



The ketogenic diet modulates tumor-associated neutrophil polarization via the AMOT-YAP/TAZ axis to inhibit colorectal cancer progression

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ABSTRACT

Despite significant advances in the diagnosis and treatment of colorectal cancer (CRC), the prognosis for late-stage patients remains poor, highlighting the urgent need for new preventive and therapeutic strategies. Recent studies have focused on the ketogenic diet (KD) and its metabolite, β-hydroxybutyrate (BHB), for their tumor-suppressive effects and modulation of inflammatory responses. Using the azoxymethane (AOM) / dextran sulfate sodium (DSS)-induced mouse CRC model, we found that the ketogenic diet and BHB inhibit pro-tumor N2-type tumor-associated neutrophils (TANs) while promoting the polarization of TANs towards the anti-tumor N1 type. This shift in TANs polarization affects tumor growth and metastasis. The underlying mechanism involves BHB acting on the intracellular receptor histone deacetylases 3 (HDAC3), which modulates the activation of the AMOT-YAP/TAZ axis, leading to the inhibition of pro-carcinogenic factor transcription and release. Moreover, clinical cohort data corroborate these findings, showing that CRC patients with elevated BHB levels have significantly lower rates of lymph node involvement, which is associated with a higher infiltration ratio of anti-carcinogenic N1-type TANs in the tumor microenvironment (TME). These results suggest that BHB levels could serve as a prognostic biomarker for CRC. In conclusion, our findings indicate that BHB derived from KD regulates TANs polarization in CRC via the HDAC3-AMOT-YAP/TAZ axis, effectively inhibiting tumor growth and metastasis. These insights establish a novel theoretical basis for employing the KD in the treatment of CRC and for developing cancer adjuvant immunotherapy strategy based on the polarization of neutrophils.

1. Introduction

Colorectal cancer (CRC) is among the most prevalent malignant tumors globally, characterized by high incidence and mortality rates[1]. Despite significant advancements in CRC diagnosis and treatment, the prognosis for patients with advanced stages remains poor, highlighting the urgent need for novel preventive and therapeutic approaches.

Recent research has underscored that cancer growth, invasion, and metastasis being heavily influenced by stromal conditions[2]. Compared to directly targeting tumor cells, modulating the tumor microenvironment (TME) has emerged as a crucial factor in CRC progression.

The ketogenic diet (KD) is a high-fat, low-carbohydrate dietary regimen designed to induce a state of ketosis, where the body predominantly uses ketone bodies as an energy source[3,4]. Specifically, as the

Abbreviations: CRC, colorectal cancer; BHB, β-hydroxybutyrate; TANs, tumor-associated neutrophils; BMDNs, bone marrow-derived neutrophils; AMOT, Angio-motin; KD, ketogenic diet; ND, normal diet; ROS, reactive oxygen species; NETs, neutrophil extracellular traps; TME, tumor microenvironment; HDAC3, histone deacetylases 3.

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most prominent ketone body resulting from KD, β -hydroxybutyrate (BHB) can regulate gene expression and signal transduction through multiple pathways such as inhibiting histone deacetylases (HDACs) and activating G-protein-coupled receptors like hydroxycarboxylic acid receptor 2 (Hcar2)[5]. Research has demonstrated that KD and its metabolite BHB not only contribute to weight loss and seizure control but also exhibit anti-tumor potential[6]. For instance, Levy et al. observed that BHB inhibited CRC cell proliferation via the Hcar-HOPX axis[7]. Additionally, BHB modulates immune cell functions in the TME, exerting anti-tumor effects. Murphy et al. demonstrated that KD and BHB enhanced the efficacy of immune checkpoint blockade (ICB) by regulating CD8⁺ T cell chemotaxis, rebalancing M1/M2 macrophages, promoting monocyte differentiation into antigen-presenting cells, and influencing neutrophil infiltration in the prostate cancer TME, thereby improving tumor prognosis[8,9].

Neutrophils, as integral components of the TME[10], are now recognized as pivotal elements of the innate immune system, performing complex dual roles within the TME[11]. Neutrophils can both promote anti-tumor immune responses by releasing cytokines and chemokines, and support tumor growth and metastasis by polarizing into distinct subtypes and secreting specific cytokines and substances[12]. N1 neutrophils possess anti-tumor activity, inhibiting tumor growth by enhancing the functions of cytotoxic T cells and natural killer (NK) cells. In contrast, N2 neutrophils exhibit pro-tumor characteristics, supporting tumor progression and metastasis through the secretion of various immunosuppressive factors and the promotion of angiogenesis[13]. However, the role of KD in modulating neutrophil polarization in the tumor microenvironment, particularly the specific mechanisms by which BHB influences this process, remains unclear.

Our research aims to investigate how the KD and BHB influence the polarization of neutrophils, particularly focusing on the polarization of tumor-associated neutrophils (TANs) within the TME and its impact on CRC progression. These findings provide a new theoretical basis for using the ketogenic diet as an adjunctive strategy for CRC treatment and offer new insights for developing tumor immunotherapy strategy based on neutrophil polarization.

2. Materials and methods

2.1. Patient samples

All procedures was approved by the First Affiliated Hospital of Soochow University (Ethics Number: 2023127). Fifty CRC patients who had their serum BHB concentration tested were included in this study. These patients underwent colonoscopy, pathological examination, relevant clinical indicators testing, and surgical treatment for colorectal malignancies at the First Affiliated Hospital of Soochow University between February 2023 and June 2024. All patients were selected according to strict inclusion and exclusion criteria. Inclusion Criteria: (1) Patients must be confirmed to be diagnosed with CRC through colonoscopy and pathological examination. (2) Complete preoperative CT imaging and laboratory data must be available. (3) Patients should be stable enough to undergo necessary assessments and procedures (e.g., blood tests, tumor tissue collection, immunohistochemical analysis). Patients must have measurable serum BHB levels. Exclusion Criteria: (1) Patients who are currently receiving chemotherapy, radiotherapy, or immunotherapy to treat CRC and other cancers. (2) Patients with severe or uncontrolled comorbidities that could affect study outcomes, such as advanced cardiovascular disease, severe liver or kidney dysfunction, or active infections. (3) Pregnant or breastfeeding women should be excluded to mitigate potential risks to the fetus or infant and to account for hormonal changes that may impact study results. (4) Patients taking medications known to significantly affect immune function, neutrophil activity, or BHB metabolism (e.g., corticosteroids, immunosuppressants) should be excluded. Tumor tissues were paraffin-embedded for subsequent immunohistochemical analysis. Two experienced surgeons

independently collected, recorded, and reviewed the clinical characteristics of the patients. This study was conducted in accordance with the principles of the Declaration of Helsinki, and all patients signed written informed consent forms.

2.2. Mouse models

All experimental procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of Soochow University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Ethics Number: 2023127). Specific Pathogen-Free (SPF) male C57BL/6 mice (4–6 weeks old, GemPharmatech Co., China) were fed sterile food and water and maintained in a sterile isolator. The mice were randomly assigned to different groups. The sample size was determined based on previous studies using C57BL/6 mice to satisfy the 3 R principles and sample size calculations. To establish the colitis-colorectal cancer (CAC) mouse model, mice were divided into ND and KD groups. The diet's macronutrient and micronutrient components were matched, differing only in the fat-to-carbohydrate ratio. All KD experiments involving a 90 % fat content utilized a diet composed of plant-based fat sources, along with sufficient fiber and essential micronutrients (supplementary material). The KD mice were pre-treated with KD for 7 days for conversion and adaptation. Subsequently, the mice were intraperitoneally injected with the CRC mutagen AOM (Aladin, China) at a dosage of 10 mg/kg. On days 1, 21, and 41, mice were given 2.5 % DSS (Sigma Aldrich, Germany) in drinking water for 5 consecutive days, followed by regular water.

BHB levels were measured in mouse serum. Blood was obtained via orbital vein bleeding and collected in heparin-coated tubes. Serum was separated by centrifugation at 1000 g for 10 minutes at 4 °C. Serum samples were frozen at −80 °C until BHB measurement by colorimetric assay. On the day 60, the evaluation of tumor formation in mice's colons was conducted. Tumor counts were determined via endoscopic examination of the mice's intestines. Observations and quantitative comparisons included changes in the mice's body weight throughout the study period, total colon length, and both the number and size of tumors. Tissue samples were collected for further analysis.

2.3. Subcutaneous tumor model

4–6 week-old male NOD.CB17-Prkdc^{scid}Il2rg^{tm1}/Bcgen mice (Biotogen Pharmaceuticals Co., China) were maintained under specific pathogen-free conditions. The mice were divided into four groups based on previous studies: Control group: MC38 cells (2×10⁶) (Fuheng Biotechnology Co., Shanghai) were injected subcutaneously into the left upper abdomen of NOD.CB17-Prkdc^{scid}Il2rg^{tm1}/Bcgen mice on day 0. KD group: Mice were fed KD as above. TANs group: MC38 cells (2×10⁶) co-injected with TANs (5×10⁵) into the subcutaneous space of the left upper abdomen, with TANs re-injected at designated times. KD+TANs group: Same as TANs group but fed the KD. All mice were housed in the Animal Service Center of Soochow University, maintained at 22–23 °C, relative humidity < 70 %, 12-hour light/dark cycle, with free access to standard mouse diet and water.

