

Research Article

Gut Microbiota-Derived Butyrate Induces Trained Immunity in Intestinal Macrophages to Potentiate Antitumor Immunity

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Abstract

The gut microbiota metabolite butyrate plays an important role in maintaining intestinal immune homeostasis, but it's still unclear whether it can induce trained immunity in intestinal macrophages and thus affect anti-tumor immune responses. This study aimed to investigate the ability of butyrate to induce trained immunity in intestinal macrophages and its function and mechanism in anti-tumor immunity. We used an MC38 colorectal cancer mouse model, combined with strategies such as broad-spectrum antibiotics to clear gut microbiota, butyrate supplementation, colonization with butyrate-producing bacteria, macrophage depletion, and adoptive transfer. In vitro, we pretreated bone marrow-derived macrophages and intestinal lamina propria macrophages with butyrate, allowed them to rest, then gave a secondary stimulus to assess cytokine secretion, phagocytic function, metabolic reprogramming, and epigenetic modifications. RNA-seq, ATAC-seq, and CUT&Tag were used to analyze the molecular mechanisms. Butyrate pretreatment induced trained immunity in macrophages, showing significantly enhanced TNF- α , IL-6, and IL-1 β secretion after a second stimulus, increased glycolysis, and enriched H3K4me3 modifications at key inflammatory gene promoters. Mechanistically, butyrate drives epigenetic reprogramming and metabolic remodeling in macrophages by inhibiting histone deacetylase activity and activating the GPR43-mTOR-HIF-1 α signaling axis. In tumor-bearing mice, butyrate supplementation or colonization with butyrate-producing bacteria significantly enhanced the anti-tumor phenotype of intestinal macrophages, promoted CD8⁺ T cell infiltration and activation in tumors, and suppressed tumor growth; this effect was markedly weakened in mice with macrophage-specific trained immunity deficiency. Gut microbiota-derived butyrate induces trained immunity in intestinal macrophages, reshapes the tumor immune microenvironment,

and enhances anti-tumor immune responses, offering new ideas for tumor immunotherapy targeting the microbiota metabolite-macrophage trained immunity axis.

Keywords

Gut microbiota, Butyrate, Trained immunity, Intestinal macrophages, Antitumor immunity

1. Introduction

The gut microbiota, as an important “metabolic organ” of the body, profoundly regulates the development, maturation, and function of the host immune system through its metabolic products, and has become a research frontier in tumor immune regulation. A large body of evidence shows that the composition of the gut microbiota and its metabolic profile are closely related to the occurrence and development of various tumors and their response to immunotherapy. Specific microbial metabolites can reshape the function of immune cells in the tumor microenvironment either directly or indirectly, affecting the strength and quality of antitumor immune responses. Among these, short-chain fatty acids (SCFAs)-especially butyrate-are the main metabolites produced from dietary fiber by gut microbiota fermentation, playing multiple immune-regulating roles such as maintaining intestinal immune homeostasis, regulating T cell differentiation, promoting regulatory T cell generation, and enhancing gut barrier function. Butyrate mainly works by inhibiting histone deacetylases (HDACs) and activating G protein-coupled receptors like GPR43 and GPR109A, thereby regulating at the epigenetic and signaling levels. However, the long-term functional reprogramming effects of butyrate on gut innate immune cells, especially macrophages, are still far from fully understood.

Trained immunity is an important breakthrough concept in the field of innate immunity in recent years. It refers to the phenomenon where innate immune cells, after first being exposed to certain stimuli (like β -glucan, BCG vaccine, oxidized LDL, etc.), go through metabolic reprogramming and epigenetic modifications, and can maintain an enhanced secondary response even after the stimulus is gone. Macrophages, as one of the core effector cells in trained immunity, develop this trained state through increased glycolysis, accumulation of TCA cycle intermediates, and enrichment of activating histone marks like H3K4me3 at key pro-inflammatory gene promoters. The concept of trained immunity challenges the traditional idea that ‘innate immunity has no memory’ and offers new strategies for boosting anti-tumor immunity by regulating innate immune cells. Studies have shown that inducing a trained immunity phenotype in tumor-associated macrophages can enhance their anti-tumor activity and promote T cell-mediated tumor clearance, showing significant translational potential.

It's worth noting that macrophages in the gut's lamina propria are long-term residents in a microenvironment rich in microbial metabolites, constantly exposed to SCFAs like butyrate. This

suggests they might naturally exist in a kind of ‘trained-like’ state shaped by these microbial metabolites. However, whether butyrate can directly trigger classic trained immunity in macrophages, the molecular mechanisms behind it, and whether this trained immunity can be harnessed to boost anti-tumor immune responses are still not well studied. Particularly, butyrate has traditionally been seen as an anti-inflammatory metabolite, so whether it can actually promote trained immunity under certain conditions is an important scientific question. Answering this could not only deepen our understanding of microbiota-innate immune interactions but also offer new targets for cancer immunotherapy based on microbial metabolites.

Based on the research background mentioned above, this study proposes the hypothesis that butyrate derived from gut microbiota can induce metabolic reprogramming and epigenetic remodeling of intestinal macrophages by activating the GPR43-mTOR-HIF-1 α signaling axis and inhibiting HDAC activity, thereby establishing a trained immune state and enhancing their anti-tumor immune function. To test this hypothesis, we used the MC38 colorectal cancer-bearing mouse model, combined with methods like microbiota depletion, butyrate supplementation, colonization with butyrate-producing bacteria, and macrophage-specific interventions, to systematically explore the induction effect of butyrate on trained immunity in intestinal macrophages and its role and mechanism in anti-tumor immunity both in vivo and in vitro. This study is expected to reveal a new regulatory network between microbiota metabolites-macrophage trained immunity-anti-tumor immunity, providing theoretical basis and experimental support for tumor immunotherapy strategies targeting the gut microbiota.

2. Literature Review

Gut microbiota, through their metabolic products, play a deep role in maintaining host immune balance and regulating the progression of diseases, and this idea has gained wide acceptance (Belkaid & Hand, 2014; Honda & Littman, 2016; Liu, 2025). In recent years, several landmark studies have revealed a close link between gut microbiome composition and tumor immunotherapy responses: in patients with melanoma and epithelial tumors, certain microbial features can predict the effectiveness of anti-PD-1 immunotherapy, and fecal transplants or colonization with specific strains can restore or boost the anti-tumor effects of immune checkpoint inhibitors (Matson et al., 2018; Routy et al., 2018). Besides, the gut microbiome also plays a key role in regulating the effectiveness of anti-CTLA-4 treatment (Vétizou et al., 2015). It shows that the gut microbiota-immune-tumor axis has become an important research direction in tumor immunology (Zitvogel et al., 2015). However, the molecules and cellular mechanisms through which the microbiome regulates anti-tumor immunity are still far from being fully understood, especially the role of microbiome metabolites in shaping the long-term functional state of innate immune cells hasn’t received much attention yet.

Short-chain fatty acids (SCFAs) are the main metabolic products produced when dietary fiber is fermented by gut bacteria, and butyrate has become a research focus because of its wide-ranging immune-regulating activities (Koh et al., 2016; Louis et al., 2014). At the level of adaptive immunity, butyrate has been shown to promote histone acetylation at the Foxp3 gene site by inhibiting HDAC activity, inducing the differentiation of colonic regulatory T cells (Tregs), and thereby maintaining intestinal immune tolerance (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). At the level of innate immunity, butyrate can suppress inflammatory responses by activating receptors like GPR43 (Maslowski et al., 2009). At the same time, it regulates the gene expression programs of gut macrophages through HDAC inhibition, affecting their antibacterial and inflammatory functions (Chang et al., 2014). Interestingly, Schulthess and others (2019) found that butyrate can alternatively activate macrophages and give them enhanced antibacterial programs. This “imprinting” effect suggests that butyrate might have long-term functional reprogramming abilities beyond just acute regulation. In addition, activating the butyrate receptor GPR109A can suppress colon inflammation and cancer development (Singh et al., 2014). It further highlights the dual role of butyrate signaling in gut immunity and tumor development.

