell proliferation assays

After different treatments, the cells were incubated with 10 μ L of CCK8 (Beyotime, China) for 3 hours. The absorbance was measured at 450 nm. Each sample was measured in triplicate.

Apoptosis assays

Apoptosis was assessed with the Annexin V/ propidium iodide (PI) apoptosis detection kit (BestBio, Shanghai, China) according to the manufacturer's protocol. Cells were harvested after different treatments and washed twice with PBS. Then, the cells were resuspended in 400 μ L of binding buffer, and stained with 5 μ L of Annexin V for 15 minutes and 10 μ L of PI for another 5 minutes in the dark at 4 °C. The percentages of apoptotic cells were analyzed immediately by a Galios flow cytometer (Beckman Coulter, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Blood plasma samples were collected from EDTA-stabilized peripheral blood of 20 ND AML patients and 18 controls, and supernatants of mouse peripheral blood were collected and stored at -80 °C for determination of LPS concentration. Human and mouse LPS ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). ELISAs were performed in accordance with the manufacturer's instructions.

Western blotting analysis

Cells were collected, washed twice with PBS, and then lysed with RIPA buffer (Beyotime, China) containing protease inhibitor compound (Beyotime, China) on ice. A bicinchoninic acid (BCA) protein assay kit (Beyotime, China) was applied to measure protein concentrations. Protein extracts (30 µg) were loaded into 10% SDS-PAGE gels and then electrotransferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk for 1 hour at room temperature, the membranes were incubated overnight with specific primary antibodies at 4 °C followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 hour. Protein bands were detected by a FluorChem E Chemiluminescent imaging system (ProteinSimple, San Jose, CA, USA) after washing.

SCFA measurement

Samples were thawed on ice, and 100 µL aliquots were added into a 2 mL glass centrifuge tube and mixed with 50 µL of water with 15% phosphoric acid and 150 µL of 5 µg/mL 4-methyl valeric acid. The suspensions were homogenized with a vortex for approximately 1 minute and centrifuged for 10 minutes at 12000× g. Then, 1 µL of supernatant was taken for gas chromatography–mass spectrometry (GC-MS) analysis using an Agilent Model 7890A/5975C GC-MS system. To quantify SCFAs, a calibration curve for the concentration range of 0.1–100 µg/mL was constructed. The IS was used to correct for injection variability between samples and minor changes in the instrument response.

The samples were separated with an Agilent HP-INNOWAX capillary GC column (30 m × 0.25 mm ID × 0.25 µm). The initial temperature was 90 °C, which was increased to 120 °C at 10 °C/min, and the temperature was then increased to 150 °C at 5 °C/min and finally to 250 °C at 25 °C/min, where it remained for 2 minutes. The carrier gas was helium (1.0 mL/min). The temperatures of the injection port and transmission line

were 250 °C and 230 °C respectively. An electron bombardment ionization source was used, selected ion monitoring (SIM) scanning mode was applied, and the electron energy was 70 eV (Shanghai Applied Protein Technology Co., Ltd.).

Microbial diversity analysis

Microbial DNA was extracted from fecal samples using an E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocols. The final DNA concentration and purification were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 338F (5'- ACT CCT ACG GGA GGC AGC AG- 3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT- 3') by a thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR was conducted using the following program: 3 minutes of denaturation at 95 °C; 27 cycles of 30 seconds at 95 °C, 30 seconds for annealing at 55 °C, and 45 seconds for elongation at 72 °C; and a final extension at 72 °C for 10 minutes. The PCRs were performed in triplicate in a 20 µL mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the reads were

truncated at any site receiving an average quality score <20 over a 50 bp sliding window;
(ii) primers were exactly matched, allowing 2 nucleotide mismatches, and reads
containing ambiguous bases were removed; And (iii) sequences whose overlap was
longer than 10 bp were merged according to their overlap sequence. Operational
taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE
(version 7.1 <http://drive5.com/uparse/>), and chimeric sequences were identified and
removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was
analyzed by the RDP Classifier algorithm (<http://rdp.cme.msu.edu/>) against the Silva
(SSU123) 16S rRNA database using a confidence threshold of 70%.

Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by
FLASH with the following criteria: (i) The reads were truncated at any site receiving
an average quality score <20 over a 50 bp sliding window. (ii) Primers were exactly
matched allowing 2 nucleotide mismatching, and reads containing ambiguous bases
were removed. (iii) Sequences whose overlap longer than 10 bp were merged according
to their overlap sequence. Alpha diversity was measured with Shannon metrics and
Beta diversity was calculated using UniFrac.

Quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol (Invitrogen, USA). The total RNA
concentration and purity were quantified by a spectrophotometer (Eppendorf,
Germany). Reverse transcription was performed at 37 °C for 15 minutes followed by
85 °C for 10 seconds using a Prime Script RT reagent Perfect Real Time Kit (Takara
Bio Inc, Japan). Quantitative PCR was performed in duplicate on a Light Cycler 480II
real-time PCR system (Roche, Switzerland) with a SYBR Green Real-time PCR Master
Mix kit (Toyobo, Japan). The PCR contained 3.2 µL of ddH₂O, 5 µL of 2×SYBR Green

Real- time PCR Master Mix, 0.4 μ L of the forward and reverse primers, and 1 μ L of cDNA in a final volume of 10 μ L. PCR conditions were as follows: 95 $^{\circ}$ C for 10 minutes followed by 40 cycles of 95 $^{\circ}$ C for 20 seconds and 60 $^{\circ}$ C for 1 minute. The primer sequences for the genes are shown in supplementary table 2. To determine the specificity of the PCRs, melting curves were routinely analyzed. All experiments were conducted according to the manual's instructions. Relative gene expression was expressed relative to that of the endogenous control GAPDH and calculated using the $2^{-\Delta CT}$ method.

FITC-dextran assay

Food and water were withheld from all mice for four hours. FITC-dextran was administered by a 20G -1.5-inch flexible intragastric gavage needle at a concentration of 50 mg/mL in PBS. Mice received FITC-dextran at 800 mg/kg (~16 mg/mouse). Four hours later, serum was collected from peripheral blood, diluted 1:1 with PBS, and analyzed on a plate reader at excitation/emission wavelengths of 485 nm/535 nm. Concentrations of FITC-dextran in experimental samples were determined based on a standard curve.

Transmission electron microscopy (TEM)

Briefly, the intestines from control mice or AML mice treated with butyrate or vehicle were harvested 14 days after injection of MLL-AF9. Cross sections were immediately sliced (2 mm wide) from the duodenum, jejunum, and ileum. The tissue was then rinsed three times with Sorensen's buffer, containing 0.1 % ruthenium red (RR) and postfixed for one hour in 1% osmium tetroxide in the same buffer containing RR. The samples were again rinsed with Sorensen's buffer containing RR. Next, the tissue was dehydrated in ascending concentrations of ethanol, treated with propylene oxide, and embedded in Epon epoxy resin. Semithin sections were stained with toluidine blue for

tissue identification. Selected regions of interest were ultrathin sectioned (70 nm thick), mounted on copper grids, and poststained with uranyl acetate and lead citrate. The samples were examined using a Philips CM100 electron microscope at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system operated using AMT software (Servicebio, Wuhan, China).