2.4. Hematoxylin and eosin (H&E) staining and histologic analysis

The tissue samples were fixed in formalin immediately after euthanasia and embedded in paraffin. The paraffin-embedded tissues were sectioned at a thickness of 3 μ m and mounted on slides. For H&E staining, sections were deparaffinized in xylene and rehydrated through graded ethanol and water washes. Hematoxylin staining was applied, followed by differentiation, bluing, and rinsing. Sections were then counterstained with eosin after graded dehydration. Finally, sections were dehydrated again through ethanol and xylene and mounted with neutral resin. The histological score is determined by blinded pathologists based on histological scoring criteria [14,15], incorporating

assessments of inflammatory cell infiltration (0–3) and mucosal architecture (0–3). For mucosal architecture: 0, normal mucosal structure with no lesions; 1, mild changes, including slight villous atrophy or minimal inflammation; 2, moderate changes, such as noticeable villous atrophy accompanied by moderate inflammation; 3, severe changes, marked by significant villous atrophy or loss and extensive inflammation. For inflammatory cell infiltration: 0, normal, with no abnormal infiltration; 1, slight infiltration limited to the mucosal layer; 2, intermediate infiltration extending into both the mucosal and submucosal layers; 3, severe infiltration affecting the mucosal, submucosal, and transmural layers.

2.5. Adaptive transfer study

Bone marrow-derived neutrophils (BMDN) were extracted from the bone marrow of healthy C57BL/6 J mice and induced *in vitro* to form N2-type TANs. They were then transplanted into tumor-bearing NOD.CB17-*Prkdc^{scid}Il2rg^{tm1}*/Bcgen mice. After CRC subcutaneous tumor establishment on days 10, 15, 20, and 25, N2-type TANs (1×10^6 cells/mouse) were injected via the tail vein into recipient mice, and mice were sacrificed on day 27. These time points were chosen because neutrophil infiltration at inflammatory sites decreases after 5 days. Tumor volume was measured with calipers using the formula $(0.5 \times a \times b^2)$, where “a” represents the long diameter and “b” represents the short diameter.

2.6. Multiplex immunofluorescence

The tissue samples were fixed in formalin immediately after euthanasia, and embed in paraffin. Cut the paraffin-embedded tissues into 3 μ m thick sections and mount them on slides. The sections were subjected to a series of deparaffinization and rehydration steps, and antigen retrieval was performed in a microwave using Tris-EDTA buffer (Bio-sharp, USA). After 5 % BSA blocking, apply the first primary antibody to the sections and incubate them overnight at 4 °C. Wash the sections in PBS to remove unbound antibodies, then incubated the sections with a fluorophore-conjugated secondary antibody. Repeat the staining process for additional markers by sequentially repeating the primary and secondary antibody incubation steps. Use distinct fluorophores for each primary antibody to ensure clear visualization. Capture images using a inverted fluorescence microscope. Analyze the images with ImageJ software to quantify fluorescence signals.

2.7. RNA sequencing

Tumor samples for RNA sequencing (RNA-seq) were rapidly frozen in liquid nitrogen immediately after harvest. The samples were prepared and analyzed by LC-Bio Technology CO., Ltd., (China). Total RNA was extracted using Trizol reagent (ThermoFisher, 15596018) following the manufacturer's procedure. The total RNA quantity and purity were analysis of Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA, 5067–1511), high-quality RNA samples with RIN number > 7.0 were used to construct sequencing library. We used HISAT2 (<https://daehwankimlab.github.io/hisat2/>, version: hisat2–2.2.1) to align reads from all samples to the <research species> reference genome, obtaining clean data aligned to the mouse genome (ftp://ftp.ensembl.org/pub/release-101/fasta/mus_musculus/dna/). The mapped reads of each sample were assembled using StringTie (<http://ccb.jhu.edu/software/stringtie/>, version: stringtie-2.1.6) with default parameters. Gene ontology (GO) enrichment analysis was conducted using the OmicStudioKits package (version 3.40.0). GO terms with a corrected p-value of less than 0.05 were considered significantly enriched among differentially expressed genes. The raw sequence data have been deposited in the NCBI Gene Expression Omnibus (GEO) dataset under accession number <GEO accession> or the NCBI Short Read Archive (SRA) dataset under accession number <SRA accession>. Differentially expressed genes (DEGs) were analyzed using DESeq2 for gene

expression differences between two groups (with edgeR used for two-sample comparisons). Genes with a false discovery rate (FDR) parameter of less than 0.05 and an absolute fold change ≥ 2 were considered differentially expressed. Subsequently, DEGs underwent GO functional and KEGG pathway enrichment analysis.

2.8. Cell culture and isolation

2.8.1. BMDNs isolation

To isolate BMDNs, bones from healthy C57BL/6 J mice were collected, filtered through the 70 μ m nylon mesh, and centrifuged with Ficoll-Hypaque. The cell pellet was resuspended in sorting buffer and stained with biotin-Ly6G antibody (Biolegend, USA), incubated at 4 °C, and centrifuged again to remove unbound antibodies. Streptavidin MicroBeads (Milteny, Germany) were added, incubated, and centrifuged to remove unbound beads. The cells were resuspended in sorting buffer and subjected to magnetic separation using LS columns. The bound cells were collected, centrifuged at 1900 rpm, resuspended in medium, and plated for subsequent experiments.

2.8.2. TANs isolation

For TANs isolation, subcutaneous tumor tissues from NOD.CB17-*Prkdc^{scid}Il2rg^{tm1}*/Bcgen mice were digested with collagenase IV (Sigma-Aldrich, Germany), DNase I (Thermo Fisher, USA) and FBS. The cell suspension were filtered, centrifuged with Ficoll-Hypaque at 1900 rpm, and the cell layer collected for further analysis.

2.8.3. Co-culture of BMDNs and MC38

BMDNs and MC38 cells were co-cultured in a 24-well plate with transwell inserts with a system ratio of 1:2. After 2 hours, BMDN were digested with trypsin, and TANs markers Ly6G/CD11b were detected.

2.9. Molecular docking

SDF format files of the drugs were obtained from the PubChem database. Homologous protein structures were optimized using PyMOL software by removing water molecules and small-molecule ligands from the target. The optimized structures were subsequently processed using AutoDock Tools for hydrogen addition and charge assignment, and saved in pdbqt format. Key targets served as receptors, while their corresponding active ingredients were utilized as ligands. Molecular docking was conducted using Vina within the PyRx software to calculate binding energies and generate the results. The molecular configuration of HDAC3 was retrieved from the Protein Data Bank (PDB ID: 4A69). The final visualization of the results was accomplished using PyMOL software. The affinity (kcal/mol) value indicates the binding affinity between the receptor and the ligand, where a lower value denotes a more stable interaction. Discovery Studio 2020 Client (<https://discover.3ds.com/discovery-studio-visualizer-download>) was employed for additional visual analysis.

2.10. Transwell migration assay

Transwell migration assays were performed by 24-well plates with inserts. A layer of matrigel coating is applied to the transwell membrane and incubated at 37 °C for 1–2 hours to solidify. The lower chamber contained medium with 10 % FBS, while the upper chamber contained 1×10^5 MC38 cells in serum-free medium. Cells were allowed to migrate for 48 hours, fixed, stained, and counted under a microscope.

2.11. Cell wound scratch assay

MC38 cells were co-cultured and then seeded into a six-well plate at a density of 1×10^5 cells per well. The cells were cultured in complete medium supplemented with 10 % FBS in an incubator at 37°C with 5 % CO2 until they reached 80–90 % confluence. A 200 μ L sterile pipette tip

was used to create the straight line across the center of the cell monolayer in each well, ensuring consistent scratch placement for reproducibility. Subsequently, the cells were gently washed twice with PBS to remove debris generated by the scratching. Before treatment (0 hours), images of each scratch area were captured using an inverted microscope to document the initial scratch width, maintaining consistency by selecting the same positions for subsequent imaging. The cells were then incubated at 37 °C with 5 % CO₂ for 24 and 48 hours. At each time point, the healing of the scratches was observed under an inverted microscope, and images were taken accordingly. The width of the scratched region at each time point was measured using ImageJ software. The percentage of scratch healing was calculated using the following formula:

$$\text{Scratchhealingpercentage} = \frac{(\text{Initialscratchwidth} - \text{Finalscratchwidth})}{\text{Initialscratchwidth}} \times 100\%$$

2.12. Flow cytometry

Cells were harvested and prepared as a single-cell suspension. After blocking, they were stained with fluorochrome-conjugated antibodies and washed. Flow cytometry was performed using a calibrated instrument, with compensation controls for spectral overlap. Flow cytometry was performed as previously described with the following antibodies: PE/Cyanine7 anti-Ly6G (Biolegend, USA), PE anti-CD206 (Biolegend, USA), PE anti-iNOS (Biolegend, USA), FITC anti-Arginase 1 (Bioss, China). Intracellular staining was performed using a Cytofix/Cytoperm kit (BD Biosciences, USA). The events were acquired using a Attune Nxt Acoustic Focusing Flow Cytometer. Data were analyzed by FlowJo software. These values represented the geometric mean fluorescence intensity of an appropriate channel.