Macrophages are one of the most abundant immune cells in the tumor microenvironment, and they are highly versatile. They can boost anti-tumor immunity by releasing inflammatory factors and presenting antigens, but under the influence of signals in the tumor microenvironment, they can also shift to an immune-suppressive state and help tumor progression (Noy & Pollard, 2014). So, reshaping the functional state of tumor-associated macrophages has become an important strategy in cancer immunotherapy. However, current research on regulating macrophage functions mostly focuses on acute activation or chronic polarization, and our understanding of whether they can be induced into a lasting ‘enhanced response’ state for anti-tumor immunity is still very limited.

The concept of trained immunity offers a brand-new perspective on the functional flexibility of innate immune cells. Netea and colleagues (2016; 2020) clearly explained the definition of trained immunity: after innate immune cells are first exposed to stimuli like β -glucan or BCG, they gain an enhanced ability to respond a second time through metabolic reprogramming and epigenetic modifications. Mechanistically, the establishment of trained immunity relies on enhanced glycolysis, accumulation of tricarboxylic acid cycle metabolites (like fumarate), and activation of the mTOR-HIF-1 α signaling pathway (Cheng et al., 2014; Arts et al., 2016). Besides terminally differentiated cells, the induction of trained immunity can also happen at the level of bone marrow hematopoietic progenitor cells, which can reshape myeloid hematopoiesis and lead to lasting changes in innate immune responses (Mitroulis et al., 2018). In the field of cancer treatment, Kalafati and others (2020) confirmed that trained immunity induced by β -glucan can promote granulopoiesis and boost anti-tumor activity, suggesting that trained immunity has potential to be turned into a cancer

immunotherapy strategy.

Although the research mentioned above has made significant progress, there are still some shortcomings in the existing literature: First, while butyrate is widely reported to have anti-inflammatory and immune tolerance-inducing functions, it's still unclear whether it can induce classical trained immunity in macrophages under certain conditions; second, the mechanisms by which microbial metabolites reprogram the long-term functions of intestinal macrophages, especially the causal relationship between metabolic reprogramming and epigenetic modifications, haven't been systematically analyzed; third, whether trained immunity induced by microbial metabolites can enhance anti-tumor immune responses in solid tumor models still lacks direct functional evidence; fourth, most existing trained immunity studies use classical inducers like β -glucan, paying little attention to the role of metabolites from normal gut microbiota.

The contribution of this study is that it systematically shows for the first time that butyrate from gut microbes can directly trigger trained immunity in intestinal macrophages. It also reveals that this process relies on the GPR43-mTOR-HIF-1 α signaling axis and HDAC inhibition to drive metabolic and epigenetic mechanisms. On the *in vivo* level, it confirms that butyrate-induced trained immunity in macrophages can reshape the tumor microenvironment and boost anti-tumor immunity, providing new theoretical and experimental support for tumor immunotherapy targeting the gut microbe metabolite–innate immune memory axis.

3. Materials and Methods

3.1. Experimental animals

SPF-grade male C57BL/6J mice (6-8 weeks old, weighing 18-22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in a barrier environment with constant temperature (22 $\pm$ 1 $^{\circ}$ C), constant humidity (55% $\pm$ 5%), a 12-hour light/dark cycle, and free access to food and water. Macrophage-specific HIF-1 α knockout mice (Hif1a^{fl/fl} Lyz2-Cre, hereafter referred to as HIF-1 α^{Δ Myc) and control littermates (Hif1a^{fl/fl}) were bred and maintained in our lab.

3.2. Cell lines and cell culture

The mouse colon cancer cell line MC38 (from the Cell Bank of the Chinese Academy of Sciences) is cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μ g/mL streptomycin, and kept in a 37 $^{\circ}$ C, 5% CO₂ incubator. Cells are passaged every two days, and cells in the logarithmic growth phase are used for *in vivo* injections. Bone marrow-derived macrophages (BMDMs) are obtained by differentiating mouse bone marrow cells with 20 ng/mL recombinant mouse M-CSF (PeproTech) for 7 days and are cultured in RPMI 1640 medium with 10% FBS. Intestinal lamina propria macrophages are isolated using Collagenase D

(Roche) digestion, and after CD11b magnetic bead (Miltenyi Biotec) positive selection, they are used for in vitro experiments.

3.3. Strain culturing and butyrate preparation

The butyrate-producing strain *Faecalibacterium prausnitzii* (ATCC 27768) was cultured in YCFA medium in an anaerobic workstation (Don Whitley Scientific, 85% N₂, 10% H₂, 5% CO₂). Cells were collected during the logarithmic growth phase, washed with sterile PBS, and resuspended to 5×10⁹ CFU/mL, freshly prepared before gavage. Sodium butyrate (Sigma-Aldrich) was prepared in sterile PBS as a 200 mg/mL stock solution and diluted to the required concentration before use. β-Glucan (Sigma-Aldrich, from brewer's yeast) was prepared in PBS at 10 mg/mL, with a working concentration of 1 μg/mL.

3.4. Animal models and in vivo interventions

Take logarithmically growing MC38 cells and resuspend them in serum-free DMEM to 2×10⁶ cells/100 μL, then inject subcutaneously into the right back of mice. Tumor volume is calculated as $V = (\text{length} \times \text{width}^2)/2$. When the tumor is palpable (about 3-5 mm), randomly group the mice and start the treatment. Mice were allowed to freely drink water containing broad-spectrum antibiotics (ampicillin 1 g/L, vancomycin 0.5 g/L, neomycin 1 g/L, metronidazole 1 g/L) for 2 weeks to clear their gut microbiota. Afterwards, they were used for butyrate supplementation or butyrate-producing bacteria colonization experiments. The effectiveness of microbiota clearance was verified by fecal 16S rRNA qPCR. Mice in the butyrate supplementation group were given daily oral gavage of sodium butyrate (200 mg/kg, 200 μL) starting one week before tumor inoculation. Mice in the butyrate-producing bacteria colonization group were gavaged with *F. prausnitzii* (1×10⁹ CFU/200 μL) every other day starting one week before tumor inoculation. The control group was gavaged with the same volume of PBS. Macrophage clearance: Mice were injected intraperitoneally with clodronate liposomes (200 μL per mouse, Liposoma B.V.) one day before tumor implantation and every 5 days afterward, while the control group received the same amount of PBS liposomes. Adoptive transfer: BMDMs trained in vitro with butyrate or β-glucan were injected into tumor-bearing mice via the tail vein at 5×10⁶ cells per mouse every 5 days, for a total of 3 times.

3.5. In vitro macrophage training for immune activation

BMDMs or intestinal lamina propria macrophages were seeded at 5×10⁵ cells per well in 12-well plates and left overnight. The training group was pretreated with sodium butyrate (0.5 mM) or β-glucan (1 μg/mL) for 24 hours, while the control group received an equal volume of culture medium. After removing the stimuli, the cells were rested in complete medium for 5 days. After the resting period, they were re-stimulated for 24 hours with lipopolysaccharide (LPS, 100 ng/mL) or heat-killed

E. coli (MOI=10), and the supernatants and cells were collected for further analysis.

To verify the role of the GPR43-mTOR-HIF-1 α signaling axis, the following inhibitors were added 30 minutes before butyrate pretreatment: GPR43 antagonist GLPG0974 (10 μ M, MedChemExpress), mTOR inhibitor rapamycin (100 nM, Sigma-Aldrich), and HIF-1 α inhibitor PX-478 (50 μ M, Selleck Chemicals). Supernatants were collected after a second stimulation to detect cytokines. Additionally, an HDAC inhibitor, trichostatin A (TSA, 50 nM, Sigma-Aldrich), was used as a positive control to confirm the epigenetic mechanism.