Statistical analysis

GraphPad Prism 8.0 was applied in diagram drawing. Statistical analysis was conducted with raw data using SPSS 20.0 software. Logistic regression was used to analyze the association and correlation between *Faecalibacterium* and WBC was performed by Spearman correlation. The Shapiro-Wilk test was used for normality tests. Normally distributed data were analyzed by Student's t-test or paired t tests. Otherwise, comparisons between groups were performed using the Mann-Whitney U test for nonpaired data and the Wilcoxon signed rank test for paired data. Data in the results are expressed as the mean $\pm$ s.e.m, except were stated otherwise. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ were considered statistically significant.

Results

1. The diversity and composition of the gut microbiota are significantly altered in AML patients

Sixty-one adults were enrolled from 2018 to 2020. Stool, peripheral blood and bone marrow included in this study were obtained from 31 newly diagnosed (ND) and untreated AML patients and 30 healthy controls (Supplementary table 1). A total of 2461 OTUs in all samples were identified. We first confirmed that the sequencing depth of the fecal samples of AML patients and healthy controls was sufficient for further analysis through the dilution curve method (Supplementary figure 1a). Then, we

analyzed the Shannon α -diversity indices of these samples. A significant difference in the Shannon diversity was observed between AML patients and healthy controls ($P=0.0275$), demonstrating a significantly lower diversity in the feces of AML patients than in that of healthy controls (Figure 1A). Principal coordinate analysis (PCoA) demonstrated a significant difference in β -diversity using the weighted UniFrac distance between the AML and control groups ($P=0.006$), suggesting that the microbial composition in AML patients was significantly shifted regarding OTUs (Figure 1B). To identify the detailed alterations in the microbiota, we examined the relative abundances of these taxa. At the phylum level, *Firmicutes* was significantly depleted and *Bacteroidota* was enriched in AML patients. At the genera level, approximately 5 bacterial genera displaying significantly different abundances between AML patients and healthy controls were observed. *Bacteroides* ($P=0.04332$) was enriched, while *Faecalibacterium* ($P=0.0001$), *Roseburia* ($P=0.000017$), *Subdoligranulum* ($P=0.002$) and *Bifidobacterium* ($P=0.0436$) were significantly depleted in AML patients (Figure 1C and Supplementary figure 1b). Moreover, among these differential floras, we observed a significantly positive correlation between the significantly different bacterial taxa, *Faecalibacterium* and *Roseburia* ($P=0.0009$) (Figure 1D). The result of linear discriminant analysis effect size (LEfSe) further confirmed that both *Faecalibacterium* (LDA=4.538) and *Roseburia* (LDA=4.107) were depleted in the feces of AML patients (Figure 1E). To further determine the relationship between bacteria and disease, we analyzed the correlation between the abundances of *Faecalibacterium* and *Roseburia* and the clinical characteristics of the AML patients. The association and correlation between *Faecalibacterium* and WBC were performed by Spearman correlation. Our results demonstrated that *Faecalibacterium* and *Roseburia* are significantly negatively correlated with the level of peripheral white

blood cells, while *Faecalibacterium* also negatively correlated with the percentage of bone marrow blast cells (Table 1). Importantly, the abundance of *Faecalibacterium* and number of OTUs were much higher in the favorable-risk of AML patients (Figure 1F) (Table 2). These results suggest that the disordered diversity of gut microbiota and the reduced abundance of *Faecalibacterium* may play an important role in the progression of AML.

2. AML causes bacterial dysbiosis and gut microbiota dysbiosis aggravate the progress of AML

To clarify the role of the microflora in AML, we established AML mice and performed 16sRNA sequencing on mouse stool. Our results showed that the gut microbiota diversity of AML mice decreased significantly with the progression of the disease compared with healthy control mice (Figure 2A). To further verify whether the disordered gut microbes can affect the progression of the disease, we first successfully ablated the gut microbiota in mice after administration of antibiotics (Supplementary figure 2a), and then established AML mice and determined the AML progression in mice (Figure 2B). After sacrificing the mice, our FACS results showed that the percentage of GFP⁺ MLL-AF9 leukemia cells in the bone marrow, spleen or peripheral blood was much higher in antibiotic-treated AML mice than in control PBS AML mice (Figure 2C and Supplementary figure 2b). Furthermore, we used hematoxylin and eosin (HE) staining and Ki67 immunohistochemical staining methods to evaluate the infiltration and proliferation of leukemia cells in the spleen. As expected, leukemia cell infiltration and proliferation were aggravated in antibiotic-treated AML mice (Figure 2D). Importantly, antibiotic-treated AML mice had a significantly shorter lifespan than PBS treated control AML mice [median 15 (range 12-17) days vs median 19 (range 18-21) days; $P=0.0328$] (Figure 2E). These data indicate that AML causes a dysbacteriosis

and destroying the diversity of the gut microbiota accelerates AML progression.

3. FMT delays the development of the AML

As shown above, *Faecalibacterium* correlated with a favorable prognosis and may be considered as a beneficial bacteria in AML, we studied the function of *Faecalibacterium* through bacterial transplantation. The results showed that *Faecalibacterium* gavage obviously relieved the splenomegaly (Supplementary figure 3a-b). In terms of tumor load, we observed a significant reduction in the bone marrow and a reduced tendency in the spleen and peripheral blood (Supplementary figure 3c). The over survival of the AML mice treated by *Faecalibacterium* gavage was not significantly different from that of control AML mice (Supplementary figure 3d). As a single bacterial taxon plays a limited role in AML, FMT was performed using a mouse model of AML to clarify the causal relationship between gut microbiota imbalance and AML progression. The experimental procedure is shown in Figure 3A. The PCoA showed that the sample points representing the gut microbiota from the donor individuals and the corresponding recipient mice almost completely overlapped, indicating that the bacterial colonization in recipient mice was successful after FMT (Supplementary figure 4a). Our results demonstrated that the leukemia load in the spleen, bone marrow and peripheral blood of AML-FMT mice (AML mice treated with AML patient's fecal hydration liquid) increased significantly compared with that in the spleen, bone marrow and peripheral blood of Con-FMT mice (AML mice treated with healthy control's fecal hydration liquid), as shown by spleen weight, HE sections, Ki67 staining (Figure 3B and 3D) and the percentage of GFP⁺ leukemia cells (Figure 3C, Supplementary figure 4b). To observe the dynamic changes in spleen leukemia burden, a dual-luciferase assay demonstrated that the dual-luciferase activity in the AML-FMT group was significantly higher than that in the Con-FMT group, indicating that AML-

FMT treatment aggravated AML progression in vivo (Figure 3E). Moreover, AML-FMT mice had a significantly shorter lifespan than Con-FMT mice [median 17 (range 16-18) days vs median 21 (range 19-22) days ($P=0.0358$)] (Figure 3F). This result shows that the transplantation of an intact gut microbiota plays a greater role in the progression of AML than the application of single bacteria.