2.13. Co-immunoprecipitation

For co-immunoprecipitation (Co-IP), cell lysates were prepared using a lysis buffer containing protease inhibitors. The lysates were incubated with specific primary antibodies (Anti-IgG anti-HDAC3) overnight at 4 °C, followed by the addition of protein A/G agarose beads and further incubation for 2 hours at 4 °C. The immune complexes were washed extensively with lysis buffer, then eluted by boiling in SDS sample buffer. The samples were subjected to SDS-PAGE and analyzed by Western blot using appropriate secondary antibodies. Control IgG was used as a negative control to ensure specificity of the immunoprecipitation.

2.14. Protein extraction and Western blot analysis

Cells were harvested and lysed using RIPA buffer (Beyotime Biotechnology, China) with 1 % phosphatase and protease inhibitor mixture (Beyotime Biotechnology, China). Protein concentrations were quantified using the BCA assay (Beyotime Biotechnology, China). The samples were heated at 90 °C for 10 minutes, then separated on a 4–20 % precast polyacrylamide SDS-PAGE gel (Genscript, USA) and transferred onto a PVDF membrane. Following blocking with 3 % BSA in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T), the membranes were incubated overnight at 4 °C with the following primary antibodies: anti-YAP (Cell Signaling, USA), anti-P-YAP (Cell Signaling, USA), anti-TAZ (Cell Signaling, USA), anti-P-TAZ (Cell Signaling, USA), anti-HDAC3 (Proteintech, China), anti-Angiomotin (Proteintech, China). After washing, the membranes were incubated with horseradish

peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (Bioss, China) at room temperature (20 °C) for 1 hour. The ECL signal was visualized using a Bio-RAD imaging system with a hypersensitive ECL chemiluminescence kit (NCM Biotech, China). The images were converted to grayscale and analyzed for densitometric values through ImageJ software.

2.15. Statistical analysis

Data were analyzed using GraphPad Prism 9.0 software and presented as mean ± SEM. Statistical significance was determined using t-tests for comparisons between two groups, and one-way ANOVA fol-

lowed by multiple comparisons tests. $P < 0.05$ was considered to indicate statistically significant differences.

3. Results

3.1. The KD inhibits the development of CRC in the AOM/DSS mouse model

To evaluate the impact of the KD on CRC development, male C57BL/6 J mice were fed either ND or KD with fat content at 90 %. During the dietary regimen, mice were injected with AOM and subjected to three cycles of DSS induction (Fig. 1A). During this period, serum levels of BHB were significantly elevated in KD-fed mice. In contrast, the ND-fed AOM/DSS mice exhibited significant weight loss, whereas KD-fed mice displayed less weight reduction post AOM/DSS induction, resembling normal control mice without AOM/DSS treatment more closely (Figs. 1B, 1C). Prior to harvest, the number of tumors and polyps observed under colonoscopy was significantly reduced in KD-fed mice while ND-fed AOM/DSS models showed (Figs. 1D, 1E). These findings indicate that KD can modulate systemic metabolic states and improve overall health markers [16].

Additionally, studies have indicated that elevated levels of BHB exerts anti-inflammatory and anti-proliferative properties, contributing to tumor suppression [17]. At harvest, KD-fed mice demonstrated colon lengths closer to normal controls than ND-fed mice, with markedly suppressed numbers and sizes of colorectal tumors (Figs. 1F, 1G). This observation is supported by existing literature suggesting that ketone bodies, particularly BHB, can inhibit cancer cell growth and induce cancer cell apoptosis through multiple mechanisms, including the inhibition of HDACs and the modulation of oxidative stress [18,19].

Moreover, KD-fed mice exhibited significantly improved colon inflammation compared to ND-fed counterparts, consistent with reports that KD can reduce colonic inflammation and improve intestinal barrier function (Fig. 1I, S1A). Histological examination using H&E staining revealed that the intestinal tissues of mice in the ND group exhibited varying degrees of adenocarcinoma, high-grade atypical hyperplasia, and low-grade atypical hyperplasia. In stark contrast, the KD group demonstrated a significant reduction in pathological changes, predominantly showing only a few instances of low-grade atypical hyperplasia, with a markedly lower incidence of both adenocarcinoma and high-grade atypical hyperplasia (Figs. 1H, 1J). This corroborates findings that dietary interventions, such as KD, can alter the tumor microenvironment and reduce the progression of pre-cancerous lesions to malignant tumors [20]. These findings consistently demonstrate that KD effectively inhibits colorectal tumor growth and may offer a viable dietary strategy for CRC prevention and therapy.

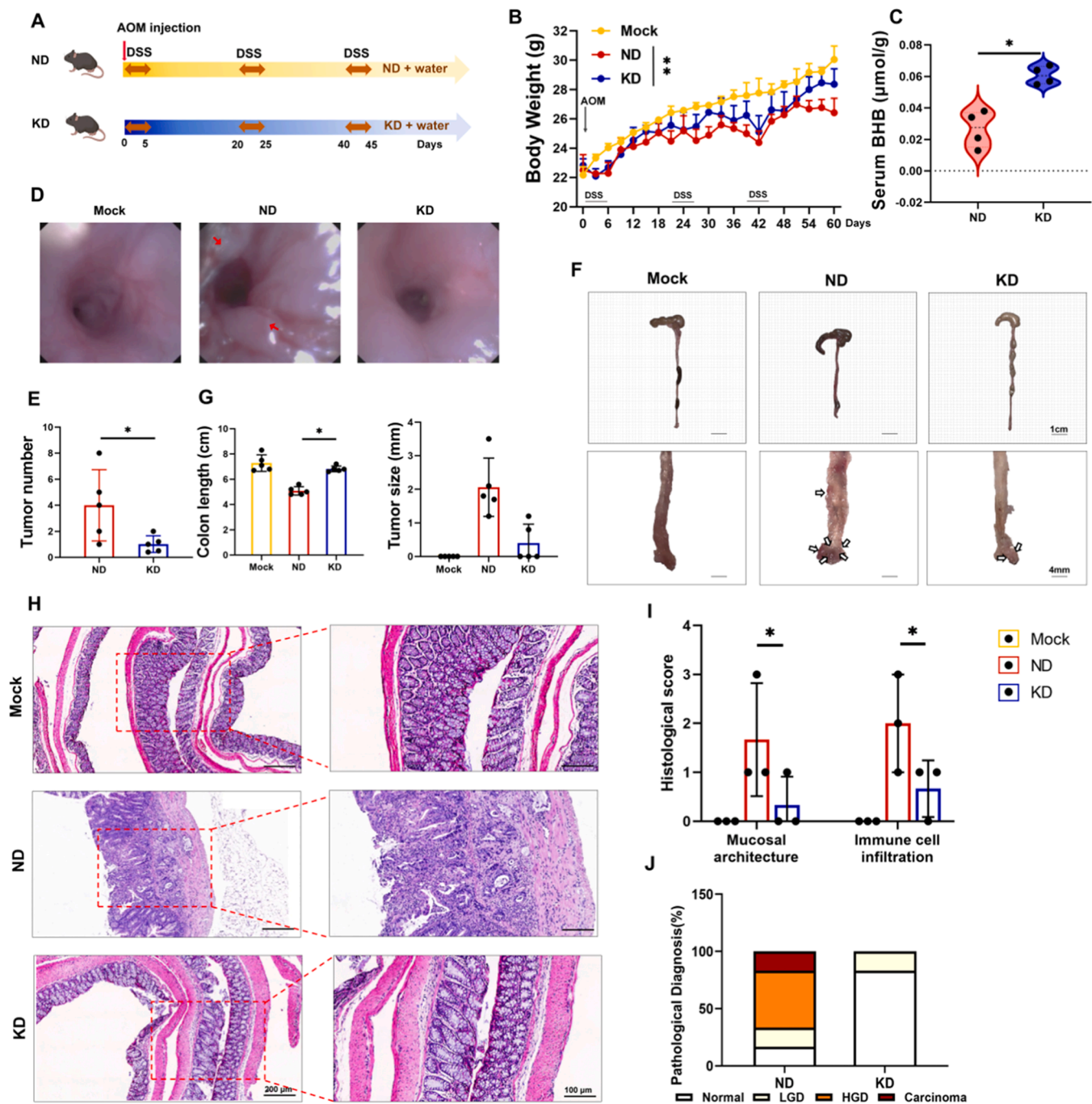


Fig. 1. KD inhibits the development of CRC in the AOM/DSS mouse model. (A) Experimental design of the AOM/DSS-induced CRC mouse model under different diets. (B) Body weight change curves of mice fed with ND and KD during the model construction period. (C) Determination of serum BHB levels in AOM/DSS mouse model (n = 5). (D) Representative images monitored by colonoscopy on day 60. (E) Tumor count based on colonoscopy. (F) Macroscopic representative images of the colon. (G) Quantification of the total colon length and tumor size in mice fed with ND and KD. (H) H&E staining for pathological diagnosis of mouse colon. (I) Quantitative analysis of histological score based on the mucosal architecture and immune cell infiltration. (J) Quantitative analysis of pathological scores based on the following criteria: 0, normal; 1, LGD; 2, HGD; 3, cancer. *, $P < 0.05$; **, $P < 0.01$.