3.6. Detection of cytokines and effector molecules

The concentrations of TNF- α , IL-6, and IL-1 β in the cell culture supernatant were measured using ELISA kits (R&D Systems) following the instructions. Cell phagocytosis was assessed by labeling with pHrodo Red *E. coli* bioparticles (Thermo Fisher) and analyzed by flow cytometry, with the phagocytic index calculated as the percentage of positive cells $\times$ mean fluorescence intensity. Reactive oxygen species (ROS) levels were measured by flow cytometry using the DCFH-DA probe (Beyotime).

3.7. Metabolic analysis

BMDMs were seeded at 1×10^5 per well in a Seahorse XF96 cell culture plate and handled according to the Seahorse XF Glycolysis Stress Test Kit (Agilent) instructions. Glucose (10 mM), oligomycin (1 μ M), and 2-deoxyglucose (50 mM) were added sequentially, and the extracellular acidification rate (ECAR) was monitored in real time. Glycolytic capacity = ECAR after oligomycin – baseline ECAR.

Cells were collected after resting and before secondary stimulation, quenched with liquid nitrogen, and extracted with 80% methanol. Intracellular levels of metabolites like lactate, fumarate, succinate, and acetyl-CoA were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS, AB Sciex 6500) and corrected with corresponding isotope internal standards. The data were processed using MultiQuant software.

3.8. Flow cytometry analysis

Tumor tissues were digested with collagenase IV (1 mg/mL, Sigma-Aldrich) and DNase I (0.1 mg/mL) at 37°C for 30 minutes, and then filtered through a 70 μ m strainer to get a single-cell suspension. After blocking with Fc receptor blocker (anti-CD16/CD32, BD Biosciences), cells were stained on the surface with the following antibody panel: CD45-AF700, CD11b-BV510, F4/80-PE-Cy7, MHC-II-APC, CD86-BV421, CD206-FITC (all from BioLegend). After fixation and permeabilization, cells were stained with CD8a-PerCP-Cy5.5, IFN- γ -PE, TNF- α -BV605 (BioLegend). Cells were acquired on a BD LSRFortessa flow cytometer and analyzed with FlowJo v10.8.

Macrophages were gated as CD45⁺CD11b⁺F4/80⁺, M1-like as CD86⁺MHC-II⁺, and M2-like as CD206⁺. Tumor-infiltrating CD8⁺ T cell function was assessed by the percentage of IFN- γ ⁺TNF- α ⁺ double-positive cells.

Take a segment of the colon, remove the Peyer's patches, and cut it longitudinally. Use EDTA-DTT to remove the epithelial layer, and digest with collagenase to obtain lamina propria cells. Staining is done the same way as for tumor tissue. Evaluate macrophage trained immunity markers for the proportion of TNF- α IL-6⁺ double-positive cells after secondary stimulation and the average fluorescence intensity of H3K4me3 staining (Cell Signaling Technology, clone C42D8).

3.9. Epigenetics and transcriptome analysis

BMDMs were rested for 5 days and then stimulated a second time with LPS for 6 hours, after which total RNA was extracted using TRIzol. RNA integrity was checked with an Agilent 2100 Bioanalyzer (RIN > 8.0). Libraries were prepared with the NEBNext Ultra II RNA Library Prep Kit and sequenced on the NovaSeq 6000 platform with PE150. Raw data were quality-checked with FastQC, aligned to mm10 using HISAT2, quantified with featureCounts, and differential expression analysis was done with DESeq2 ($|\log_2FC| > 1$, $p_{adj} < 0.05$). KEGG and GO enrichment analyses were done using clusterProfiler.

Take 5×10^4 resting cells after training, prepare a transposase library according to the Omni-ATAC protocol, check the library quality using ViiA 7 real-time qPCR, and sequence on the NovaSeq platform with PE50. The data are aligned with Bowtie2, peaks are called with MACS2, differential open regions are analyzed using diffbind, and ChIPseeker is used to annotate the genomic distribution.

Take 1×10^5 resting cells after training and use the CUT&Tag kit (Vazyme) to detect H3K4me3 modifications. Primary antibody: H3K4me3 (Cell Signaling Technology, 1:100), secondary antibody: goat anti-rabbit IgG. After library construction, sequence on NovaSeq PE150. Data were aligned with Bowtie2, peaks called with SEACR, H3K4me3 enrichment differences between groups compared using DiffBind, and transcription factor binding motif enrichment analyzed with Homer.

3.10. Protein immunoblotting

Total protein was extracted from cells using RIPA lysis buffer and quantified by the BCA method. After separation by SDS-PAGE, the proteins were transferred to a PVDF membrane, blocked with 5% skim milk, and incubated with primary antibodies: p-mTOR (Ser2448), mTOR, HIF-1 α , HDAC1, HDAC3, and β -actin (all from Cell Signaling Technology, 1:1000) overnight at 4°C. The membrane was then incubated with an HRP-conjugated secondary antibody at room temperature for 1 hour, developed with ECL, and the band intensities were analyzed using ImageJ.

3.11. Chromatin immunoprecipitation (ChIP)-qPCR

Collect BMDMs at rest after butyrate treatment, crosslink with 1% formaldehyde for 10 min, then quench with glycine. Sonicate until the DNA fragments reach 200-500 bp, and perform immunoprecipitation using H3K4me3 antibody (Cell Signaling Technology) or control IgG. After elution and reverse crosslinking, purify the DNA and use qPCR to detect the promoter regions of Tnf, Il6, and Il1b (Tnf: F 5'-CCCTCCAGTTCTAGTTCCT-3', R 5'-GGGTGTCCCAAGGAAAGATG-3'; Il6: F 5'-GACATGCTGGTGACAACCAC-3', R 5'-GCCTCCGACTTGTGAAGTGG-3'; Il1b: F 5'-GCAACTGTTCTGAACTCAACT-3', R 5'-ATCTTTTGGGGTCCGTCAACT-3'). Results are shown as a percentage of the Input.

3.12. Statistical analysis

All data are shown as mean $\pm$ SEM and statistical analysis was done using GraphPad Prism 9.0. Comparisons between two groups were done with an unpaired Student's t-test; for multiple groups, one-way ANOVA followed by Tukey's multiple comparison test was used; tumor growth curves were analyzed with two-way ANOVA followed by Bonferroni post hoc test. Each experiment was independently repeated at least three times, and $P < 0.05$ was considered statistically significant. Flow cytometry data were analyzed using FlowJo v10.8, and group comparisons were done with the Mann-Whitney U test.

4. Research Results

4.1. Butyrate pretreatment induces a trained immunity-like enhanced response in macrophages

To figure out whether butyrate can trigger trained immunity in macrophages, we used the classic 'training-rest-re-stimulation' in vitro model. BMDMs were pretreated with 0.5 mM sodium butyrate for 24 hours, then the stimulus was washed away and the cells were allowed to rest for 5 days, followed by a second stimulation with LPS (100 ng/mL) for 24 hours. The results showed that, compared to the untrained control group, macrophages trained with butyrate secreted about 2.4 times more TNF- α , 2.3 times more IL-6, and 2.1 times more IL-1 β (Figure 1A, Table 1), similar to the classic trained immunity inducer β -glucan (1 μ g/mL). At the same time, butyrate-trained macrophages had a notably higher ability to engulf E. coli bioparticles, with the phagocytic index increasing by around 1.8 times (Figure 1B). These findings suggest that butyrate pretreatment can keep macrophages in a heightened inflammatory and phagocytic state even after the initial stimulus has faded, matching the key features of trained immunity.