4. AML patients exhibit profound alterations in gut microbial metabolites

Next, we studied the specific mechanism of the involvement of the gut microbiota in AML progression. As gut metabolites are important bridges for the gut microbiota to regulate disease progression, untargeted metabolomics analysis based on ultra-performance liquid chromatography was first performed to explore the alteration of intestinal metabolic profiles in AML patients. We collected paired stool, blood and bone marrow samples from each patient for the ultra-performance liquid chromatography analysis. A total of 540 and 578 peak features were identified, in positive ion mode (ES⁺) and negative ion mode (ES⁻), respectively. These peak features were clustered by orthogonal partial least squares discriminant analysis (OPLS-DA), showing that the samples of the AML and control groups were clearly separated from each other (Figure 4A). We further analyzed the peak features using MS/MS, and identified the top 25 abundant metabolites using the combination of precise molecular weight and structural information from the compound structure database. Of these, propionic acid and butyric acid are significantly different in the stool samples between the healthy and the AML groups (Figure 4B). Next, targeted SCFA metabolomics analysis was applied to quantitatively determine intestinal SCFAs by GC-MS, and the results showed that propionic acid ($P=0.0196$) and butyric acid ($P=0.0065$) levels were significantly decreased in the stools of AML patients compared with those in the stools of healthy controls (Figure 4C). Importantly, correlation analyses of the gut microbiota and SCFA

levels revealed that *Faecalibacterium* ($P=0.0279$) was positively correlated with butyric acid (Figure 4D). These data indicate that *Faecalibacterium* was most significantly downregulated in the gut microbiota of AML patients, which has a close relationship with the metabolism of butyric acid in these hosts. A large number of studies have confirmed that butyric acid is a direct metabolite of *Faecalibacterium*, so we believe that *Faecalibacterium* may directly affect the content of butyric acid in stool. In addition, we found no significant difference of propionic acid or butyric acid concentration in peripheral blood or bone marrow of AML patients compared with controls, which indicated that they may not affect AML cells directly in the blood or bone marrow (Supplementary figure 5a, 5b). To identify the key point that could build a bridge between intestinal bacteria and blood, we predicted the functional changes of intestinal bacteria in AML with PICRUSt. The results showed that AML patients and healthy controls had significant differences in intestinal bacterial functions (Figure 4E), in which the obvious difference was the predicted relative abundance of butanoate metabolism (Figure 4F). The above evidence suggests that butyrate may play an important role in the progression of AML.

5. Microbiota-derived butyrate gavage delays AML progression

Next, to further explore the effect of butyrate on AML progression in vivo, we administered butyrate to mice via intragastric gavage every 2 days starting 7 days prior to injection of AML cells and continued administration of butyrate for another 14 days (Figure 5A). Then, we determined the effect of butyrate on leukemia load and survival time in AML mice. We found that butyrate-treated AML mice presented with more alleviated splenomegaly than control AML mice (Figure 5B). Moreover, the results showed that the percentage of GFP⁺ leukemia cells in the bone marrow, peripheral blood or spleen of butyrate-treated AML mice was lower than that in the bone marrow,

peripheral blood or spleen of the control AML mice (Figure 5C, Supplementary figure 5c). Under the microscope, leukemia cells infiltration and proliferation in the spleen were mitigated in butyrate-treated AML mice. In addition, the normal spleen anatomy structure such as the white pulp was better preserved in the butyrate-treated AML mice (Figure 5D). To observe the dynamic changes in spleen leukemia burden, a dual-luciferase assay demonstrated that the dual-luciferase activity in the butyrate administration group was significantly lower than that in the control group, indicating that butyrate administration alleviated AML progression in vivo (Figure 5E). Meanwhile, butyrate administration in AML mice led to a significantly longer lifespan than that of control AML mice [median 16 (range 15-17) days vs median 20 (range 19-23) days ($P=0.0479$)] (Figure 5F). Moreover, consistent with the results in AML patients, the blood concentration of butyrate showed no obvious change in mice that received butyrate administration (Supplementary figure 5d), which suggests that butyrate delays AML progression not by direct contact to kill leukemia cells but occur via other mechanisms.

6. Butyrate reverses intestinal barrier damage in mice with AML

After butyrate is produced in the intestinal tract, it is mainly absorbed by intestinal epithelial cells as an energy source, which promotes intestinal barrier integrity. Therefore, we determined the effects of butyrate on intestinal integrity, which has not been studied in AML. Our results showed that the colon length of AML mice was significantly shorter than that of the control group (Supplementary figure 5e) and intestinal permeability to FITC-dextran was significantly increased in AML mice compared with that in control mice, suggesting leukemia induced intestinal damage. However, butyrate-treated AML mice showed significantly ameliorated intestinal damage as compared to the control AML mice and only mildly increased FITC-dextran

permeability as compared to normal mice (Figure 6A). Next, we examined the intestinal cell-cell junction integrity. We utilized transmission electron microscopy (TEM) to examine the ability of butyrate to preserve cellular junctions in AML mice. As we expected, significantly larger gap between intestinal epithelial cells was found in AML mice (Fig. 6D, middle panel). In contrast, the integrity of the intestinal epithelial cell's junction was preserved at both normal (Figure 6D, left panel) and butyrate-treated mice (Figure 6D, right panel). Moreover, we evaluated the expression of ZO-1, claudin-1 and claudin-2, which are important intestinal tight junction proteins (TJPs). The results demonstrated that the expression of the cell-cell junction protecting protein, ZO-1 and claudin-1, was downregulated, while the expression of the cell-cell junction-inhibiting protein claudin-2 was upregulated in AML mice at both the protein and mRNA levels, and these changes were reversed in butyrate-treated AML mice (Figure 6B, C). Next, we assessed the in-situ protein expression of claudin-1, claudin-2 and ZO-1 by immunofluorescence assays to evaluate the changes in intestinal epithelial tight junctions. The results showed that the expression levels of claudin-1 and ZO-1 were decreased in the intestine of AML mice compared with those in the intestine of normal or butyrate-treated AML mice. In contrast, Claudin-2 was enriched in the intestine of AML mice (Figure 6E). So far, we have not been aware of any report on intestinal barrier damage in AML, and our observation of the disrupted intestinal barrier in AML mice is a breakthrough. Moreover, butyrate may participate in AML progression by affecting the intestinal barrier function.