3.2. The KD impacts the polarization of neutrophils in the TME

To further elucidate the mechanisms through which the ketogenic diet influences CRC progression, transcriptome analysis was performed on tumor specimen from ND or KD-fed mice (Fig. S1B-D). Pathway enrichment analyses revealed differential expression of genes involved in innate immune response functions in the tumor tissues of KD-fed mice compared to ND-fed counterparts (Figs. 2A, 2B). Indicated that KD influences inflammation and tumors by modulating innate immune cells

and their metabolic pathways[21]. Furthermore, we observed that CRC mouse models fed KD exhibited significantly reduced neutrophil infiltration in tumorous intestinal tissue compared to those on ND. These findings were corroborated by RNA-seq data analysis using QUANTISEQ and TIMER estimations in situ tumor tissues of mice (Fig. 2C). Specifically, compared to ND-fed mice, KD-fed mice exhibited significant reductions in chemokines associated with neutrophil infiltration, particularly cytokines that regulate neutrophil pro-tumorigenic N2 polarization, such as CCL3, CXCL5, and CXCL2 (Fig. 2D). Chemotaxis

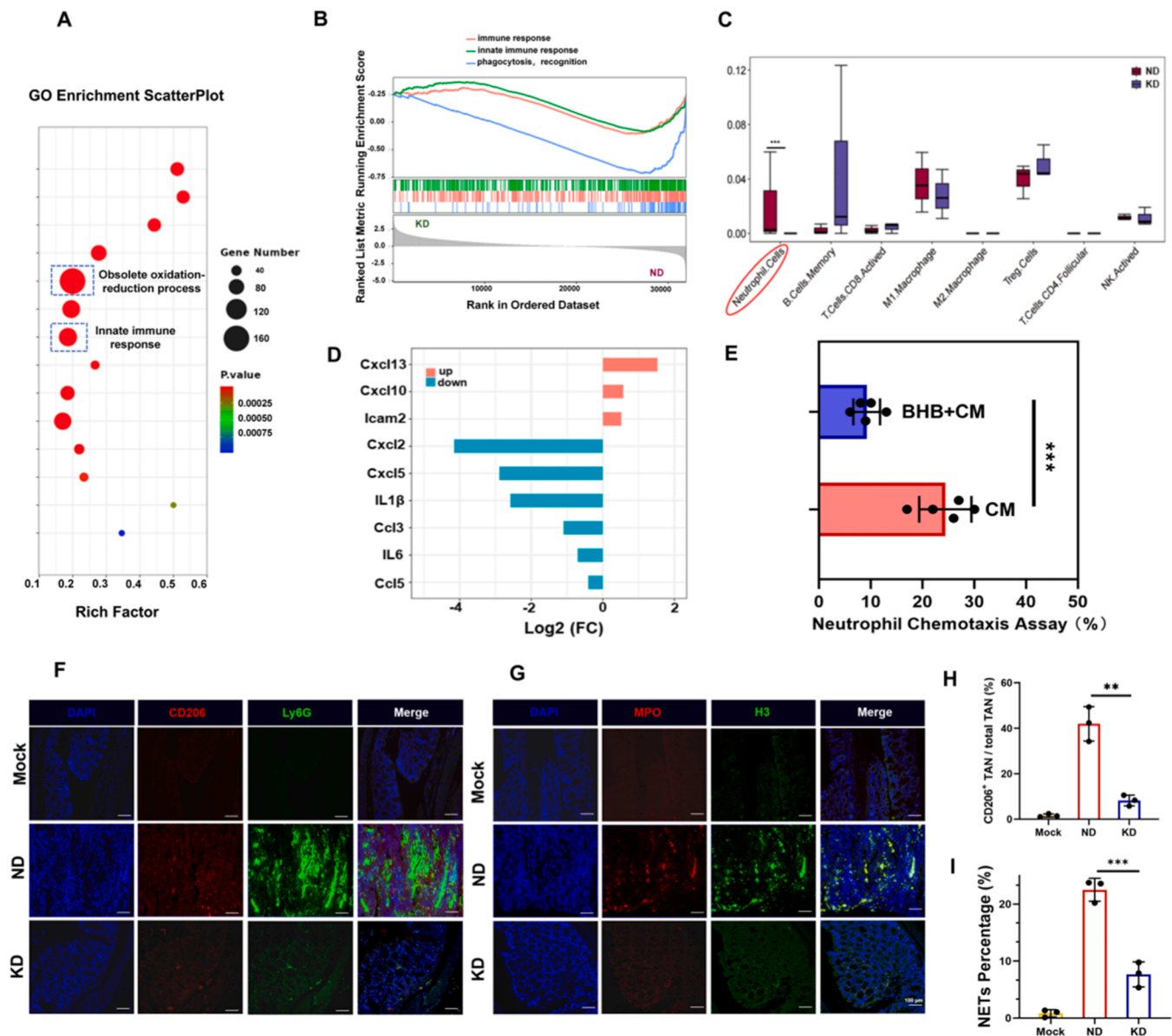


Fig. 2. KD impacts N1/N2 polarization of neutrophils in the TME. (A) Gene ontology analysis of regulatory functions in the AOM/DSS mouse model following KD and ND interventions, assessed via RNA-seq. (B) Gene Set Enrichment Analysis (GSEA) plots illustrating the cumulative enrichment scores of gene sets in the ranked gene list of the two sample groups based on GO analysis. (C) Estimation of the relative percentages of various immune and cellular subtypes in CRC tissue samples from KD and ND groups using CIBERSORT analysis based on RNA-seq data. (D) The fold change (FC) values of gene expression representing N1 (Cxcl13, Cxcl10, and Icam2) versus N2 (Cxcl2, Cxcl5, IL-1 β , Ccl3, IL-6, and Ccl5) type TANs in the AOM/DSS mouse model are displayed. (E) Conditioned medium (CM) with or without BHB on neutrophil recruitment in colorectal cancer cells *in vitro*. (F, H) Immunofluorescence staining images showing the proportion of N2 (CD206⁺/Ly6G⁺) phenotype TANs in intestinal tumor tissue samples from the AOM/DSS mouse model (F). Quantitative analysis of the proportion of N2 neutrophil fluorescence within the total neutrophil fluorescence (H). (G, I) Representative confocal microscopy images of NETs, demonstrating the colocalization of fluorescence from DNA (blue), histone H3 (green), and myeloperoxidase (red) (G). Quantitative analysis of the proportion of NETs colocalized by fluorescence (I) (n = 3). **, $P < 0.01$; ***, $P < 0.001$.

assays *in vitro* demonstrated a significantly reduced ability of CRC tumor cells treated with BHB to recruit neutrophils actively (Fig. 2E).

Interestingly, despite a general trend towards reduced neutrophil infiltration in the TME following KD feeding, there was significant upregulation of cytokines associated with neutrophil anti-tumorigenic N1 polarization, including CXCL10, CXCL13, and ICAM2 (Fig. 2D). Hence, we hypothesize that KD not only inhibits recruitment of neutrophils to tumor cells but also induces phenotypic and functional changes in TANs within the TME. Current research indicates that TANs have two distinct phenotypes: N1, which promotes anti-tumor activity by inducing apoptosis, and N2, which fosters tumor growth through factors like matrix metalloproteinase-9 (MMP9) and vascular

endothelial growth factor (VEGF) [22,23].