Table 1

Effects of Butyrate Training on Macrophage Secondary Response and Metabolism (mean $\pm$ SEM, n=6)

Testing Indicators	control group	Butyrate Training Group	β -Glucan Training Group
TNF- α (pg/mL)	512 $\pm$ 45	1236 $\pm$ 112	1488 $\pm$ 135
IL-6 (pg/mL)	835 $\pm$ 76	1902 $\pm$ 168	2105 $\pm$ 187
IL-1 β (pg/mL)	218 $\pm$ 25	462 $\pm$ 41	518 $\pm$ 47
Devouring Index	32.4 $\pm$ 3.1	58.7 $\pm$ 5.2	62.3 $\pm$ 5.8
Basic ECAR (mpH/min)	18.2 $\pm$ 1.6	29.5 $\pm$ 2.3	33.1 $\pm$ 2.8
Maximum glycolysis capacity (mpH/min)	28.7 $\pm$ 2.4	60.4 $\pm$ 5.1	66.8 $\pm$ 5.7
Lactic acid (nmol/10 ⁶ cells)	12.6 $\pm$ 1.2	21.4 $\pm$ 1.9	23.5 $\pm$ 2.0

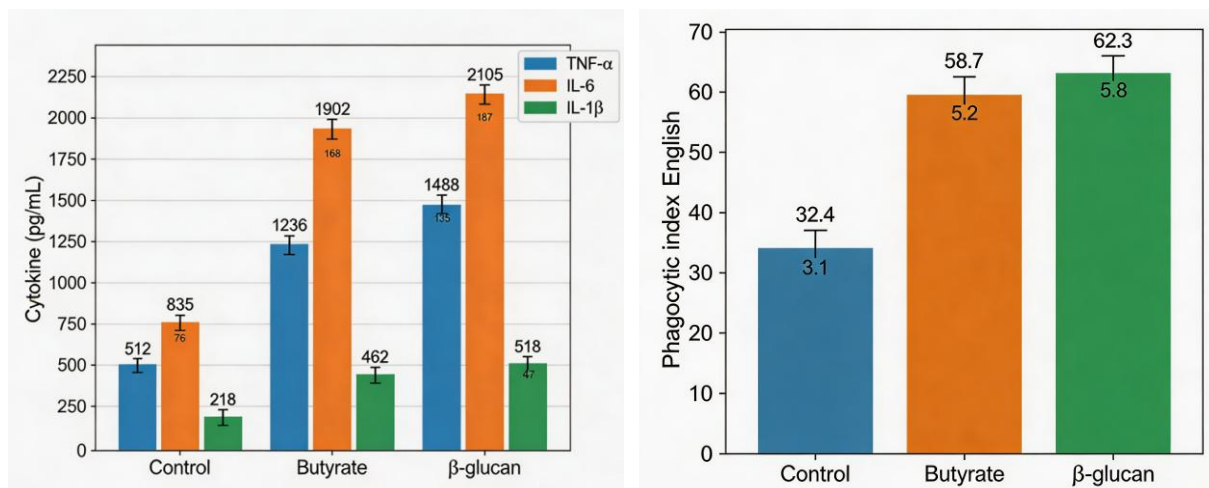
Note. P < 0.05 vs control group (one-way ANOVA, Tukey post hoc)

Further metabolic analysis showed that macrophages in the butyrate-trained group had significantly upregulated glycolysis levels when re-stimulated with LPS after resting. The baseline extracellular acidification rate (ECAR) and maximum glycolytic capacity increased by 1.6-fold and 2.1-fold, respectively (Figure 1C), and intracellular lactate content increased by 1.7-fold (Table 1). This suggests that butyrate training is associated with persistent metabolic reprogramming, with enhanced glycolysis providing energy and building blocks for the cells.

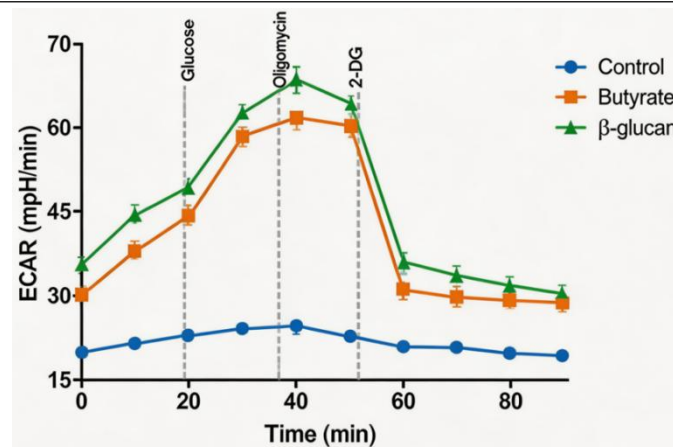
Figure 1

A: Cytokine secretion bar plot

B: Phagocytic index bar plot



C: ECAR glycolysis stress test curve



4.2. Butyrate trains immunity through GPR43-mTOR-HIF-1 α signaling axis and HDAC inhibition

To figure out how butyrate induces trained immunity at the molecular level, we added the GPR43 antagonist GLPG0974 (10 μ M), the mTOR inhibitor rapamycin (100 nM), or the HIF-1 α inhibitor PX-478 (50 μ M) during the butyrate pretreatment phase. The results showed that all three inhibitors could significantly block the enhanced TNF- α and IL-6 secretion caused by butyrate training, bringing them back close to untrained control levels (Figure 2A, Table 2), with rapamycin and PX-478 having especially noticeable effects. At the same time, p-mTOR (Ser2448) and HIF-1 α protein levels were significantly increased in macrophages from the butyrate-trained group, while GLPG0974 pretreatment could block this upregulation (Figure 2B), suggesting that butyrate activates the mTOR–HIF-1 α pathway through GPR43.

Table 2

Effect of inhibitors on TNF- α secretion in butyrate-trained macrophages (mean $\pm$ SEM, n=5)

Treatment group	TNF- α (pg/mL)	Percentage relative to the butyric acid training group (%)
Untrained control	498 $\pm$ 42	39.5
Butyrate training	1261 $\pm$ 109	100
Butyric + GLPG0974	682 $\pm$ 58	54.1
Butyrate Rapamycin	575 $\pm$ 49	45.6
Butyric + PX-478	543 $\pm$ 44	43.1
Butyric + TSA (Alternative Butyric Acid)	1148 $\pm$ 96	91.0

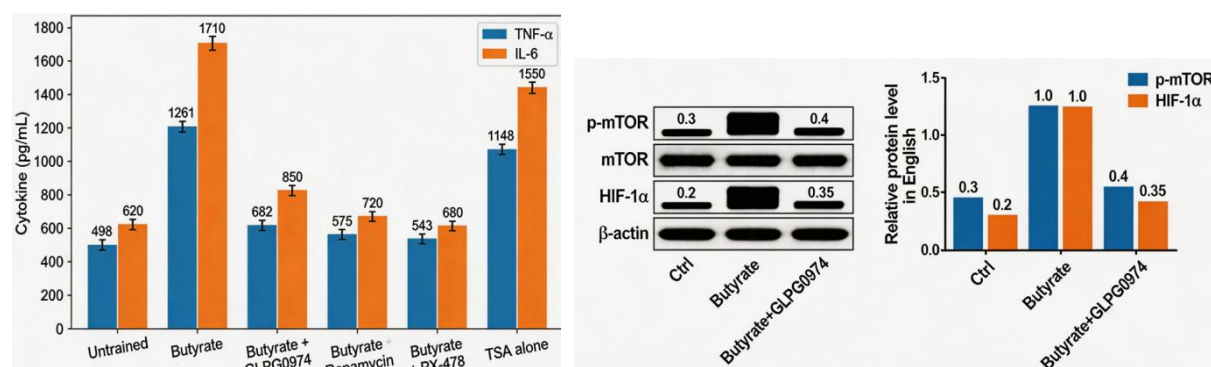
Note. $P < 0.05$ vs untrained control; $P < 0.05$ vs butyrate training group (one-way ANOVA, Tukey post hoc)

In addition, as an HDAC inhibitor, does the training effect of butyrate depend on epigenetic regulation? We checked the enrichment levels of H3K4me3 in the promoter regions of key pro-inflammatory genes in resting macrophages after butyrate training. ChIP-qPCR showed that the enrichment of H3K4me3 at the *Tnf*, *Il6*, and *Il1b* promoters increased by 2.3, 2.0, and 1.8 times in the butyrate-trained group (Figure 2C); pre-treatment with the broad-spectrum HDAC inhibitor TSA (50 nM) could also mimic the training effect of butyrate and boost TNF- α secretion (Table 2), suggesting that HDAC inhibition-mediated epigenetic remodeling plays a key role in butyrate-trained immunity. ATAC-seq further showed that chromatin accessibility at the *Tnf* and *Il6* gene sites significantly increased in the butyrate-trained group (Figure 2D), providing a structural basis for enhanced transcription.

