Butyrate Impedes the Recruitment of MDSCs to Alleviate CAC Development by Inhibition of the TLR2/MyD88/NF-κB Signaling Pathway

Tengfei Xiao  
Yancheng Third People's Hospital

Yi Chang  
Yancheng Third People's Hospital

Hongmei Chen  
Yancheng Third People's Hospital

Jingjing Kang  
Yancheng First Hospital

Mingzhong Sun (✉ syjykzr@163.com)  
Yancheng Third People's Hospital  https://orcid.org/0000-0003-0025-4986

Research Article

Keywords: Butyrate, Colitis-associated colorectal cancer, Tumor microenvironment, Myeloid-derived suppressor cells, TLR2

Posted Date: December 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1186467/v1

License: ☒ ☑ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

Background: The colitis-associated colorectal cancer (CAC) with inflammatory bowel disease (IBD) serving as its prelude often has a poor prognosis due to the hysteretic diagnosis. As a representative of short chain fatty acids (SCFAs), butyrate has been proved to have obvious antitumor effect. Here, we aimed to examine its effect on CAC and possible mechanism in tumor microenvironment (TME).

Method: The establishment of CAC mouse model was mainly based on the combination of AOM intraperitoneal injection and DSS three cycle. HE staining was used to analyze the degree of colonic inflammation and tumor dysplasia. The proportion of MDSCs population was mainly evaluated by flow cytometry assay. RT-PCR, immunohistochemical staining and western blot analysis was carried out to detect protein molecular expression.

Results: In our current study, the AOM-DSS induced CAC mouse model was utilized to evaluate the effect of butyrate on CAC. The administration of butyrate significantly improved the weight loss, falling survival rate, higher DAI index and anal prolapse caused by the AOM-DSS during the CAC modeling process. Anatomical results including the size and number of tumors and histological results including the abnormal hyperplasia shown by HE staining also confirmed the inhibitory effect of butyrate on CAC. In addition, the proportion of myeloid-derived suppressor cells (MDSCs) assisting tumor immune escape in tumor microenvironment (TME) decreased under the intervention of butyrate. And inflammatory mediators including CCL2, IL-6 and TNF-α in TME that induce the recruitment of MDSCs showed the same trend as MDSCs. Toll-like receptor 2 (TLR2) as a receptor molecule related to inflammation and immune function was also up-regulated in CAC, accompanied by the synchronous up-regulation of downstream Myd88 and NF-κB molecules, while the use of butyrate significantly inhibited the up-regulation of these molecules.

Conclusions: Butyrate might reduce the release of CCL2, IL-6 and TNF-α in TME by inhibiting TLR2/MyD88/NF-κB signaling pathway to reduce the recruitment of MDSCs in TME, which eventually weakened the immune escape of tumors and retarded the progress of CAC.

Background

Colorectal cancer (CRC) due to concealment and high mortality has become the third large malignancy with the changes of contemporary eating habits and lifestyle worldwide[1]. Colitis-associated colorectal cancer (CAC) as a subtype of CRC is the result of interleaving by multifactor including infections, immune response, pro-inflammatory mediators and gut microbiota dysbiosis, etc. And accumulated evidence strongly indicated chronic inflammation is a fundamental factor that contributes to the initiation and progression of CAC. [2]. Due to repetitive cycles of damage and repair in the intestinal epithelium, clinically inflammatory bowel disease (IBD) patients covering ulcerative colitis (UC) and Crohn's disease (CD) are accompanied by a higher risk of developing CAC compared to the general population [3]. Therefore, anti-inflammatory treatment is the most frequently chosen treatment means to contain the
development of CAC. For instance, regular usage of aspirin as a kind of non-steroid anti-inflammatory drugs (NSAID) can effectively reduce CRC incidence and international guidelines has recommended aspirin as a primary preventive drug for CRC in specific populations, which reflects the association between chronic inflammation and CRC from the side [4, 5].