To further ascertain how KD and BHB regulate neutrophils in the TME and mitigate CRC progression, we performed multi-color fluorescence staining to identify CD206⁺/Ly6G⁺ cells as N2 TANs and iNOS⁺/Ly6G⁺ cells as N1 TANs (Fig. 2F, S1E). In ND-fed mice, whereas N2-state TANs comprised nearly 50 % of Ly6G⁺ cells within colon tumor tissues. In contrast, KD-fed mice had N2-state TANs accounting for less than 10 % of Ly6G⁺ cells (Fig. 2H). Interestingly, while the overall neutrophils infiltration decreased following the KD diet, the proportion of N1 polarization significantly increased among the total infiltrating neutrophils (Fig. S1E). Functionally, co-localization immunofluorescence assays on colon cancer tissues from mice fed different diets revealed

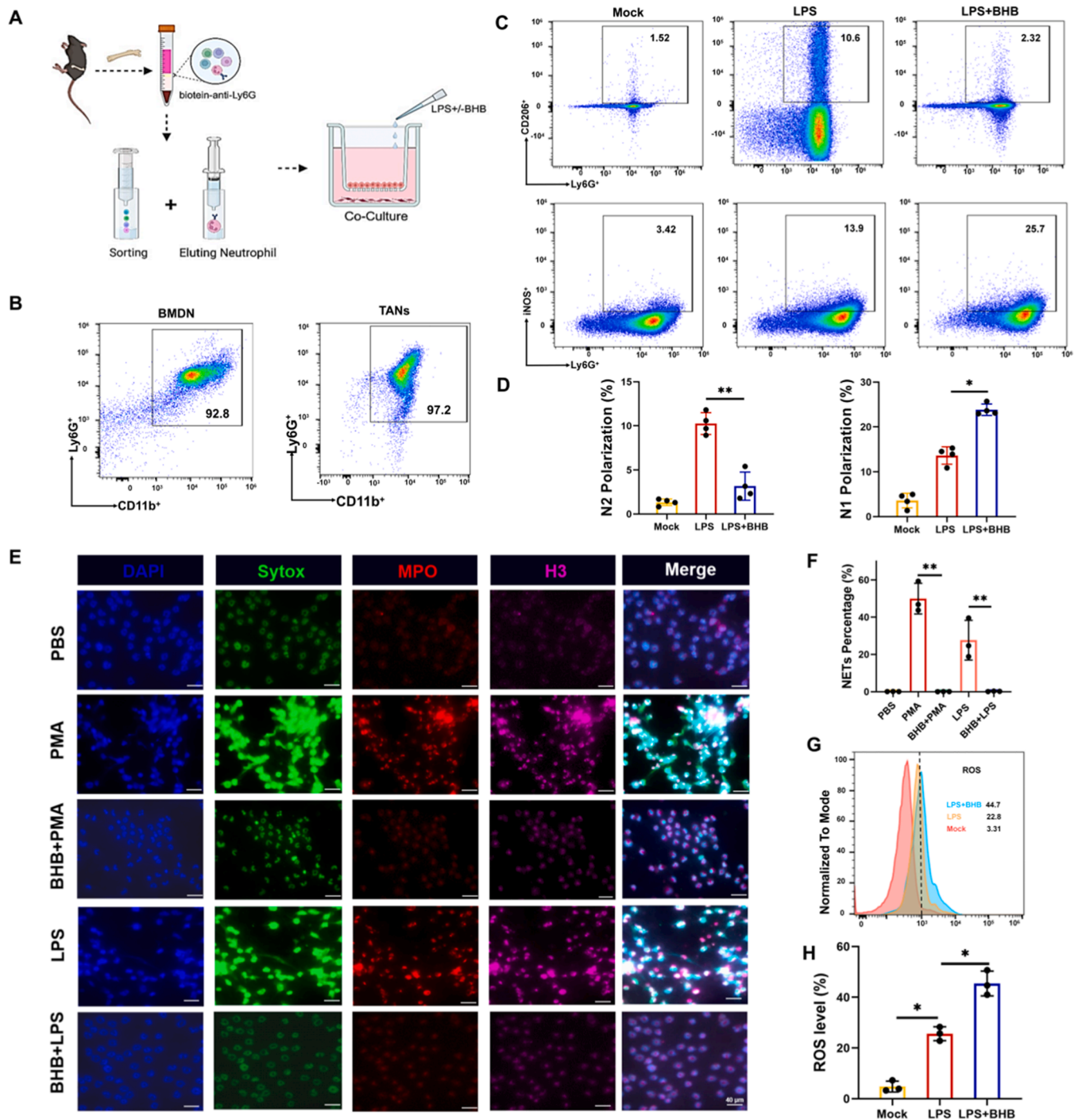


Fig. 3. BHB regulates the phenotype and function of N1/N2 polarization of neutrophils. (A–B) Isolation and extraction of BMDNs from mouse bone marrow using magnetic beads, and induced them into TANs *in vitro*. Followed by dual-marker (Ly6G⁺/CD11b⁺) flow cytometry validation of extraction purity. (C, D) Flow cytometry was employed to assess the levels of N2 (Ly6G⁺/CD206⁺) and N1 (Ly6G⁺/iNOS⁺) polarization in TANs, along with quantitative analysis. (E, F) TANs were exposed to various treatment conditions: PBS, PMA (100 nM) for 3 hours, BHB (6 mM) for 3 hours followed by PMA (100 nM), and BHB (6 mM) for 2 hours followed by LPS (10 μg/mL). Representative fluorescence colocalization images showing NETs formation under these conditions (n = 4), along with quantitative analysis. (G, H) Use flow cytometry with a DCFH-DA probe to detect reactive oxygen species levels under different treatment conditions, along with quantitative analysis. *, *P* < 0.05; **, *P* < 0.01.

significant NETs formation and release in ND-fed model mice, while no prominent NETs release was observed in KD-fed mice (Figs. 2G, 2I). Collectively, these results consistently suggest that KD and its principal ketone body, BHB, mitigate CRC progression by modulating neutrophil infiltration, phenotype, and function within the TME.

3.3. BHB regulates the phenotype and function of N1/N2 polarization of neutrophils

To further validate the effects of KD and its primary effector, BHB, on neutrophils, magnetic bead separation techniques were employed to isolate BMDNs from mice for culture (Fig. 3A). BMDNs were induced into TANs using conditioned medium from MC38 cells, as confirmed by

flow cytometry with double staining for Ly6G⁺/CD11b⁺ (Fig. 3B). Subsequently, purified TANs were treated with LPS to simulate the polarization status within the CRC tumor microenvironment, either with or without BHB supplementation. LPS-treated neutrophils exhibited significant N2 polarization, which was markedly inhibited by BHB addition, with BHB even promoting polarization towards the N1 phenotype (Figs. 3C, 3D). Functionally, neutrophils treated with PBS did not release NETs, whereas those treated with phorbol 12-myristate 13-acetate (PMA) or LPS exhibited significant NET formation. Interestingly, the addition of BHB significantly reduced NETs formation induced by PMA or LPS (Figs. 3E, 3F). Based on the previous flow cytometry results of TANs polarization, we found that the pro-carcinogenic N2-polarized TANs significantly decreased, accompanied by a marked reduction in NETs release. Thus, we speculate that NETs release may also play an important role in the pro-carcinogenic effects of N2-polarized TANs. Notably, N1-polarized TANs are known to release higher levels of reactive oxygen species (ROS), which suppress tumor progression [24]. Flow cytometric analysis revealed that TANs treated with BHB exhibited elevated ROS levels compared to controls (Figs. 3G, 3H).

These results demonstrate that BHB protects TANs from pro-carcinogenic polarization and NETs formation, while inhibiting neutrophil polarization towards the N2 phenotype and promoting polarization towards the N1 phenotype [25]. These findings highlight the potential role of BHB in regulating TANs polarization, which is crucial for understanding the implications of KD in cancer therapy.

3.4. BHB regulates the N1/N2 polarization of neutrophil via the HDAC3-AMOT-YAP/TAZ axis

To elucidate the potential mechanisms by which BHB regulates neutrophil polarization in the TME, we conducted transcriptome sequencing analysis on TANs treated with LPS alone or in combination with BHB (Fig. S2). Significant differences in gene expression were observed in TANs after BHB treatment compared to those induced by LPS alone. KEGG pathway enrichment analysis revealed that the Hippo signaling pathway, primarily regulated by AMOT, was notably down-regulated following BHB intervention (Fig. 4A-C). AMOT is known to influence YAP phosphorylation through various pathways, thereby modulating Hippo pathway activation. Increasing evidence suggests that downstream effectors of the Hippo pathway, such as YAP/TAZ transcriptional activity, play crucial roles in tumor immune regulation [26].

Further validation at the protein level revealed that following LPS induction, TANs exhibited increased levels of HDAC3, AMOT, and YAP/TAZ proteins. In contrast, BHB treatment led to a synchronized decrease in these proteins (Fig. 4D). Previous studies have shown that BHB primarily acts through modulation of the key effector HDAC3 [27]. We further conducted a molecular docking study between BHB and its target, HDAC3. The binding free energy of BHB with HDAC3 was calculated to be -8.5 kcal/mol, indicating a strong affinity and favorable interaction between the two. Moreover, a higher number of hydrogen bonds is typically associated with stronger apparent binding affinity between molecules. Intriguingly, our docking analysis revealed that BHB forms nine hydrogen bonds with HDAC3, suggesting robust binding activity within HDAC3-related signaling pathways (Fig. 4E). Through co-immunoprecipitation, we found strong interaction between HDAC3, the primary intracellular receptor for BHB, and AMOT proteins in neutrophils (Fig. 4F).