Figure 2

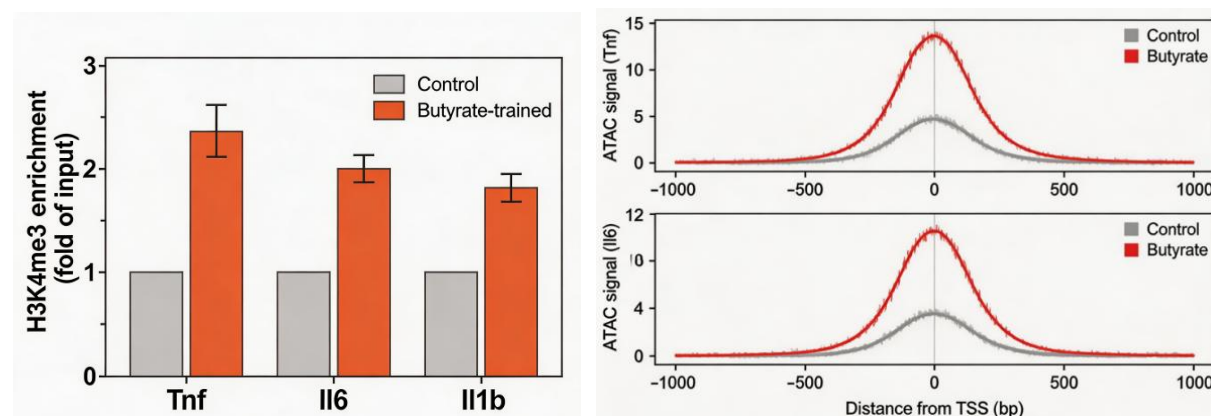
A: Inhibitor effects on TNF- α and IL-6

B: Western blot and quantification



C: ChIP-qPCR H3K4me3 enrichment

D: ATAC-seq signal tracks



4.3. Butyrate training remodels macrophage transcriptome and enriches

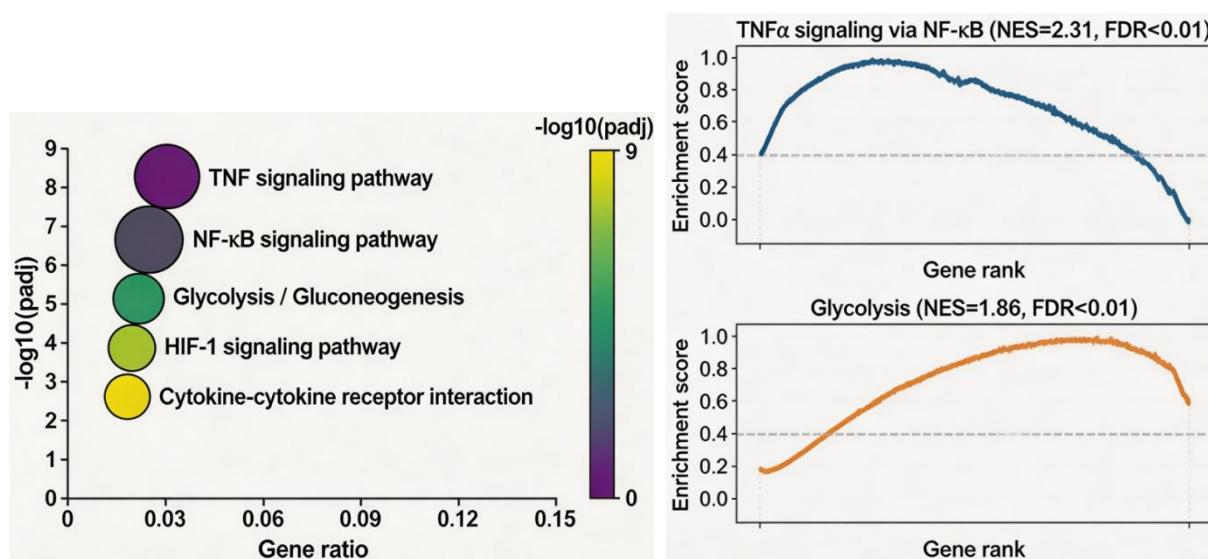
inflammatory and metabolic pathways

We performed RNA-seq analysis on BMDMs that were rested after butyrate training and then stimulated with LPS, identifying a total of 1,247 differentially expressed genes ($|\log_2FC| > 1$, $p_{adj} < 0.05$), with 786 upregulated and 461 downregulated. KEGG enrichment analysis showed that the upregulated genes were significantly enriched in the TNF signaling pathway, NF- κ B signaling pathway, glycolysis/gluconeogenesis, and HIF-1 signaling pathway (Fig. 3A), which is highly consistent with the metabolic and inflammatory features of trained immunity. GSEA analysis further confirmed that the TNF α signaling via NF- κ B and Glycolysis gene sets were significantly enriched in the butyrate-trained group (NES = 2.31 and 1.86, FDR < 0.01) (Fig. 3B). In addition, joint analysis of ATAC-seq and RNA-seq showed that chromatin accessibility in about 32% of the promoters of differentially expressed genes increased simultaneously, suggesting that epigenetic remodeling is closely related to transcriptional activation (Fig. 3C).

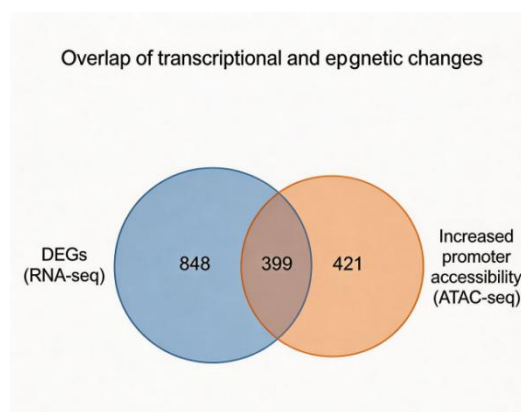
Figure 3

A: KEGG enrichment bubble plot

B: GSEA enrichment plots



C: Overlap of ATAC-seq and RNA-seq



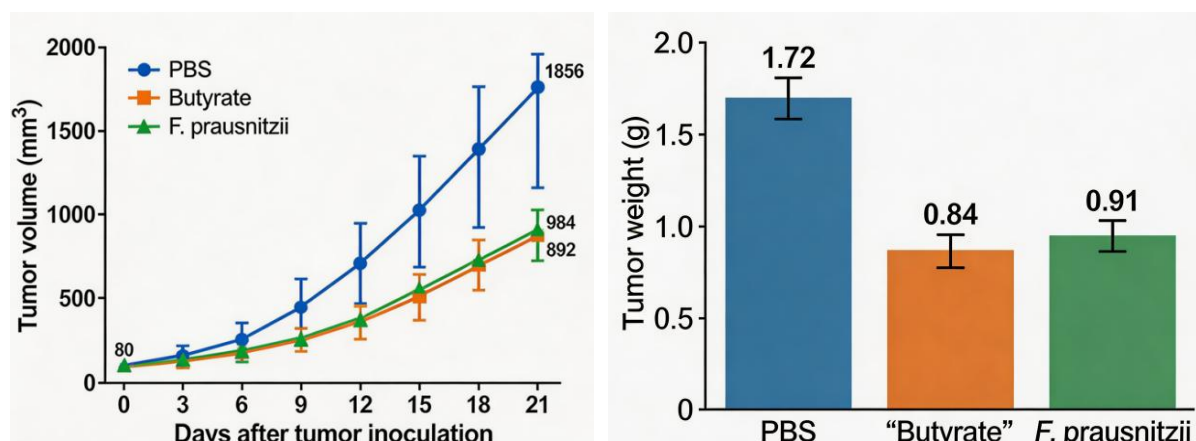
4.4. Butyrate supplementation or colonization with butyrate-producing bacteria boosts anti-tumor immunity through gut macrophages