Although the exact mechanisms of chronic inflammation eventually developing into CAC remain to be elucidated, the continuous evolution of chronic inflammation into malignant tumor is bound to be accompanied by the changes of tumor microenvironment (TME) [6]. The important components of TME including some recruited various immune cells and inflammatory mediators, play an indispensable regulatory role in tumor formation, proliferation, apoptosis, and migration [7][7]. As a heterogeneous group of immature myeloid cells, myeloid-derived suppressor cells (MDSCs) can usually be further divided into monocytic MDSCs (Mo-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs) in both mice and humans, which support tumor development by suppressing antitumor immunity and help tumor cells to evade immune surveillance in the TME[8, 9]. Moreover, this immunosuppressive effect of MDSCs can be mediated by means of the activation of two enzymes including inducible No Synthase (iNOS) and Arginase-1 (Arg1)[10, 11]. On the other hand, growing evidence suggest various inflammatory mediators play a key role in promoting IBD as well as inducing CAC through similar mechanism, largely because the inflammatory environment has a similar cytokine composition to TME [12]. For instance, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are obviously elevated in CRC in the serum of CRC patients and make a contribution to CRC development and prognosis [13, 14]. And CC chemokine ligand 2 (CCL2) is considered to promote colorectal carcinogenesis by recruiting PMN-MDSCs [15].

Toll like receptors (TLRs) as a kind of pattern recognition receptors (PRRs), can recognize conserved antigen molecules in the evolution of pathogenic microorganisms, that is, pathogen-associated molecular patterns (PAMPs), and then exert the corresponding functions in innate immune responses [16]. Signals triggered by TLRs are transduced by downstream Myd88/NF-κB signaling pathway to recruit pro-inflammatory cytokines, and finally promote inflammatory responses and tumor formation [17]. TLR2 as an important part of TLRs family, is a type I transmembrane protein receptor composed of 784 amino acids. Studies have shown that the occurrence of CRC is closely related to the increased TLR2 expression level [18]. Existing studies have proved that TLR2 expression in CRC patients was obviously higher in cancerous tissue than in noncancerous tissue by TaqMan RT-PCR and immunohistochemical analysis [19, 20]. C. Xin, et al. found that TLR2 was detected as a direct target of miR-154 as a tumor suppressor in CRC cells. Moreover, overexpression of TLR2 could reverse the tumor suppressive effects of miR-154 on CRC cells [21]. Taken together, TLR2 has been considered as a crucial molecule in the mechanism of mediating CRC formation.

Short chain fatty acids (SCFAs) represented by butyrate are the metabolic end products synthesized from the fermentation of undigested dietary fiber, and the metabolites are evaluated as a nutritional support to maintain the balance between gut microbiota and host [22]. SCFAs has attracted extensive attention of many researchers in view of its positive roles including anti-inflammatory and anti-cancer effects, providing energy source for colonocytes, reducing oxidative stress and maintaining the integrity of
intestinal epithelium [23]. The increased risk of CRC is closely related to the alterations in the composition of the intestinal flora and the reduction in the SCFAs content [24]. Butyrate as the most studied short-chain fatty acid, can suppress the proliferation and induce apoptosis of colon cancer cell lines [25]. In addition, as a histone deacetylase (HDAC) inhibitor, the main anti-cancer mechanism of butyrate is to promote histone acetylation and ultimately reduce the risk of enteritis and CRC by largely activating the immunomodulatory activity of various tumor suppressors [26]. However, the effect of single butyrate on CAC is lack of direct experimental evidence in vivo, and the exact mechanisms remain to be clarified. We constructed a mouse model mimicking human CAC by the exposure of azoxymethane (AOM) and dextran sulfate sodium (DSS) to evaluate the alleviating effect of butyrate on intestinal inflammation and transition to CAC. Our main research focus is to determine whether butyrate can affect the components of TME to retard the progress of CAC by inhibition of the TLR2/MyD88/NF-κB signaling pathway.

**Materials And Methods**

**Animals and induction of CAC murine model**

Six to eight-week-old male BALB/c mice were purchased from SPF (Beijing) Biotechnology Co., Ltd. All animal experiments were approved by the Ethics Committee of Jiangsu Vocational College of Medicine (Yancheng, China), and strictly comply with experimental animal operation specifications. All mice were housed in plastic cages under standard laboratory conditions (23 ± 2°C, humidity of 50 ± 5%, and a 12-hour light/dark cycle). Mice were pre-adapted to the environment for one week and provided free access to standard diet and sterile water at regular intervals. The induction of CAC murine model mainly refers to previous studies [27]. All mice (n=10 for each group) were randomly and medially assigned into three groups including control group, model group and butyrate group. Mice of model group and butyrate group were injected intraperitoneally with a single dose of AOM (10mg/kg, Sigma-Aldrich, USA) in PBS on day 0, and followed by three DSS (2.5%, MP Biomedicals, Canada, M.W.=36000-50000 kDa) cycles. In addition, mice in butyrate group were treated butyrate (2%, Sigma-Aldrich, USA) in the drinking water throughout the process. In view of the stability of butyrate, the butyrate solution was renewed weekly. Correspondingly, the control group mice were only given normal drinking water without any treatment (Fig. 1A).