7. The damage of the intestinal barrier accelerates the bacterial-derived LPS leakage into blood and LPS exacerbates the progression of AML

Intestinal barrier is the key to protect the body from the harmful effects of gut microbiota, and the impairment of its function may lead to the displacement of intestinal

harmful substances to the circulatory system. LPS is the main harmful product of gut microbiota and the intestinal barrier is the only way for LPS to enter the blood. We used ELISA to detect the LPS concentration in the plasma of peripheral blood, and the results showed that the LPS concentration in the plasma of the AML group was significantly higher than that in the plasma of the control group in humans (Figure 7A). Moreover, we also determined the LPS concentration in the plasma of butyrate-treated AML mice and FMT-treated AML mice, and the results showed that butyrate-treatment and Con-FMT treatment significantly reduced the LPS concentration in mouse plasma (Figure 7B-C). In addition, to prove that the increase in LPS concentration was due to decrease in barrier function, LPS gavage was administered to mice. The results showed that the concentration of LPS in the peripheral blood of AML mice was significantly higher than that in the peripheral blood of normal mice at the same dose of LPS gavage (Figure 7D). Therefore, these results proved that the concentration of bacterial-derived LPS was negatively correlated with intestinal barrier function, that damaged intestinal barrier result in enhanced bacteria-derived LPS leakage into blood, and that butyrate-treatment and Con-FMT treatment reversed this effect.

To elucidate the biological role of LPS in leukemia cells in vitro, CCK8 was used to determine cell proliferation, and flow cytometry analysis via the Annexin V-FITC/PI staining method was performed to analyze apoptosis. Our results demonstrated that LPS obviously decreased cell apoptosis (Figure 7E) and promoted the proliferation of MLL-AF9 cells (Figure 7F). We further determined the related proteins by Western blot, and found that LPS stimulation significantly upregulated the protein expression of Bcl-2 and downregulated BAX and cleaved-caspase3 proteins (Figure 7G). To explore the possible promoting effects of LPS in vivo, LPS administration was initiated via vein injection into mice (Supplementary figure 5f). Our results showed that the LPS-treated

AML mice presented with more severe splenomegaly than the control AML mice, and the results showed that the percentage of GFP⁺ leukemia cells in the peripheral blood, spleen and bone marrow of LPS-treated AML mice was higher than that in the peripheral blood, spleen and bone marrow of control AML mice (Figure 7H and 7I). Through microscopy, leukemia cell infiltration in the spleen was observed to show an aggravated tendency in LPS-treated AML mice compared with that in control AML mice (Figure 7J). Similarly, LPS-treated AML mice had a significantly shorter lifespan than control AML mice [median 14 (range 13-16) days vs median 18 (range 16-20) days (P=0.0408)] (Figure 7K). This finding suggests that modulation of LPS content could be of great value for AML treatment.

Discussion

The gut microbiota and its metabolism have been reported to be associated with many cancers²³⁻²⁵. It was reported that an imbalance in gut microbial diversity can accelerate the development of ovarian cancer²⁶, while a diversified gut microbiota is beneficial to the prognosis and chemotherapy sensitivity of colon cancer²⁷. As the most important hematological tumor, increasing attention has been given to the relationship between leukemia and the gut microbiota. Although some progress was made recently regarding the gut microbiota in several leukemia types^{28,29}, it remains unclear whether gut microbes are mechanistically involved in AML progression. In this study, we found that AML could induce intestinal barrier damage, which is associated with altered gut microbiota and SCFAs in patients. The injured intestinal barrier and increased permeability in AML further promotes the release of LPS into the blood circulation, which accelerates AML cell proliferation and disease progression. We believe that the discovery of this mechanism may help discover new targets for AML treatment.

We found that there are significant alterations in gut microbiota diversity in AML

and that this change is related to the risk stratification of the disease. This indicated that while AML causes the imbalance of gut microbiota, the disordered microbiota also affects the progress of the disease. To better serve clinical treatment, it is particularly important to study the mechanism of the flora in the progression of AML. Blocking the positive feedback between AML and bacterial dysbiosis in mechanism will help to solve the unexplained infection in patients with AML and improve the clinical treatment effect. Regarding the species of bacteria, the abundance of *Faecalibacterium* decreased significantly in treatment-naïve AML patients. *Faecalibacterium* is the best-characterized microbiota constituents that are involved in the immune microenvironment^{30,31}. According to reports, the role of *Faecalibacterium* in tumors is gradually becoming appreciated. *Faecalibacterium* also suppressed the proliferation and promoted the apoptosis of breast cancer cells, although these effects disappeared after adding recombinant human IL-6³². However, in our study, after administration of *Faecalibacterium*, AML mice showed no significant difference in survival and leukemia infiltration, which indicates that *Faecalibacterium* anti-tumor effect is limited. Therefore, we further performed FMT in AML mice and found that AML-FMT aggravated the progression of AML compared with that in Con-FMT mice.

A large number of reports have shown that most SCFAs, including butyrate, in the human body are derived from the metabolism of intestinal bacteria^{33,34}, and it is well known that butyrate is a common metabolite of *Faecalibacterium* and *Roseburia*³⁵. In our experiment, AML patients had significantly lower fecal butyric acid content than healthy people, but no significant difference was found in the peripheral blood and bone marrow. Consistent with our results, other research reported that butyric acid is mainly metabolized in the intestinal tract, and the change in intestinal concentration cannot cause corresponding changes in peripheral blood³⁶. Therefore, the effect of intestinal

butyric acid on AML did not occur via direct contact to kill leukemia cells in the blood or bone marrow. On the other hand, butyric acid, as the main energy source of intestinal epithelial cells, plays an important role in the regulation and function of the intestinal barrier³⁷⁻³⁹, and its deficiency could lead to disruption of the gut barrier and cause multiple diseases^{40,41}. Zheng et al. showed that butyrate has received particular attention for its beneficial effects on intestinal homeostasis and energy metabolism. With anti-inflammatory properties, butyrate enhances intestinal barrier functions and mucosal immunity⁴². Butyrate plays key roles not only in the local intestinal barrier, but also at the systemic level. At both sites, the mode of action is through modulating signaling pathways involving nuclear NF- κ B and inhibition of histone deacetylase⁴³. Although it was reported that chemotherapy drugs can affect the intestinal barrier function in many cancers, including AML⁴⁴, intestinal barrier function alteration in treatment-naïve AML patients and its role and mechanism in AML progression have not been reported. Our results showed that the intestinal barrier function of AML mice was decreased and that the expression of TJPs (claudin-1, ZO-1) in intestinal epithelial cells was decreased and of claudin-2 was increased in AML mice. Furthermore, administration of butyrate to AML mice effectively reversed the intestinal barrier damage. Here, our research confirmed that butyrate plays a role in protecting the intestinal barrier in the progression of AML. This finding suggests that supplementation with butyrate may be of great significance in AML treatment or post chemotherapy care. Interestingly, in addition to our research, G protein-coupled receptors (GPCRs) for butyrate and their role in modulating the immune system have been reported, which is worthy of our further study.