To see if butyrate-induced trained immunity in macrophages has anti-tumor effects in the body, we set up an MC38 subcutaneous tumor model. Tumor-bearing mice started receiving daily oral gavage of sodium butyrate (200 mg/kg) or every-other-day gavage of butyrate-producing bacteria *F. prausnitzii* (1×10^9 CFU) one week before tumor inoculation. The results showed that tumor growth was significantly slowed in both groups (Figure 4A). By the end of the experiment (day 21), tumor volumes in the butyrate group and bacteria group were reduced by about 52% and 47% compared to the PBS control group, and tumor weights were also correspondingly lower (Figure 4B, Table 3). Flow cytometry analysis of tumor-infiltrating immune cells showed that the proportion of CD8⁺ T cells in the tumor microenvironment was significantly increased in both the butyrate and bacteria groups (control 5.1% vs butyrate 12.8%, $P < 0.01$), and the proportion of functional IFN- γ ⁺TNF- α ⁺ CD8⁺ T cells increased about threefold (Figure 4C, Table 3). At the same time, among tumor-associated macrophages, the proportion with the CD86⁺MHC-II⁺ M1-like phenotype increased, while the CD206⁺ M2-like phenotype decreased (Table 3), indicating enhanced anti-tumor polarization of macrophages.

Figure 4

A: Tumor growth curves

B: Terminal tumor weight/volume



C: Infiltrating immune cells

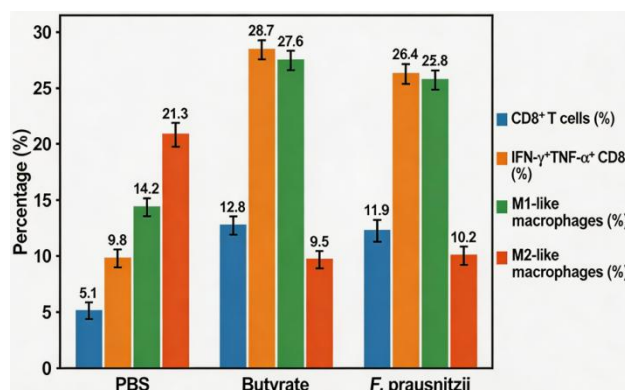


Table 3

Tumor growth and immune infiltration indicators in mice groups (mean $\pm$ SEM, n=8)

Indicator	PBS control group	Butyrate Supplement Group	Butyrate-producing bacteria colonization group
Final tumor volume (mm ³)	1856 $\pm$ 214	892 $\pm$ 103	984 $\pm$ 118
Final tumor weight (g)	1.72 $\pm$ 0.19	0.84 $\pm$ 0.10	0.91 $\pm$ 0.11
CD8 ⁺ T cells (%)	5.1 $\pm$ 0.6	12.8 $\pm$ 1.4	11.9 $\pm$ 1.3
IFN- γ ⁺ TNF- α ⁺ CD8 ⁺ T cells (%)	9.8 $\pm$ 1.1	28.7 $\pm$ 2.6	26.4 $\pm$ 2.4
M1-like Macrophages (%)	14.2 $\pm$ 1.5	27.6 $\pm$ 2.8	25.8 $\pm$ 2.5
M2-like Macrophages (%)	21.3 $\pm$ 2.1	9.5 $\pm$ 1.0	10.2 $\pm$ 1.1

Note. P < 0.05 vs PBS control group (one-way ANOVA, Tukey post hoc)

4.5. Macrophages are a necessary and sufficient mediator of the anti-tumor effects of butyrate

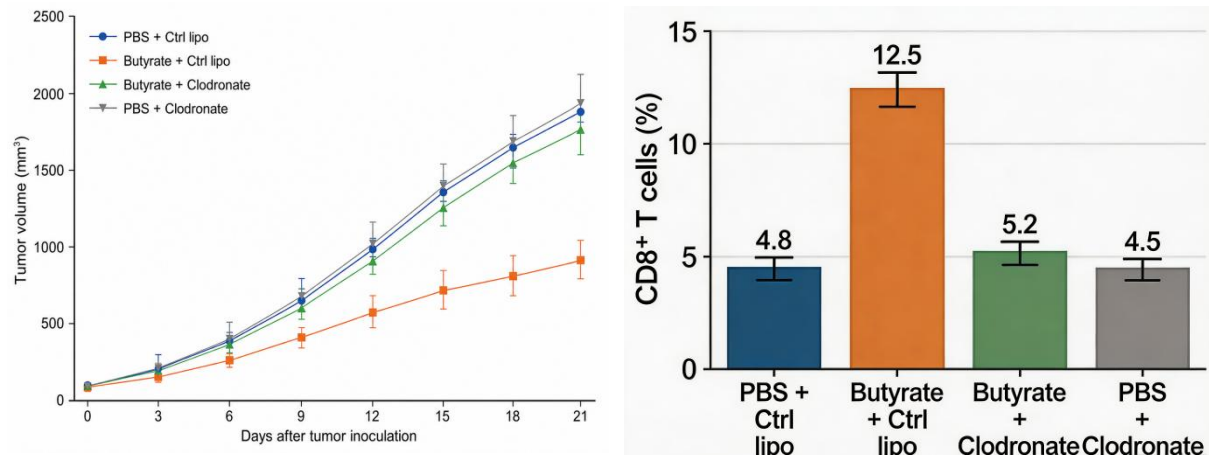
To clarify the key role of macrophages in butyrate's anti-tumor effects, we used clodronate disodium liposomes to deplete macrophages in mice. The results showed that after macrophage depletion, the anti-tumor effect of butyrate supplementation almost completely disappeared, and tumor growth returned to the level of the control group (Fig. 5A), with no significant increase in tumor-infiltrating CD8⁺ T cells (Fig. 5B). On the other hand, adoptive transfer of BMDMs trained with butyrate in vitro into tumor-bearing mice significantly suppressed tumor growth (Fig. 5C) and increased functional CD8⁺ T cell infiltration within the tumor (Fig. 5D), an effect similar to direct butyrate supplementation. Moreover, in macrophage-specific HIF-1 α knockout (HIF-1 α^{Δ} Myc) mice,

the tumor-suppressing effect of butyrate supplementation was significantly weakened (Fig. 5E), and the increase in M1-like polarization of tumor-associated macrophages and CD8⁺ T cell infiltration was noticeably lower than in wild-type mice (Fig. 5F), further proving that the HIF-1 α signaling pathway in macrophages is essential for butyrate-induced trained immunity and anti-tumor effects.

Figure 5

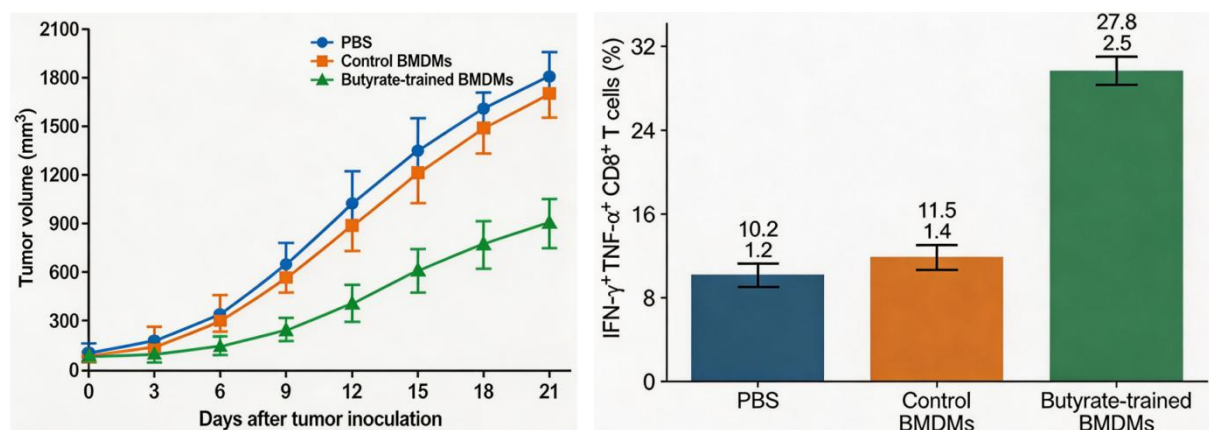
A: Tumor growth after macrophage depletion