The clinical symptoms of all mice were assessed and recorded weekly during the CAC modeling process, including body weight, diarrhea, bloody stools, death rate, and tumor formation. In addition, the specific calculation of the disease activity index (DAI) was mainly based on previous experiments according to the three parameters of stool consistency, gross bleeding and weight loss [28]. Mice were sacrificed at day 70 by cervical dislocation. The target colorectal segment (from the ileocecal junction to the anal verge) were removed and cut open along the longitudinal axis. The contents were washed off with PBS (pH 7.4). The three groups of colons were photographed. The number and size of tumors in mouse colon were measured and statistically analyzed by Image J software. After cutting the colon into parts, one part was fixed in 10% formalin for histology, and the remaining intestinal tissue was used for total protein and
RNA extraction respectively. A similar procedure was used for the spleen and the spleen weight was additionally recorded.

**Histopathological And Immunohistochemical Analysis**

The obtained intestinal and spleen tissue (4 µm thick) was fixed with 10% formalin and embedded in paraffin to make paraffin sections, followed by hematoxylin and eosin (H&E) staining according to standard procedure. The degree of tumor atypia and inflammation of CAC mice was graded. The pathological scoring criteria of HE staining in mouse colon was based on the previous standards [29]. For immunohistochemistry analysis, paraffin sections were dewaxed in water, and then antigen retrieval was carried out by antigen repair solution in a pressure cooker. After the sections were cooled to room temperature, they were incubated with 3% hydrogen peroxide and then blocked with 10% goat serum, so as to exclude non-specific background staining. Each step was followed by 3 washes of PBS, 5 min each. Then the sections were covered with the diluted primary antibody including TLR2 (1:200, ab209216, Abcam, U.K.), Ki67(1:200, ab279653, Abcam, U.K.), BCL2(1:200, ab182858, Abcam, U.K.), CD31(1:200, ab182981, Abcam, U.K.), and incubated overnight in a 4 °C refrigerator. The next day, after washing the sections with PBS for three times, add 1:200 diluted HRP labeled secondary antibody (Abcepta, China) and incubate at room temperature for 30 minutes. Finally, the sections were visualized by means of the chromogenic reaction of diaminobenzidine (DAB) substrate, followed by hematoxylin counterstaining for microscopy. Images were captured by fluorescence inverted microscope (Nikon, Japan).

**Flow Cytometric Analysis**

After sacrifice, the removed intestinal tissue was cut along the longitudinal axis, and then the luminal contents were washed with PBS. The intestinal tissue was cut into small 1 cm pieces, followed by with collagenase IV digestion (Worthington, USA). The digested cells were then dispersed into flocs with a pipette and filtered through 100-µm strainers. Finally, the complete single cell suspension was obtained by 40% percoll solution separation solution. As for spleen, splenocytes are mainly obtained by grinding, filtering, and then lysing erythrocytes with ACK lysis buffer (Elabscince, China).

The single cell suspension was counted, and the appropriate numbers of cells were selected for blocking with rat serum for 10min and fluorescent antibody staining at 4°C for 30 min. (5ul / test). These antibodies mainly include anti-mouse CD11b-FITC (E-AB-F1081C, Elabscince, China) and anti-mouse Gr1-APC antibodies (E-AB-F 1120E, Elabscince, China). After washing twice with PBS, the cells were analyzed on a BD FACS Verse flow cytometer (BD Biosciences). Data were analyzed by FlowJo (Tree Star Inc.).

**Reverse Transcriptionquantitative Polymerase Chain Reaction (Rtqpcr)**
Total RNA was extracted mainly by Trizol reagent. The extracted RNA was reverse transcribed into cDNA by reverse transcription kit (RK20402, ABclonal, China), which could be stably stored in the refrigerator at -80 °C. The specific procedures were as follows: 25°C for 5 min, 42°C for 15 min, 85°C for 5 s, and finally hold at 12°C. The mRNA expressions of target gene were detected by 2X Universal SYBR Fast qPCR Mix (RK21203, ABclonal, China) with β-actin as an internal control. The amplification procedure was as follows: pre-denaturation at 95 °C for 3 min, 1 cycle; then 45 cycles of 95°C for 5 s, 60°C for 30 s; finally end at 25°C. The primer sequences were listed in table 1.