To further validate the association between HDAC3 and the AMOT-YAP/TAZ axis, we pretreated neutrophils with the HDAC3 inhibitor (RGFP966) (Fig. 4G). Interestingly, after treatment with BHB combined with RGFP966, the number of CD206⁺ TANs did not decrease, even following BHB intervention. Similarly, there was no observed increase in the number of iNOS⁺ TANs. Therefore, after adding an HDAC3 inhibitor, BHB's ability to promote the phenotypic shift from N2 to N1 TANs was reversed (Fig. 4H). Furthermore, under these conditions, neither LPS nor

LPS/BHB treatment significantly affected AMOT protein expression. Additionally, YAP/TAZ expression did not decrease significantly even after BHB treatment, and the levels of p-YAP/p-TAZ did not increase accordingly. This indicates that the inhibitory effect of BHB on the AMOT-YAP/TAZ axis is nullified when HDAC3 is specifically inhibited (Fig. 4I), indicating that BHB exerts its suppressive effect on TANs polarization towards the pro-carcinogenic N2 type in an HDAC3-AMOT-YAP/TAZ axis-dependent manner.

Collectively, these results indicate that BHB suppresses the polarization of TANs towards the pro-tumorigenic N2 phenotype while promoting their polarization towards the anti-tumorigenic N1 phenotype by regulating the expression of the AMOT-YAP/TAZ axis through HDAC3.

3.5. The KD and BHB influence the progression of CRC by regulating the polarization of TANs both in vivo and in vitro

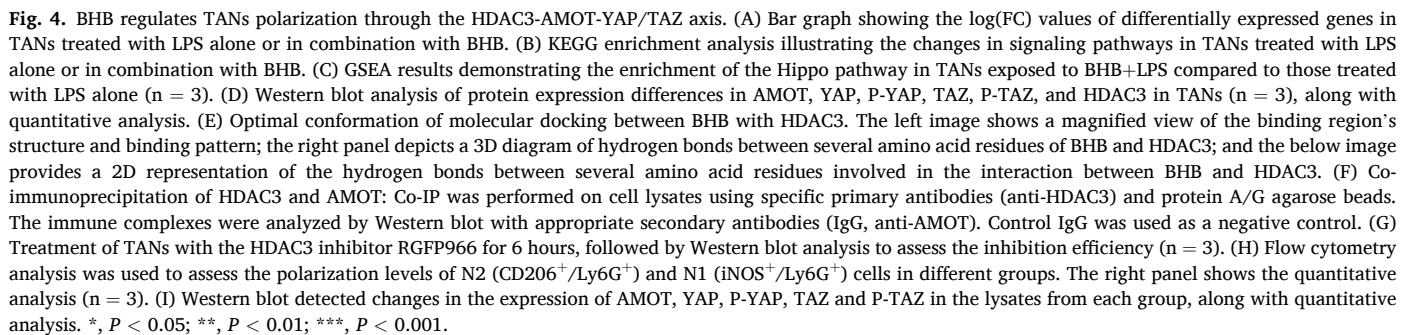
Initially, we employed an adoptive transfer approach in a mouse subcutaneous model to confirm that KD suppresses tumor growth by inhibiting pro-tumor N2 TANs and promoting anti-tumor N1 TANs. We injected N2 TANs, induced *in vitro* by LPS, into NOD.CB17-Prkdc^{scid}/Jl2r^{tm1}/Bcgen mice bearing MC38 tumors, with injections repeated on days 10, 15, 20, and 25 (Fig. 5A). The mice were divided into two groups and fed either ND or KD. We found that N2 TANs significantly promoted tumor growth in mice on ND. In contrast, there was no significant difference in subcutaneous tumor size between KD-fed mice, regardless of whether they received N2 TANs. Furthermore, tumors in KD group were significantly smaller compared to those in ND group (Figs. 5B, 5C).

Flow cytometry analysis of TANs extracted from subcutaneous tumors revealed that the proportion of pro-tumor N2 TANs (CD206⁺/Arginase 1⁺) was significantly higher in ND group and positively correlated with tumor size and proliferation. Conversely, in mice on KD, the proportion of anti-tumor N1 TANs (iNOS⁺) was significantly higher, even following the exogenous injection of N2 TANs. Despite increased neutrophil infiltration in the subcutaneous tumors, the proportion of N1 TANs remained higher, with this proportion negatively correlating with tumor size and proliferation (Fig. 5D, S3E). Furthermore, the proliferation of subcutaneous tumors was significantly reduced in mice fed on KD (Figs. 5E, 5F). Multiplex immunofluorescence staining of subcutaneous tumor tissues further corroborated these findings. Compared to ND group, the KD group exhibited a significant decrease in N2 TANs and an increase in N1 TANs within TME (Figs. 5G, 5H).

Additionally, we employed a transwell assay to co-culture MC38 cells with LPS-treated TANs polarized to the N2 state and TANs whose polarization state was modulated by the addition of BHB, to evaluate the migration capacity of MC38 cells after co-culture. Subsequently, we performed a transwell assay and a wound healing assay to assess migration and invasion capabilities of MC38 cells following co-culture treatment (Fig. S3A-D). The results indicated that TANs polarized to the N2 state by LPS significantly enhanced the invasion and migration capabilities of MC38 cells. In contrast, TANs treated with BHB did not enhance these abilities in MC38 cells and actually inhibited their invasion and migration. These findings indicate that the KD and BHB, promote N1 polarization within the TME and exert anti-tumor functions.

3.6. The relationship between serum BHB level and neutrophil counts and prognosis in CRC patients

To investigate the role of BHB levels as a prognostic indicator, we measured circulating BHB concentrations in a cohort of 50 CRC patients. Results showed that blood BHB level alters patients' white blood cell count, neutrophil count, neutrophil percentage, lymphocyte percentage, and bile acid levels (Fig. 6A, Table 1). Notably, patients with elevated serum BHB concentrations exhibited significantly reduced white blood cell and neutrophil counts, while their lymphocyte counts were increased (Fig. 6B). This clinical observation aligns with the trends observed in our prior research findings. Additionally, BHB