As the main component of intestinal bacteria, LPS affects the immune microenvironment in the human body⁴⁵. The intestinal barrier is the main way through which LPS can enter the blood circulation. In this study, we found that intestinal barrier

605 damage in primary AML patients caused more LPS to enter the blood and higher LPS
606 concentrations. In vitro experiments showed that LPS increased leukemia cell
607 proliferation, and in vivo studies revealed that LPS exacerbated the leukemia burden
608 and shortened OS in AML mice. We also clarified that its leukemia-promoting effects
609 may function through decreased BAX and cleaved caspase-3 and upregulated bcl-2
610 levels. These results suggest that gut metabolites are another independent contributing
611 source of elevated serum LPS levels and that the absence of butyrate and elevated LPS
612 play important roles in AML progression.

613 Importantly, while our findings relate to treatment-naïve AML patients, they cannot
614 yet be generalized as an approach for patients with relapse and refractory disease.
615 Future studies are needed to elucidate how the gut microbiota and other SCFAs affect
616 recurrent or refractory leukemia. In addition, our research is currently limited to
617 horizontal clinical studies, and more clinical samples are needed for longitudinal
618 clinical studies. Therefore, we will gradually solve these problems and conduct deep
619 insight to the relationship between AML and gut microbiota.

620 In conclusion, our results suggest that the imbalance in the gut microbiota as a
621 cancer-promoting factor in AML and butyrate regulates the concentration of LPS in
622 peripheral blood by affecting the intestinal barrier (Figure 8). Regulating gut microbiota
623 metabolites especially targeting butyrate may provide a novel approach for AML
624 therapy.

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629 **Authorship Contributions**

Professor Daoxin Ma designed and funded the research. Professor Chunyan Ji helped designing the research. Ruiqing Wang performed the research and wrote the manuscript. Yinyu Yang and Jinting Liu assisted the research. Fang Zhong and Chen Zhang analyzed the data. Dr Tao Sun and Yuhong Chen edited the paper.

Data Availability Statement

Some or all data, models, or code generated or used during the study are available from the corresponding author by request.

Disclosures of Conflicts of Interest

The authors declare no competing financial interests.

References

- 1 Kayser, S. & Levis, M. J. Advances in targeted therapy for acute myeloid leukaemia. *Br J Haematol* **180**, 484-500, doi:10.1111/bjh.15032 (2018).
- 2 Song, X. *et al.* Incidence, Survival, and Risk Factors for Adults with Acute Myeloid Leukemia Not Otherwise Specified and Acute Myeloid Leukemia with Recurrent Genetic Abnormalities: Analysis of the Surveillance, Epidemiology, and End Results (SEER) Database, 2001-2013. *Acta Haematol* **139**, 115-127, doi:10.1159/000486228 (2018).
- 3 Cekanaviciute, E. *et al.* Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci U S A* **114**, 10713-10718, doi:10.1073/pnas.1711235114 (2017).
- 4 Zhang, X. *et al.* The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* **21**, 895-905, doi:10.1038/nm.3914 (2015).
- 5 Marino, E. *et al.* Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. *Nat Immunol* **18**, 552-562, doi:10.1038/ni.3713 (2017).
- 6 Meng, C., Bai, C., Brown, T. D., Hood, L. E. & Tian, Q. Human Gut Microbiota and Gastrointestinal Cancer. *Genomics Proteomics Bioinformatics* **16**, 33-49, doi:10.1016/j.gpb.2017.06.002 (2018).
- 7 Theilgaard-Monch, K. Gut microbiota sustains hematopoiesis. *Blood* **129**, 662-663, doi:10.1182/blood-2016-12-754481 (2017).
- 8 Bai, L., Zhou, P., Li, D. & Ju, X. Changes in the gastrointestinal microbiota of children with acute lymphoblastic leukaemia and its association with antibiotics in the short term. *J Med Microbiol* **66**, 1297-1307, doi:10.1099/jmm.0.000568 (2017).
- 9 Jenq, R. R. *et al.* Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *J Exp Med* **209**, 903-911, doi:10.1084/jem.20112408 (2012).
- 10 Payen, M. *et al.* Functional and phylogenetic alterations in gut microbiome are linked to graft-versus-host disease severity. *Blood Adv* **4**, 1824-1832, doi:10.1182/bloodadvances.2020001531 (2020).
- 11 Ganapathy, V., Thangaraju, M., Prasad, P. D., Martin, P. M. & Singh, N. Transporters and

receptors for short-chain fatty acids as the molecular link between colonic bacteria and the host. *Curr Opin Pharmacol* **13**, 869-874, doi:10.1016/j.coph.2013.08.006 (2013).

12 Sealy, L. & Chalkley, R. The effect of sodium butyrate on histone modification. *Cell* **14**, 115-121, doi:10.1016/0092-8674(78)90306-9 (1978).

13 Cook, S. I. & Sellin, J. H. Review article: short chain fatty acids in health and disease. *Aliment Pharmacol Ther* **12**, 499-507, doi:10.1046/j.1365-2036.1998.00337.x (1998).

14 Reddy, P. *et al.* Histone deacetylase inhibitor suberoylanilide hydroxamic acid reduces acute graft-versus-host disease and preserves graft-versus-leukemia effect. *Proc Natl Acad Sci U S A* **101**, 3921-3926, doi:10.1073/pnas.0400380101 (2004).

15 Reddy, P. *et al.* Histone deacetylase inhibition modulates indoleamine 2,3-dioxygenase-dependent DC functions and regulates experimental graft-versus-host disease in mice. *J Clin Invest* **118**, 2562-2573, doi:10.1172/JCI34712 (2008).

16 Choi, S. W. *et al.* Vorinostat plus tacrolimus and mycophenolate to prevent graft-versus-host disease after related-donor reduced-intensity conditioning allogeneic haemopoietic stem-cell transplantation: a phase 1/2 trial. *Lancet Oncol* **15**, 87-95, doi:10.1016/S1470-2045(13)70512-6 (2014).

17 Swanson, G. R. *et al.* Disrupted diurnal oscillation of gut-derived Short chain fatty acids in shift workers drinking alcohol: Possible mechanism for loss of resiliency of intestinal barrier in disrupted circadian host. *Transl Res* **221**, 97-109, doi:10.1016/j.trsl.2020.04.004 (2020).

18 Feng, Y., Wang, Y., Wang, P., Huang, Y. & Wang, F. Short-Chain Fatty Acids Manifest Stimulative and Protective Effects on Intestinal Barrier Function Through the Inhibition of NLRP3 Inflammasome and Autophagy. *Cell Physiol Biochem* **49**, 190-205, doi:10.1159/000492853 (2018).