**Western blot analysis.**

The RIPA lysis buffer (P0013B, Beyotime, China) mixed with PMSF (ST506, Beyotime, China) (100:1) was added to colon tissue for total protein extraction. The protein concentration was quantified by Bicinchoninic acid (BCA). According to the molecular weight of the target protein. 10% SDS-PAGE gels were prepared by the SDS-PAGE Gel Quick Preparation Kit (P0012AC, Beyotime, China) to separate the target protein. The separated protein was transferred to PVDF membranes and blocked with 5% skim milk for 1 h at 37°C. Then the PVDF membranes were incubated with the primary antibodies at 4°C overnight. These primary antibodies included TLR2 (1:1000, ab209216, Abcam, U.K.), Myd88 (1:1000, ab28736, Abcam, U.K.), P65 (1:1000, ab32536, Abcam, U.K.) and anti-β-actin (1:3000, T0022, Affinity Biosciences, China). The next day, the blots were incubated secondary antibodies for 1 h at room temperature and visualized by chemiluminescent reagents (BL520, Biosharp, China) and finally quantified using imaging software (ImageJ2x).

**Statistical analysis**

All data was presented as mean ± SEM (standard error of the mean). The statistical significance of differences in three groups was evaluated by one-way ANOVA using GraphPad Prism 5. P value<0.05 was considered to indicate a statistically significant.

Table I. Primer sequences for RT-PCR

| Gene   | Sequences (5'3') | Forward                  | Reverse                  |
|--------|------------------|--------------------------|--------------------------|
| Actin  | AGCCATGTACGTAGCCATCC | GCTGTGGTGGTGAGCTGTA     |                          |
| TNF-α  | ATGTTCAGCCTCTTCTCACTT | GCTGTCACTCGAATTTTTGAGA |                          |
| IL-6   | CCACCAACAGACCTGCTATAC | CCATTGCACAACCTTTTCTCA   |                          |
| CCL2   | TTCCCAACACCACCTCAAGCCTC | ATTAAGGCATCACAGTGCGAGTCAC |                      |
| TLR2   | GACTCTTCACCTTAAAGGCAGTCT | AACCTGGCAAGTTAGTATCTC |                          |
Results

General observations

The CAC mouse model was performed in strict accordance with Figure 1A. We first made a macro level evaluation of the three groups of mice. The symptoms of three mice presented in Figure 1B are representative experimental results. After AOM-DSS treatment, mice showed obvious rectal prolapse and perianal tumor invasion. Interestingly, through butyrate prevention, AOM-DSS treatment did not cause this symptom compared with NC group. In addition, the mice in the experimental group died successively at the 7th and 8th weeks, while the other two groups did not (Figure 1C). We then evaluated the effect of butyrate on body weight and DAI of AOM-DSS mice. As for weight monitoring, the weight of mice in NC group increased steadily with time, while the weight of mice in experimental group and butyrate group decreased precipitously after each DSS cycle. And the treatment intervention of butyrate effectively slowed down the weight loss (Figure1D). The disease activity index (DAI), as a recognized index to evaluate colitis, increased significantly after AOM-DSS treatment. With the whole process intervention of butyrate, the index was significantly improved with significantly decreased DAI(Figure1E). These observations indicate that, butyrate administration significantly inhibited AOM-DSS-induced symptoms in CAC model mice.

Butyrate Alleviated Aom-dss Induced Cac Development

Once exposure to AOM-DSS, the colon length of model group mice was shortened in anatomy compared with the NC group, but butyrate did not significantly alleviate the shortening of colon length (Figure2A-B). However, butyrate significantly improved splenomegaly caused by AOM-DSS (Figure2E-F). Through the colon image, we noticed that AOM-DSS successfully induced colon tumors and the tumor almost involved the whole colon segment while the intestinal segment in NC group was normal anatomical structure. The intervention of butyrate obviously inhibited the growth of tumor, and few tumor bulges were seen with the naked eye (Figure2A). Furthermore, the number and size of intestinal tumors in the three groups were statistically analyzed. Regardless of the number or size of tumors, the butyrate group mice were greatly reduced (Figure2C-D). As indicated by H&E staining, the spleen nodule structure collapsed in AOM-DSS group, and butyrate effectively improved the destruction of spleen structure. In addition, the colonic structure of CAC model mice was severely disrupted with a large amount of inflammatory cells infiltration compared with NC group mice. At the same time, the crypts thickened, the goblet cells decreased, and the mucosal epithelial cells showed dendritic dysplasia after AOM-DSS treatment, and the whole process prevention of butyrate significantly weakened the trend of inflammatory infiltration and atypical hyperplasia (Figure2G). The statistics of intestinal inflammation and hyperplasia score also coincided with this conclusion that butyrate inhibited AOM-DSS induced CAC development (Figure2H-I).