B: CD8⁺ T cells after depletion



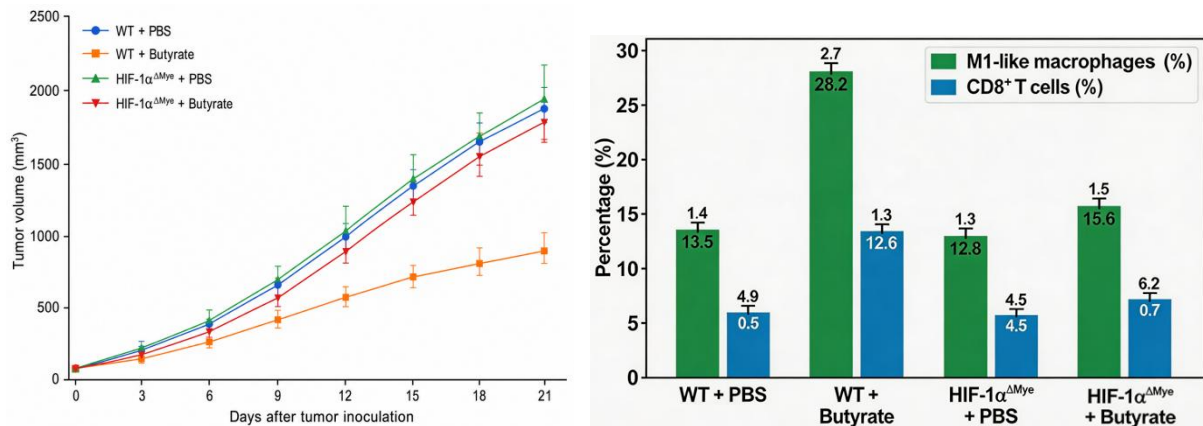
C: Tumor growth after adoptive transfer

D: Functional CD8⁺ T cells after adoptive transfer



E: Tumor growth in HIF-1 α Δ Mye mice

F: Macrophage polarization and CD8⁺ T cells in HIF-1 α Δ Mye



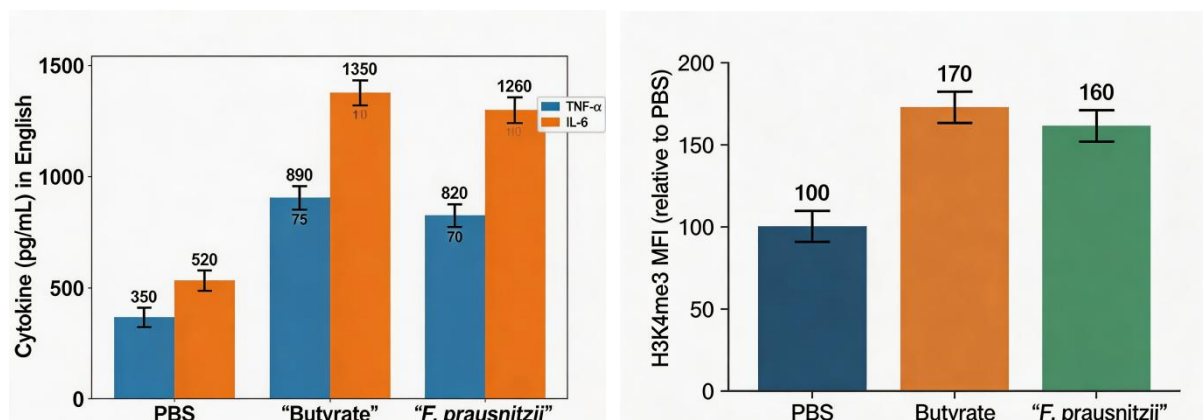
4.6. Butyrate-induced trained immunity accompanied by epigenetic remodeling of intestinal macrophages

To confirm that butyrate intervention in the body can also induce trained immunity in intestinal macrophages, we isolated colonic lamina propria macrophages from mice in each group and measured cytokine secretion and H3K4me3 levels after LPS restimulation *in vitro*. The results showed that the butyrate-supplemented group and the butyrate-producing bacteria colonized group had significantly enhanced TNF- α and IL-6 secretion in intestinal macrophages (Figure 6A), with average H3K4me3 fluorescence intensity increased by 1.7-fold and 1.6-fold, respectively (Figure 6B). At the same time, both the basal ECAR and maximal glycolytic capacity of these macrophages were also significantly elevated (Figure 6C), indicating that *in vivo* butyrate exposure similarly imparted long-lasting metabolic and epigenetic training features to intestinal macrophages. CUT&Tag analysis showed that in the butyrate-supplemented group, H3K4me3 enrichment peaks at the *Tnf* and *Il6* promoter regions of intestinal macrophages were significantly expanded (Figure 6D), consistent with the *in vitro* results, confirming that butyrate induces trained immunity in intestinal macrophages *in vivo*.

Figure 6

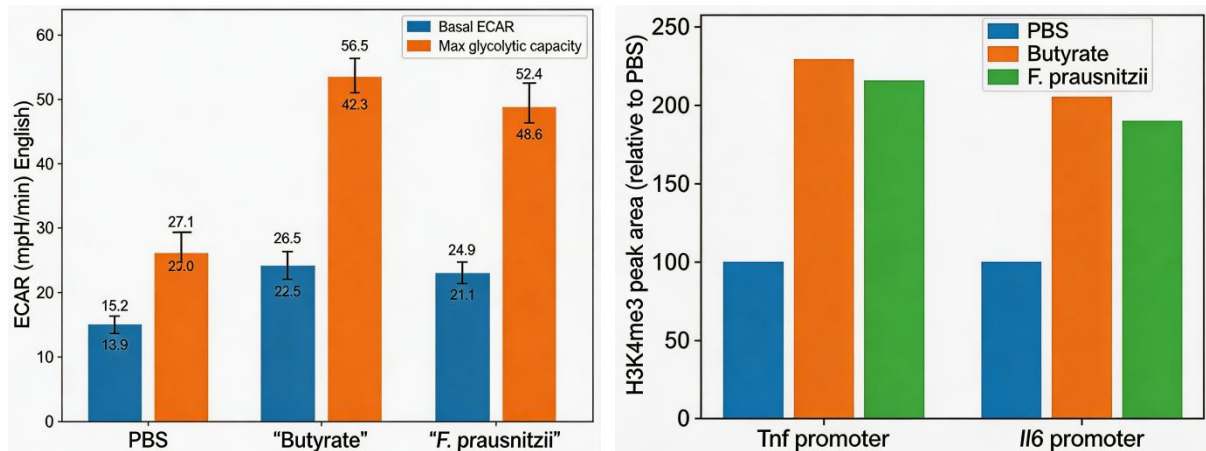
A: Cytokine secretion by intestinal macrophages

B: H3K4me3 MFI in intestinal macrophages



C: ECAR in intestinal macrophages

D: CUT&Tag peak areas at *Tnf* and *Il6* promoters



5. Discussion

This study shows for the first time that butyrate, a short-chain fatty acid from gut microbes, can directly trigger macrophages to develop what's known as trained immunity and can boost anti-tumor immune responses in the body by reshaping the tumor microenvironment. This finding not only broadens the range of factors that can induce trained immunity but also uncovers a new regulation network between microbial metabolites, innate immune memory, and tumor immunity, which is important both for basic research and potential clinical applications.