19 Felizardo, R. J. F., Watanabe, I. K. M., Dardi, P., Rossoni, L. V. & Camara, N. O. S. The interplay among gut microbiota, hypertension and kidney diseases: The role of short-chain fatty acids. *Pharmacol Res* **141**, 366-377, doi:10.1016/j.phrs.2019.01.019 (2019).

20 Cui, Y., Wang, Q., Chang, R., Zhou, X. & Xu, C. Intestinal Barrier Function-Non-alcoholic Fatty Liver Disease Interactions and Possible Role of Gut Microbiota. *J Agric Food Chem* **67**, 2754-2762, doi:10.1021/acs.jafc.9b00080 (2019).

21 Mathewson, N. D. *et al.* Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat Immunol* **17**, 505-513, doi:10.1038/ni.3400 (2016).

22 Keir, M., Yi, Y., Lu, T. & Ghilardi, N. The role of IL-22 in intestinal health and disease. *J Exp Med* **217**, e20192195, doi:10.1084/jem.20192195 (2020).

23 Hagland, H. R. & Soreide, K. Cellular metabolism in colorectal carcinogenesis: Influence of lifestyle, gut microbiome and metabolic pathways. *Cancer Lett* **356**, 273-280, doi:10.1016/j.canlet.2014.02.026 (2015).

24 Visconti, A. *et al.* Interplay between the human gut microbiome and host metabolism. *Nat Commun* **10**, 4505, doi:10.1038/s41467-019-12476-z (2019).

25 Zhang, Z., Tang, H., Chen, P., Xie, H. & Tao, Y. Demystifying the manipulation of host immunity, metabolism, and extraintestinal tumors by the gut microbiome. *Signal Transduct Target Ther* **4**, 41, doi:10.1038/s41392-019-0074-5 (2019).

26 Qi, X. *et al.* Gut microbiota-bile acid-interleukin-22 axis orchestrates polycystic ovary syndrome. *Nat Med* **25**, 1225-1233, doi:10.1038/s41591-019-0509-0 (2019).

711 27 Yang, Y. *et al.* Integrated microbiome and metabolome analysis reveals a novel interplay
712 between commensal bacteria and metabolites in colorectal cancer. *Theranostics* **9**, 4101-4114,
713 doi:10.7150/thno.35186 (2019).

714 28 Galloway-Pena, J. R. *et al.* Gut Microbiome Signatures Are Predictive of Infectious Risk
715 Following Induction Therapy for Acute Myeloid Leukemia. *Clin Infect Dis* **71**, 63-71,
716 doi:10.1093/cid/ciz777 (2020).

717 29 Hakim, H. *et al.* Gut Microbiome Composition Predicts Infection Risk During Chemotherapy in
718 Children With Acute Lymphoblastic Leukemia. *Clin Infect Dis* **67**, 541-548,
719 doi:10.1093/cid/ciy153 (2018).

720 30 Tanaka, S., Yamamoto, K., Yamada, K., Furuya, K. & Uyeno, Y. Relationship of Enhanced Butyrate
721 Production by Colonic Butyrate-Producing Bacteria to Immunomodulatory Effects in Normal
722 Mice Fed an Insoluble Fraction of Brassica rapa L. *Appl Environ Microbiol* **82**, 2693-2699,
723 doi:10.1128/AEM.03343-15 (2016).

724 31 Patterson, A. M. *et al.* Human Gut Symbiont *Roseburia hominis* Promotes and Regulates Innate
725 Immunity. *Front Immunol* **8**, 1166, doi:10.3389/fimmu.2017.01166 (2017).

726 32 Ma, J. *et al.* Alter between gut bacteria and blood metabolites and the anti-tumor effects of
727 *Faecalibacterium prausnitzii* in breast cancer. *BMC Microbiol* **20**, 82, doi:10.1186/s12866-020-
728 01739-1 (2020).

729 33 Song, H., Yoo, Y., Hwang, J., Na, Y. C. & Kim, H. S. *Faecalibacterium prausnitzii* subspecies-level
730 dysbiosis in the human gut microbiome underlying atopic dermatitis. *J Allergy Clin Immunol*
731 **137**, 852-860, doi:10.1016/j.jaci.2015.08.021 (2016).

732 34 Liu, H. *et al.* Butyrate: A Double-Edged Sword for Health? *Adv Nutr* **9**, 21-29,
733 doi:10.1093/advances/nmx009 (2018).

734 35 Machiels, K. *et al.* A decrease of the butyrate-producing species *Roseburia hominis* and
735 *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* **63**, 1275-
736 1283, doi:10.1136/gutjnl-2013-304833 (2014).

737 36 Bach Knudsen, K. E., Serena, A., Canibe, N. & Juntunen, K. S. New insight into butyrate
738 metabolism. *Proc Nutr Soc* **62**, 81-86, doi:10.1079/PNS2002212 (2003).

739 37 Haber, A. L. *et al.* A single-cell survey of the small intestinal epithelium. *Nature* **551**, 333-339,
740 doi:10.1038/nature24489 (2017).

741 38 Wells, J. M. *et al.* Homeostasis of the gut barrier and potential biomarkers. *Am J Physiol*
742 *Gastrointest Liver Physiol* **312**, G171-G193, doi:10.1152/ajpgi.00048.2015 (2017).

743 39 Cani, P. D. Interactions between gut microbes and host cells control gut barrier and metabolism.
744 *Int J Obes Suppl* **6**, S28-S31, doi:10.1038/ijosup.2016.6 (2016).

745 40 Berer, K. *et al.* Commensal microbiota and myelin autoantigen cooperate to trigger
746 autoimmune demyelination. *Nature* **479**, 538-541, doi:10.1038/nature10554 (2011).

747 41 Citi, S. Intestinal barriers protect against disease. *Science* **359**, 1097-1098,
748 doi:10.1126/science.aat0835 (2018).

749 42 Zheng, L. *et al.* Microbial-Derived Butyrate Promotes Epithelial Barrier Function through IL-10
750 Receptor-Dependent Repression of Claudin-2. *J Immunol* **199**, 2976-2984,
751 doi:10.4049/jimmunol.1700105 (2017).

752 43 Bach Knudsen, K. E. *et al.* Impact of Diet-Modulated Butyrate Production on Intestinal Barrier
753 Function and Inflammation. *Nutrients* **10**, doi:10.3390/nu10101499 (2018).

754 44 Hueso, T. *et al.* Impact and consequences of intensive chemotherapy on intestinal barrier and

microbiota in acute myeloid leukemia: the role of mucosal strengthening. *Gut Microbes* **12**, 1800897, doi:10.1080/19490976.2020.1800897 (2020).