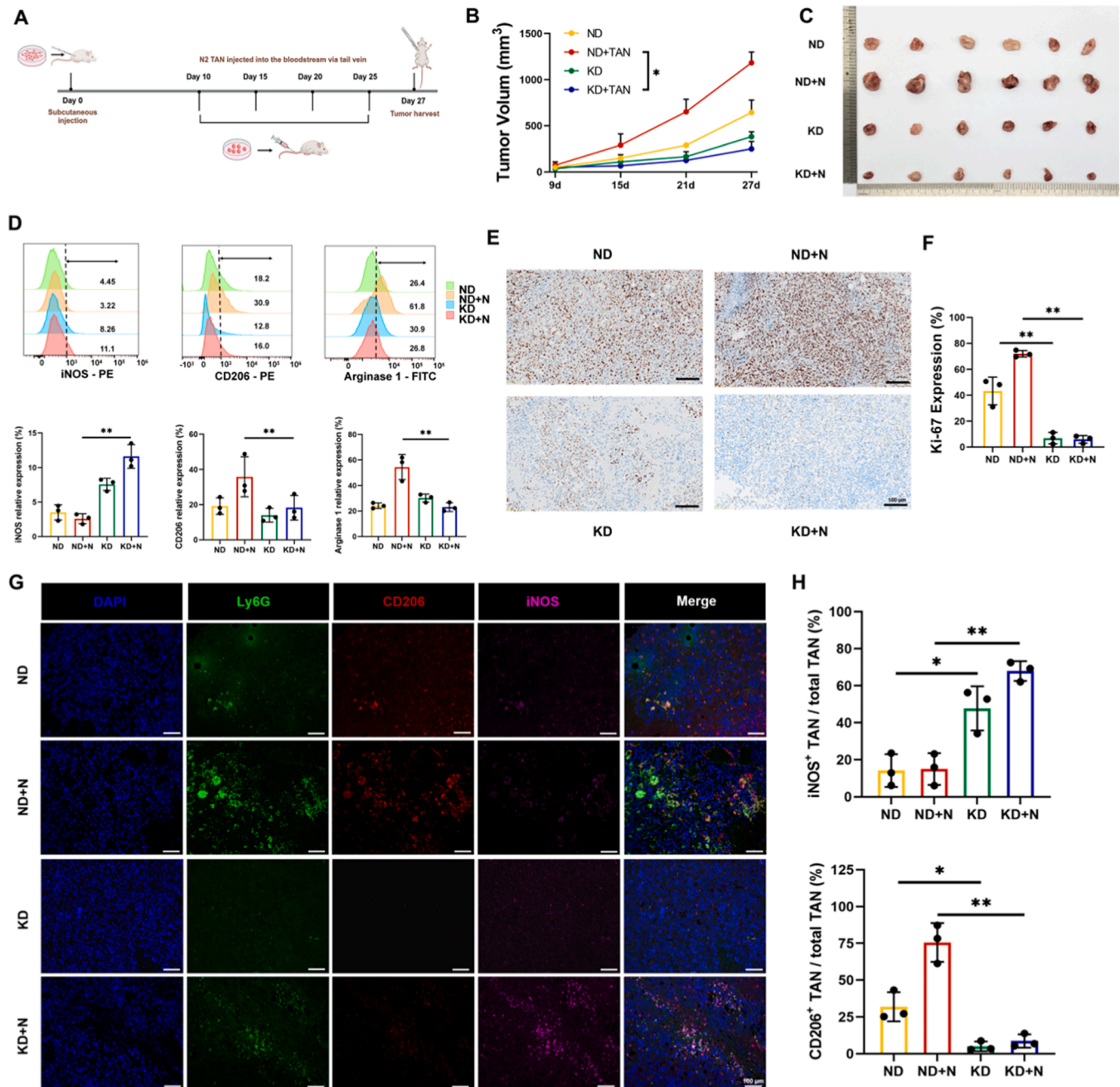


Fig. 5. KD and BHB influence the progression of CRC by regulating the polarization of TANs both *in vivo*. (A-B) Mouse Subcutaneous Tumor Experiment Schematic: On day 0, four groups of B-NDG mice were injected subcutaneously with MC38 cells, with or without TANs. ND+N2 TANs and KD+N2 TANs groups received additional TANs at specified times. ND and ND+N2 TANs groups were fed ND, while KD and KD+N2 TANs groups were fed KD. Tumor volumes were monitored every 3 days, and mice were sacrificed after 27 days. (C) Macroscopic observation of tumor samples. (D) Flow cytometry analysis of TANs: Single-cell suspensions were prepared from excised tumor tissues, and the polarization proportions of N1 (iNOS + Ly6G) and N2 (CD206 + Ly6G) TANs were assessed using flow cytometry, along with quantitative analysis. (E, F) Immunohistochemistry was performed to assess the expression levels of Ki-67 in tumor tissues from each group (E). Quantitative analysis of the proportion of Ki-67 expression in subcutaneous tumors across different groups (F). (G, H) Tumor tissues from each group were paraffin-embedded and sectioned for staining. Multiplex immunofluorescence was used to detect the polarization proportions of N1 (iNOS + Ly6G) and N2 (CD206 + Ly6G) TANs in the tumor tissues (G). Quantitative analysis of the percentage of N1/N2 polarized neutrophils within the total neutrophil population in subcutaneous tumors across different groups (H) ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

concentrations were significantly associated with the TNM staging of CRC prognosis, particularly showing a negative correlation with regional lymph node involvement (Fig. 6C, Table 2). Given that lymph node metastasis often indicates cancer dissemination, the negative correlation of BHB with such metastasis suggests that BHB may serve as a potential marker for diagnostic prognosis.

To further validate that BHB mitigates CRC progression by

modulating the polarization phenotype of TANs, we collected CRC tissue samples from patients with either normal or elevated BHB levels, indicative of a nutritional ketogenic state. Our results demonstrated that patients with elevated BHB levels exhibited reduced overall TANs infiltration in TME compared to those with normal BHB levels (Figs. 6D, 6E). This reduction primarily involved pro-tumor N2 (CD206⁺) TANs, whereas anti-tumor N1 (TNF- α ⁺) TANs were significantly increased

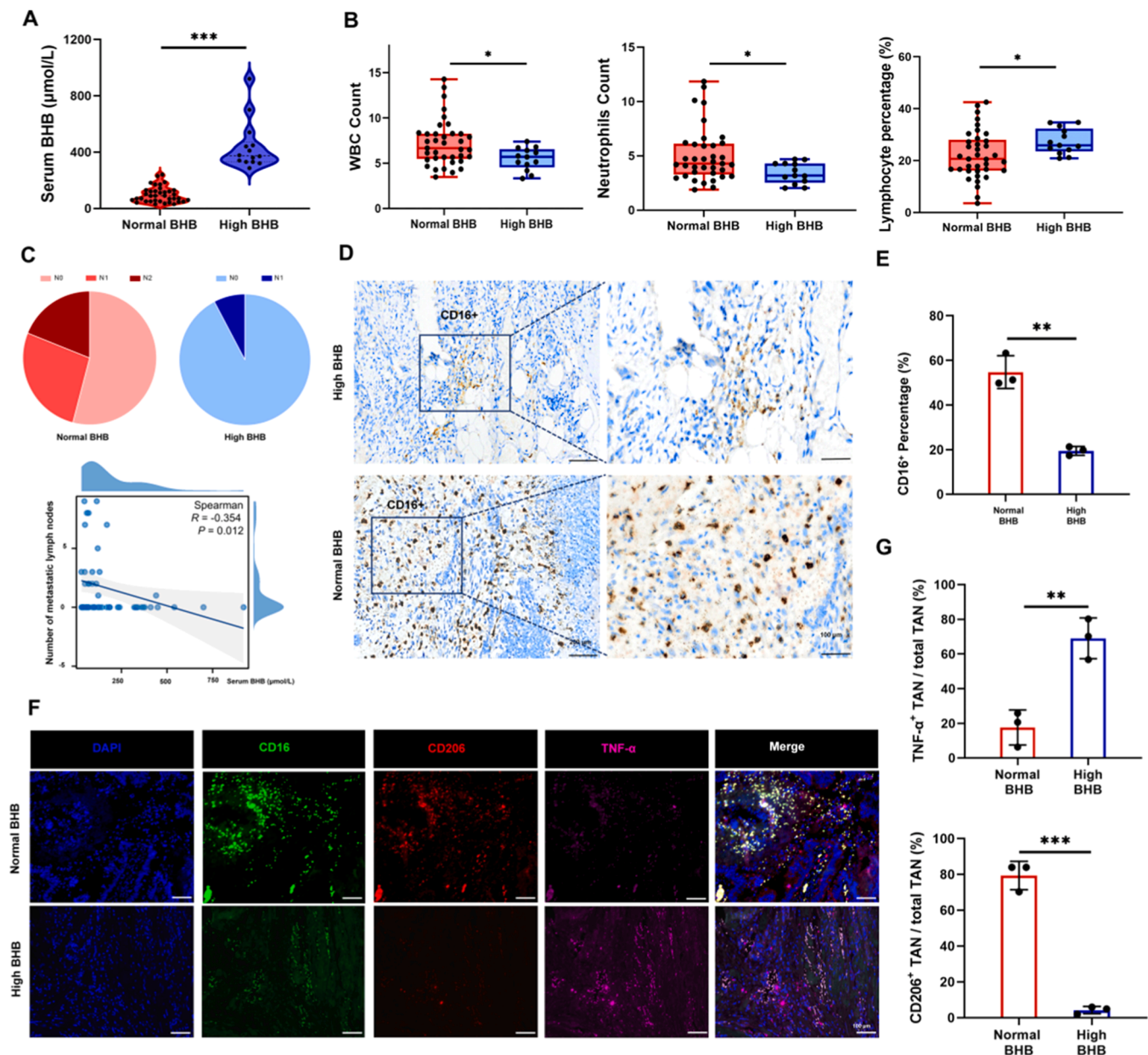


Fig. 6. Reduced neutrophil counts and negative prognosis correlation with elevated serum BHB levels in CRC patients. (A) The cohort is divided into a BHB normal group ($n = 37$) and a BHB high group ($n = 13$). (B) The box plots display the comparison of WBC counts, neutrophil counts, and the proportion of lymphocytes in serum samples from patients in the normal BHB group and the high BHB group. (C) The comparison of lymph node metastasis grading between the normal BHB group and the high BHB group in CRC patients, and the correlation analysis between the number of lymph node metastases and serum BHB concentration in CRC patients. (D, E) Immunohistochemical analysis was performed to assess neutrophil infiltration in colorectal cancer tissues from patients in both the BHB normal and high groups. Quantitative analysis was conducted on the proportion of neutrophils relative to the total cell count ($n = 3$). (F, G) Multiplex immunofluorescence staining was employed to evaluate the polarization of neutrophils into N1 and N2 phenotypes in colorectal cancer tissues from patients in the BHB normal and high groups. Quantitative analysis was performed on the proportion of N1 and N2 type neutrophils among the total infiltrating neutrophils ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Figs. 6F, 6G).

By combining experimental findings with clinical data, we have conclusively demonstrated that BHB regulates CRC progression and prognosis by influencing TANs in the tumor immune microenvironment. Our study indicates that BHB holds significant clinical application value and may serve as a potential biomarker for predicting CRC prognosis.

4. Discussion

Until now, the influence of KD and its primary metabolite, BHB, on the tumor immune microenvironment has been inadequately explored.