Traditionally, butyrate has been seen as a metabolite that mainly promotes anti-inflammatory effects and immune tolerance. It regulates immunity by encouraging Treg differentiation, inhibiting NF- κ B signaling, and maintaining the integrity of the gut barrier. However, our study found that under a 'training-rest-re-stimulation' setup, butyrate can make macrophages keep an enhanced ability to secrete TNF- α , IL-6, and IL-1 β and maintain phagocytic function even after the initial stimulus fades. The strength of this effect is comparable to the classic trained immunity inducer β -glucan. This seemingly contradictory finding suggests that butyrate's immune-modulating role is highly context-dependent: in acute exposure, it may suppress inflammation, while as a 'training signal,' it boosts macrophages' long-lasting response potential through epigenetic and metabolic reprogramming. This 'double-edged' nature aligns with research by Schulthess et al. that showed butyrate 'imprints' macrophage antibacterial programs, but our study goes further by confirming this effect within a strict trained immunity framework and extending its role to anti-tumor immunity.

On the mechanistic level, this study reveals that butyrate drives trained immunity through a combination of the GPR43-mTOR-HIF-1 α signaling axis and HDAC inhibition. GPR43, as one of the main receptors for butyrate, can trigger mTOR phosphorylation and HIF-1 α protein accumulation when activated, which in turn kicks off glycolytic metabolic reprogramming. At the same time,

butyrate acts as an HDAC inhibitor, promoting H3K4me3 enrichment and chromatin opening at key pro-inflammatory gene promoters. Both mechanisms are essential: blocking either GPR43 or the mTOR-HIF-1 α pathway alone can stop the training effect, while TSA mimicking HDAC inhibition can partly reproduce the trained phenotype. This ‘signal-epigenetic’ double mechanism shows similarities to the classic trained immunity inducer β -glucan via the dectin-1-AKT-mTOR-HIF-1 α pathway, but also has the uniqueness of a microbial metabolite—that is, it has both receptor activation and epigenetic regulation abilities. Notably, butyrate itself can serve as a substrate for fatty acid oxidation, and the establishment of trained immunity comes with increased glycolysis, suggesting that macrophages might switch their metabolism from fatty acid oxidation to glycolysis. The specific changes in metabolic flux still need to be clarified through further flux analysis.

In *in vivo* experiments, supplementing with butyrate or colonizing with butyrate-producing bacteria significantly inhibited the growth of MC38 tumors and increased the infiltration of M1-like macrophages and functional CD8⁺ T cells in the tumor microenvironment. Macrophage depletion almost completely abolished the anti-tumor effect of butyrate, while adoptively transferring butyrate-trained BMDMs was enough to reproduce the tumor-suppressing effect, indicating that macrophages are a necessary and sufficient mediator of butyrate’s anti-tumor activity. Moreover, in mice with macrophage-specific HIF-1 α knockout, the anti-tumor effect of butyrate was significantly weakened, genetically confirming the central role of the HIF-1 α signaling pathway in *in vivo* trained immunity induction. These findings echo Kalafati et al.’s conclusion that β -glucan promotes anti-tumor myelopoiesis through trained immunity, but the key point of this study is that, as a metabolite continuously produced by gut microbiota under physiological conditions, butyrate’s ability to induce trained immunity has much better translational feasibility than exogenous β -glucan. Regulating gut butyrate levels through dietary fiber intervention or colonization with butyrate-producing bacteria could be a safe and cost-effective strategy to support tumor immunotherapy.

This study also has several limitations. The butyrate concentration used in *in vitro* experiments (0.5 mM) is at the upper end of the physiological range, but the actual local concentration of butyrate in the gut microenvironment is highly heterogeneous and affected by factors like dietary fiber intake, microbiota composition, and intestinal motility. Future studies need to validate these findings in more physiologically relevant concentration gradients and primary intestinal macrophage systems. The tumor model used subcutaneous MC38 transplants, which can’t fully mimic the direct interaction between microbiota metabolites and immune cells in the colorectal cancer microenvironment. In the future, it would be better to use an AOM/DSS-induced colorectal cancer model or co-culture systems with intestinal tumor organoids. This study focused on macrophages, but the direct training effects or indirect regulation of dendritic cells, NK cells, and T cells by butyrate were not explored in depth.

Whether trained immunity involves reprogramming at the level of bone marrow hematopoietic progenitor cells also deserves further investigation. The molecular crosstalk between HDAC inhibition and GPR43 signaling is not fully understood. For example, it's unclear whether HDAC inhibition affects the acetylation status of downstream GPR43 signaling molecules or whether GPR43 activation regulates HDAC activity through metabolites, which will require more detailed biochemical studies. Finally, the durability of trained immunity and its potential synergy with existing therapies like immune checkpoint inhibitors still need to be evaluated in longer-term studies and combination therapy models.

Future research could go in a few directions: first, to further check how butyrate-trained immunity works in the body using physiological concentration gradients and in situ colon cancer models; second, to figure out how long the epigenetic marks of butyrate-trained immunity last and whether they can be passed on to progeny cells or bone marrow stem cells; third, to understand the detailed molecular conversation between HDAC inhibition and GPR43 signaling; and fourth, to look at the combined effects and real-world potential of current tumor immunotherapies, like dietary fiber treatments, butyrate-producing bacteria, or engineered probiotics with immune checkpoint inhibitors. In addition, with the deep integration of spatial transcriptomics and AI technology, using explainable spatial AI to analyze the spatial interaction patterns of tumor-immune cells is expected to further improve the accuracy of predicting immune therapy responses and discover new treatment targets (Liu et al., 2026a). At the same time, new neoantigen immunogenicity prediction tools based on multimodal foundational models also provide a new way to accurately design personalized cancer vaccines (Liu et al., 2026b). These emerging technologies, combined with the microbiome metabolite-trained immune axis revealed in this study, might bring more possibilities for optimizing cancer immunotherapy strategies.

In summary, this study looks at the whole picture from a new angle of 'microbial metabolites-trained immunity-anti-tumor immunity,' revealing the biological functions and molecular mechanisms of butyrate as an endogenous inducer of trained immunity. This finding not only deepens our understanding of how gut microbes regulate the plasticity of innate immunity, but also provides a new theoretical basis and potential targets for tumor immunotherapy based on microbial metabolites. In the future, we can further explore the value of butyrate-trained immunity in cancer vaccines, adoptive cell therapy, and combination strategies with immune checkpoint blockade, as well as assess the clinical feasibility of boosting anti-tumor immunity by precisely adjusting gut butyrate levels through tailored nutrition or engineered probiotics.

6. Conclusion

This study systematically revealed a new function of gut microbiota-derived short-chain fatty

acid butyrate as an endogenous inducer of trained immunity. In vitro experiments confirmed that butyrate could induce long-lasting enhanced inflammatory responses and phagocytic activity in macrophages through the ‘train-rest-re-stimulate’ paradigm, with effects comparable to the classic inducer β -glucan. Mechanistically, butyrate drives metabolic reprogramming via the GPR43-mTOR-HIF-1 α signaling axis and promotes H3K4me3 enrichment and chromatin opening at key pro-inflammatory gene promoters through its HDAC inhibitory activity, together establishing a trained immunity state. In vivo, the MC38 tumor model further showed that butyrate supplementation or colonization with butyrate-producing bacteria could reshape the tumor immune microenvironment in a macrophage-dependent manner, increasing M1-like macrophages and functional CD8⁺ T cell infiltration, significantly suppressing tumor growth. Macrophage depletion, adoptive transfer, and macrophage-specific HIF-1 α knockout experiments respectively confirmed the necessity, sufficiency, and signaling pathway involvement of these effects. This study is the first to establish a direct causal link between microbial metabolites and trained immunity, proposing a new regulatory paradigm of ‘microbial metabolite-macrophage trained immunity–anti-tumor immunity,’ providing both theoretical basis and experimental support for gut microbiota-targeted cancer immunotherapy.

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Author contributions

Conceptualization, Gang Liu; methodology, Jia Wang; validation, Liqiang An; formal analysis, Gang Liu and Jia Wang; investigation, Jia Wang and Liqiang An; resources, Gang Liu; data curation, Liqiang An; writing—original draft preparation, Jia Wang; writing—review and editing, Gang Liu; visualization, Liqiang An; supervision, Gang Liu; project administration, Jia Wang. All authors have read and agreed to the published version of the manuscript.

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Ethics approval



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