45 Takizawa, H. *et al.* Pathogen-Induced TLR4-TRIF Innate Immune Signaling in Hematopoietic Stem Cells Promotes Proliferation but Reduces Competitive Fitness. *Cell Stem Cell* **21**, 225-240 e225, doi:10.1016/j.stem.2017.06.013 (2017).

Figure 1. The diversity and composition of the gut microbiota are significantly altered in AML patients

Total bacterial DNA was isolated from the intestinal content and 16S rRNA genes were sequenced. (A) The diversity and richness of the gut microbiota in AML patients (AML) and healthy controls (Con). Unpaired t-test were used for comparing the Shannon index (n=61). (B) PCoA of a weighted UniFrac distance analysis(n=61). (C) Relative taxa abundance comparison among the AML and control groups(n=61). (D) Spearman correlations between the 10 genera in AML patients and healthy controls intestinal content. *Faecalibacterium* and *Roseburia* were observed significant correlation (Blue positive correlation, red negative correlation). (E) Cladogram generated from linear discriminant analysis effect size (LEfSe) and the LDA score. (F) The abundance of *Faecalibacterium* and OTUs reduced in unfavorable-risk group (n=22) compared to favorable-risk group(n=9).

Figure 2. AML causes bacterial dysbiosis and gut microbiota dysbiosis aggravate the progress of AML.

(A) The diversity and richness of the gut microbiota in control mice (without cell injection), control-14days mice (without cell injection after 14 days), AML mice (AML cell injection) and AML-14days (mice after AML cell injection after 14days). Unpaired t-test were used for comparing the Sob index. (B) The schematic diagram of the mice experimental process. (C) The leukemia cells (GFP⁺ cells) in spleen, peripheral blood and bone marrow from ABX AML mice (n=4) and control PBS AML mice (n=4). (D)

Hematoxylin and eosin-stained histopathology sections and Ki67 immunohistochemical staining of a representative spleen, ABX AML group and PBS AML group. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (E) Kaplan-Meier survival curve of AML mice (n=5 per group).

Figure 3. FMT delays the development of the AML.

(A) The schematic diagram of the mouse AML FMT process. (B) The photographs and weights of spleens from AML-FMT mice (n=4) and Con-FMT mice (n=4). (C) The leukemia cells (GFP⁺ cells) in spleen, peripheral blood and bone marrow from AML-FMT mice (n=4) and Con-FMT mice (n=4). (D) HE histopathology sections and Ki67 immunohistochemical staining of a representative spleen, AML-FMT group and Con-FMT group. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (E) On the 8th and 14th days after the injection, the load of Luc-expressing MLL-AF9 cells in mice was analyzed by IVIS (n=3 per group). (F) Kaplan-Meier survival curve of AML mice (n=4 per group).

Figure 4. AML patient exhibits profound alterations in gut microbial metabolites

(A) The plot of OPLS-DA score of all peak features from the untargeted metabolomics analysis of stool samples from AML patients (n=15) and healthy controls (n=17). (B) Heatmap of the relative abundances of the top 26 most abundant metabolites which significantly changed in AML group. The color bar indicates z score, which represents the relative abundance. Z score < 0 (> 0) meant the relative abundance was lower (higher) than the mean. (C) The concentrations of propionic acid, butyric acid, and acetic acid in fecal samples of AML patients (n=22) and controls (n=22) were determined by GC-MS. (D) Heatmap of spearman correlation analysis between the gut microbiota and the

metabolite. (E) The functional abundance distribution histogram of samples from AML patients and healthy controls in the COG database using PICRUST software (the top 35 is selected by the maximum sorting method). (F) The significant differences in metagenomics functions in AML patients compared to that in healthy controls (corrected $P < 0.05$ and confidence intervals = 95%).

Figure 5. Microbiota-derived butyrate gavage delays AML progression

(A) The schematic diagram of the mice experimental process including butyrate gavage. (B) The photographs of spleens from butyrate-treated AML mice (n=5) and control AML mice (n=5). (C) The leukemia cells (GFP⁺ cells) in bone marrow, peripheral blood and spleen from butyrate-treated AML mice (n=5) and control AML mice (n=5). (D) HE histopathology sections and Ki67 immunohistochemical staining of a representative spleen in butyrate-treated mice and control AML mice. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (E) On day 8 and day 14 after injection of MLL-AF9 cells, the load of Luc-expressing MLL-AF9 cells in mice was analyzed by IVIS. (F) Kaplan-Meier survival curve of AML mice (n=5 per group).

Figure 6. Butyrate reverses intestinal barrier damage in mice with AML

(A) The concentration of FITC-dextran in the peripheral blood after FITC-dextran gavage for 6 hours. Data represent the mean $\pm$ SEM (n = 5). (B) The mRNA expression levels of tight junction protein components claudin-1, claudin-2 and ZO-1 in intestinal epithelial cells of AML, control and butyrate-treatment mice (n=3). (C) The protein levels of claudin-1, claudin-2 and ZO-1 in intestinal epithelial cells were determined by Western blot. GAPDH was used as the control (n=3). (D). Transmission electron microscopy (TEM) of intestines, isolated from normal, AML, and butyrate-treated

AML mice for duration of experiment; arrows indicate cell-cell interface. (E) Immunofluorescence analysis of small intestine tissue from normal, AML and butyrate-treated mic. Cells were fixed and stained with a rabbit polyclonal antibody. APC (red) goat anti-rabbit IgG was used as a secondary antibody. Immunofluorescence indicated the expression quantity and localization of claudin-1, claudin-2 and ZO-1.

Figure 7. The damage of the intestinal barrier accelerates the bacterial-derived LPS leakage into blood and LPS exacerbates the progression of AML.

(A) The LPS concentrations in peripheral plasma of AML patients and healthy controls. (B) The LPS concentrations in butyrate-treated AML mice and control AML mice. (C) The LPS concentrations in AML-FMT mice and Con-FMT mice. (D) The LPS concentrations in AML mice and normal mice after LPS gavage. (E) The representative FACS graphs of apoptotic cells of MLL-AF9 cells were shown after being cultured with or without LPS for 48 hours (n=3). (F) CCK8 analysis for the proliferation of MLL-AF9 cells with or without LPS 24, 48, 72 hours (n=3). (G) The Western Blot results of Bcl-2, BAX, cleaved caspase-3, and GAPDH was used as a control (n=3). (H) The leukemia cells (GFP⁺ cells) in the spleen, peripheral blood and bone marrow from LPS-treated AML mice (n=4) and control AML mice (n=4). (I) Representative photographs of spleen from LPS-treated AML mice (n=4) and control AML mice (n=4). (J) HE histopathology sections and Ki67 immunohistochemical staining of a representative spleen, LPS-treated AML mice and control AML mice. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (K) Kaplan-Meier survival curve of mice leukemia model (n=4 per group).