This study provides a more detailed understanding by combining experimental and clinical data analyses to investigate the impact of KD on neutrophils in the CRC tumor microenvironment. Our findings reveal that KD and BHB play critical roles in modulating the tumor immune microenvironment and influencing CRC progression. Prior research has predominantly concentrated on the direct impacts of KD and BHB on tumor cells, such as their inhibition of tumor progression via the Hcar2-HOPX axis[6]. Recent studies have extended these observations, showing that KD can alter macrophage phenotypes within the TME, reduce circulating neutrophil levels, and inhibit neutrophil chemotaxis and recruitment to inflammatory sites[28]. Building on these findings,

Table 1
Comparison of clinical characteristics according to serum BHB Levels in CRC patients.

Characteristics	BHB		P value
	> 270 μmol/L (High) n = 13	20–270 μmol/L (Normal) n = 37	
Height	163.69 ± 7.8037	162.16 ± 7.9005	0.55
Weight	62.615 ± 11.155	61.611 ± 10.853	0.78
Age	66.923 ± 16.805	70.297 ± 8.2961	0.5
WBC count	5.69 (4.85, 6.41)	6.67 (5.56, 8.24)	0.02
Neutrophil count	3.18 (2.68, 4.28)	4.28 (3.4, 6.06)	0.02
Lymphocyte count	1.3054 ± 0.3935	1.5981 ± 0.66086	0.07
Monocyte count	0.42 (0.35, 0.54)	0.47 (0.39, 0.66)	0.25
Neutrophil percentage	61.6 ± 4.3761	67.203 ± 10.717	0.01
Lymphocyte percentage	27.362 ± 4.875	22.149 ± 9.2958	0.01
Monocyte percentage	8.5462 ± 2.2678	7.8892 ± 2.9791	0.47
T, stage n (%)			0.27
T1	1 (2 %)	0 (0 %)	
T2	2 (4 %)	4 (8 %)	
T3	9 (18 %)	29 (64 %)	
T4	1 (2 %)	1 (2 %)	
N, stage n (%)			0.04
N0	12 (24 %)	20(40 %)	
N1	1 (2 %)	10(20 %)	
N2	0 (0 %)	7(14 %)	
M, stage n (%)			1
M0	13 (26 %)	36(72 %)	
M1	0 (0 %)	1 (2 %)	
Bile acids	1.3 (1.1, 2.3)	3.5 (2.2, 5)	0.01
Total cholesterol	4.3169 ± 1.0549	4.4392 ± 1.0505	0.72
Blood glucoser	6.1 ± 1.5716	5.3162 ± 1.1904	0.07

our study further explores the effects of KD and BHB on neutrophil polarization within the CRC tumor microenvironment and elucidates potential mechanisms, offering new insights for adjunctive cancer immunotherapy.

Kim et al. has documented that KD can impede colorectal tumor advancement by inducing oxidative stress, inhibiting MMP-9 expression, and shifting tumor-associated macrophages (TAMs) from the M2 to the M1 phenotype[29–31]. Additionally, KD has been shown to influence the epigenetic and immune landscape of prostate cancer, affecting CD8⁺ T cell chemotaxis, balancing M1/M2 macrophages, and modulating monocyte recruitment to overcome resistance to immune checkpoint blockade[8,32]. Building on these studies, our research, utilizing RNA-seq combined with immune infiltration analysis, discovered that KD significantly regulates neutrophil infiltration in the TME of CRC.

Neutrophils are traditionally recognized as first responders in the immune system, exhibiting complex roles in tumor regulation by polarizing into two main phenotypes: N1 (anti-tumor) and N2 (pro-tumor)[33,34]. Within the TME, the polarization of TANs is influenced by various factors. Hypoxic conditions and lactate accumulation typically promote N2 polarization, thereby supporting tumor progression [35–39]. KD, by restricting carbohydrate intake, increases the concentration of ketone bodies (such as BHB) in the blood. These ketone bodies are metabolized more efficiently in tumor cells, potentially reducing lactate accumulation and thereby mitigating the hypoxic-driven

promotion of N2 polarization, which in turn may inhibit tumor progression[40–46].

Our findings highlight the potential of targeting neutrophil polarization for cancer therapy. Specifically, KD-fed mice displayed a significant reduction in N2-type neutrophil infiltration within the CRC TME and an increase in chemokines associated with anti-tumor N1 TANs. This suggests that KD’s inhibition of tumor progression is closely related to its modulation of TANs polarization. And further experiments revealed that BHB promotes the polarization of TANs towards the anti-tumor N1 phenotype while reducing pro-tumor N2 TANs. After co-cultured with BHB-treated TANs, the invasive and migratory capabilities of CRC cells diminish significantly. Subcutaneous tumor models of MC38 in NOD.CB17-*Prkdc^{scid}Il2rg^{tm1}*/Bcgen mice further corroborated these results, showing that KD combined with N2 TANs injection led to slower tumor growth and predominantly anti-tumor N1 TANs infiltration.

At the molecular level, NF-κB and STAT3 signaling pathways are known to drive the polarization of TANs towards the N2 phenotype[24, 46]. However, our research has uncovered a potential key pathway through which BHB influences TANs polarization. Interestingly, BHB inhibited the transcriptional activation of the Hippo pathway by interacting with the intracellular proteins HDAC3 and AMOT in neutrophils, thereby inhibiting the polarization of TANs towards the pro-tumor N2 type. In this study, we chose to focus on the Hippo signaling pathway because it was identified as the top canonical pathway associated with inflammation and tumors in our KEGG enrichment analysis of transcriptome sequencing results. Previous studies have demonstrated that the Hippo pathway plays a crucial role in regulating the polarization of tumor-associated macrophages. However, there is currently no literature indicating whether the Hippo pathway is involved in regulating neutrophil polarization. Based on this intriguing new finding, we ultimately selected the Hippo pathway, with AMOT as its primary regulatory component, for further investigation. In conjunction with clinical data analysis, we also found that high BHB levels in CRC patients were closely related to an increase in N1 neutrophils and a decrease in N2 neutrophils in the TME, suggesting its potential role as a biomarker for CRC prognosis.

Despite these significant advances, the study has limitations. The KD’s high-fat content and unpalatability may hinder long-term adherence[7]. Balancing the diet’s anti-cancer benefits with its practical sustainability remains a challenge. Moreover, while we elucidated the regulatory mechanisms of BHB in neutrophil polarization, further molecular-level research is necessary to fully understand these pathways. Large-scale clinical trials are required to confirm the safety and efficacy of KD for CRC treatment and to evaluate its long-term effects on other organs. Optimizing the KD’s composition to enhance its anti-cancer effects while minimizing adverse reactions is essential. Through interdisciplinary collaboration across nutrition, oncology, and immunology, the KD and BHB have the potential to emerge as viable adjunctive treatments for cancer, offering new diagnostic and therapeutic insights.

In summary, we systematically investigate the anti-tumor effects and mechanisms of KD and its primary ketone body BHB in CRC via mouse

Table 2
Associations between clinical characteristics and serum BHB levels: univariate and multivariate analysis.

Characteristics	Total (n)	OR (95 % CI)	P value	OR (95 % CI)	P value
		Univariate analysis	Univariate analysis	Multivariate analysis	Multivariate analysis
WBC count	50	1.628 (1.049 – 2.527)	0.03	1.216 (0.490 – 3.018)	0.67
Neutrophil count	50	1.844 (1.039 – 3.274)	0.04	1.391 (0.349 – 5.545)	0.64
Neutrophil percentage	50	1.073 (0.991 – 1.163)	0.08	1.017 (0.784 – 1.319)	0.90
Lymphocyte percentage	50	0.927 (0.854 – 1.006)	0.07	0.983 (0.794 – 1.215)	0.87
N, stage n	50				
No	32	Reference		Reference	
Yes	18	10.200 (1.200 – 86.691)	0.03	2.574 (0.212 – 31.307)	0.46
Bile acids	50	1.906 (1.143 – 3.179)	0.01	2.138 (0.988 – 4.628)	0.05

models and clinical cohort observations. The KD significantly inhibits tumor growth and metastasis, primarily by regulating the polarization state and functional changes of neutrophils in the TME. This reprogramming can lead to a more robust anti-tumor immune response, providing new insights for cancer treatment and demonstrating significant clinical application potential.

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CRediT authorship contribution statement

Lijun Meng: Validation, Methodology. **Huihui Yao:** Validation, Methodology. **Xinyu Shi:** Validation, Methodology. **Xiaoshan Yang:** Validation, Methodology. **Yudong Duan:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Xiuwei Mi:** Writing – review & editing, Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Songbing He:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Qingliang Tai:** Validation, Methodology. **Sheng Xiao:** Writing – review & editing, Methodology. **Jiying Sun:** Validation, Methodology. **Yizhou Yao:** Writing – review & editing, Validation, Methodology, Conceptualization. **Junjie Chen:** Validation, Methodology. **Bo Shi:** Validation, Methodology. **Diyuan Zhou:** Validation, Methodology. **Liang Sun:** Validation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.phrs.2024.107494](https://doi.org/10.1016/j.phrs.2024.107494).

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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