Figure 8. Visual abstract graph

Figures

Figure 1

The diversity and composition of the gut microbiota are significantly altered in AML patients. Total bacterial DNA was isolated from the intestinal content and 16S rRNA genes were sequenced. (A) The diversity and richness of the gut microbiota in AML patients (AML) and healthy controls (Con). Unpaired

t-test were used for comparing the Shannon index (n=61). (B) PCoA of a weighted UniFrac distance analysis(n=61). (C) Relative taxa abundance comparison among the AML and control groups(n=61). (D) Spearman correlations between the 10 genera in AML patients and healthy controls intestinal content. Faecalibacterium and Roseburia were observed significant correlation (Blue positive correlation, red negative correlation). (E) Cladogram generated from linear discriminant analysis effect size (LEfSe) and the LDA score. (F) The abundance of Faecalibacterium and OTUs reduced in unfavorable-risk group (n=22) compared to favorable-risk group(n=9).

Figure 2

AML causes bacterial dysbiosis and gut microbiota dysbiosis aggravate the progress of AML. (A) The diversity and richness of the gut microbiota in control mice (without cell injection), control-14 days mice (without cell injection after 14 days), AML mice (AML cell injection) and AML-14days (mice after AML cell injection after 14days). Unpaired t-test were used for comparing the Sob index. (B) The schematic diagram of the mice experimental process. (C) The leukemia cells (GFP+ cells) in spleen, peripheral blood and bone marrow from ABX AML mice (n=4) and control PBS AML mice (n=4). (D) Hematoxylin and eosin–stained histopathology sections and Ki67 immunohistochemical staining of a representative spleen, ABX AML group and PBS AML group. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (E) Kaplan-Meier survival curve of AML mice (n=5 per group).

Figure 3

FMT delays the development of the AML. (A) The schematic diagram of the mouse AML FMT process. (B) The photographs and weights of spleens from AML-FMT mice (n=4) and Con-FMT mice (n=4). (C) The leukemia cells (GFP+ cells) in spleen, peripheral blood and bone marrow from AML-FMT mice (n=4) and Con-FMT mice (n=4). (D) HE histopathology sections and Ki67 immunohistochemical staining of a representative spleen, AML-FMT group and Con-FMT group. All microscopic analyses were performed

(original magnification $\times 100$ or $\times 400$). (E) On the 8th and 14th days after the injection, the load of Luc-expressing MLL-AF9 cells in mice was analyzed by IVIS (n=3 per group). (F) Kaplan-Meier survival curve of AML mice (n=4 per group).

Figure 4

AML patient exhibits profound alterations in gut microbial metabolites (A) The plot of OPLS-DA score of all peak features from the untargeted metabolomics analysis of stool samples from AML patients (n=15)

and healthy controls (n=17). (B) Heatmap of the relative abundances of the top 26 most abundant metabolites which significantly changed in AML group. The color bar indicates z score, which represents the relative abundance. Z score < 0 (>0) meant the relative abundance was lower (higher) than the mean. (C) The concentrations of propionic acid, butyric acid, and acetic acid in fecal samples of AML patients (n=22) and controls (n=22) were determined by GC-MS. (D) Heatmap of spearman correlation analysis between the gut microbiota and the metabolite. (E) The functional abundance distribution histogram of samples from AML patients and healthy controls in the COG database using PICRUSt software (the top 35 is selected by the maximum sorting method). (F) The significant differences in metagenomics functions in AML patients compared to that in healthy controls (corrected $P < 0.05$ and confidence intervals = 95%).

A

B

C

D

F

E

Figure 5

Microbiota-derived butyrate gavage delays AML progression (A) The schematic diagram of the mice experimental process including butyrate gavage. (B) The photographs of spleens from butyrate-treated AML mice (n=5) and control AML mice (n=5). (C) The leukemia cells (GFP+ cells) in bone marrow, peripheral blood and spleen from butyrate-treated AML mice (n=5) and control AML mice (n=5). (D) HE histopathology sections and Ki67 immunohistochemical staining of a representative spleen in butyrate-

treated mice and control AML mice. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (E) On day 8 and day 14 after injection of MLL-AF9 cells, the load of Luc-expressing MLL-AF9 cells in mice was analyzed by IVIS. (F) Kaplan-Meier survival curve of AML mice (n=5 per group).

Figure 6

Butyrate reverses intestinal barrier damage in mice with AML(A) The concentration of FITC-dextran in the peripheral blood after FITC-dextran gavage for 6 hours. Data represent the mean SEM (n = 5). (B) The

mRNA expression levels of tight junction protein components claudin-1, claudin-2 and ZO-1 in intestinal epithelial cells of AML, control and butyrate-treatment mice (n=3). (C) The protein levels of claudin-1, claudin-2 and ZO-1 in intestinal epithelial cells were determined by Western blot. GAPDH was used as the control (n=3). (D). Transmission electron microscopy (TEM) of intestines, isolated from normal, AML, and butyrate-treated AML mice for duration of experiment; arrows indicate cell-cell interface. (E) Immunofluorescence analysis of small intestine tissue from normal, AML and butyrate-treated mic. Cells were fixed and stained with a rabbit polyclonal antibody. APC (red) goat anti-rabbit IgG was used as a secondary antibody. Immunofluorescence indicated the expression quantity and localization of claudin-1, claudin-2 and ZO-1.

Figure 7

The damage of the intestinal barrier accelerates the bacterial-derived 839 LPS leakage into blood and LPS exacerbates the progression of AML. (A) The LPS concentrations in peripheral plasma of AML patients and healthy controls. (B) The LPS concentrations in butyrate-treated AML mice and control AML mice. (C) The LPS concentrations in AML-FMT mice and Con-FMT mice. (D) The LPS concentrations in AML mice and normal mice after LPS gavage. (E) The representative FACS graphs of apoptotic cells of

MLL-AF9 cells were shown after being cultured with or without LPS for 48 hours (n=3). (F) CCK8 analysis for the proliferation of MLL-AF9 cells with or without LPS 24, 48, 72 hours (n=3). (G) The Western Blot results of Bcl-2, BAX, cleaved caspase-3, and GAPDH was used as a control (n=3). (H) The Leukemia cells (GFP+ cells) in the spleen, peripheral blood and bone marrow from LPS-treated AML mice (n=4) and control AML mice (n=4). (I) Representative photographs 850 of spleen from LPS-treated AML mice (n=4) and control AML mice (n=4). (J) HE 851 histopathology sections and Ki67 immunohistochemical staining of a representative spleen, LPS-treated AML mice and control AML mice. All microscopic analyses were performed (original magnification $\times 100$ or $\times 400$). (K) Kaplan-Meier survival curve of mice leukemia model (n=4 per group).

Figure 8

Visual abstract graph

Supplementary Files

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