Butyrate inhibited the proliferation and promoted the apoptosis of CAC induced by AOM-DSS
Next, we analyzed the tumor proliferation and apoptosis of the three groups of mice by immunohistochemistry. As a sensitive index to detect the proliferation activity of tumor cells, the positive rate of Ki67 increased significantly in AOM-DSS mice, while the use of butyrate decreased the positive rate of Ki67, indicating that butyrate has an obvious inhibitory effect on the proliferation of CAC tumor cells (Figure 3A). We then detected the apoptosis inhibitor BCL2. The positive rate of BCL2 in AOM-DSS group increased significantly, which means that the apoptosis of tumor cells decreased. The intervention of butyrate significantly reduced the positive rate of BCL2, suggesting that butyrate can inhibit the development of CAC by promoting tumor cell apoptosis (Figure 3B). In addition, we detected CD31 to evaluate tumor angiogenesis and obtained results similar to Ki67. It is inferred that butyrate inhibited the growth of CAC tumor cells by inhibiting proliferation and promoting apoptosis (Figure 3C).

**Butyrate decreases the infiltration of MDSCs (CD11b+Gr-1+) in both colon and spleen tissues of CAC mice**

As an important part of tumor microenvironment, MDSCs play an important role in supporting tumorigenesis. Therefore, we detected the proportion of MDSCs in colon and spleen of three groups mice by flow cytometry. The data showed that the proportion of MDSCs (CD11b+Gr-1+) in colon and spleen of mice in AOM-DSS group was significantly up-regulated compared with NC group, whereas the administration of butyrate reduced this increase. Moreover, CD11b+Gr-1- cell population showed a similar trend to MDSCs (Figure 4A). The results of statistical analysis suggest that butyrate can inhibit the aggregation of various immunosuppressive cells represented by MDSCs in the TME and further inhibit tumor proliferation (Figure 4B-C).

![Figure 4](image4.png)

**Butyrate inhibits the expression of inflammatory mediators in both colon and spleen tissues of CAC mice**

The inflammatory environments caused by the three cycles of DSS must have a great promoting effect on the induction of CAC, and various pro-inflammatory mediators have played a key role. Therefore, we next detected the pro-inflammatory mediators including TNF-α, IL-6 and CCL2 in colon and spleen tissues using RT-PCR.

As shown in Figure 5A-C, the AOM-DSS group mice had significantly elevated levels of TNF-α, IL-6 and CCL2, whereas butyrate treatment was associated with a significant decrease in these three cytokines.

![Figure 5](image5.png)
Butyrate inhibits AOM-DSS induced CAC by down-regulating the expression of TLR2 receptor

Colitis-associated cancer (CAC) as an inflammation-driven carcinogenesis is inseparable from the activation of toll-like receptors (TLRs) signals in inflammatory environment [30]. As one of TLRs, the relationship between TLR2 and CAC is still controversial. To explore whether butyrate can alleviate inflammation and inhibit the formation of CAC by regulating TLR2 of intestinal epithelium. Through immunohistochemical analysis, we found that intestinal epithelial dysplasia was obvious in AOM-DSS group, and TLR2 was significantly up-regulated. The intestinal hyperplasia of CAC mice treated with butyrate was alleviated, and the positive rate of TLR2 was significantly reduced (Figure 6A-B). The results of RT-PCR also showed a consistent conclusion (Figure 6C). The activation of TLR2 signal will trigger the transmission of downstream signals. MyD88 dependent signal pathway is the main signal pathway for TLR2 to play an immunomodulatory role. It can also induce the production of proinflammatory cytokines by activating NF-κB, and finally drive the transition from inflammation to cancer [31]. Therefore, we used western blot to detect the effect of butyrate on TLR2/MyD88/NF-κB signaling pathway. Western blot results showed that the use of AOM-DSS significantly activated this signal pathway, while butyrate inhibited the activation of the pathway in CAC mice [Figure 6D-G]. In conclusion, butyrate alleviated AOM-DSS induced CAC by inhibiting the expression of TLR2 in intestinal epithelium and blocking the activation of downstream MyD88 dependent signaling pathway.

Discussion

It is indisputable that chronic colitis is an important risk factor for colitis-associated colorectal cancer (CAC), and IBD is usually the precancerous basis of colorectal cancer. Clinically, IBD patients are usually accompanied by the gut microbiota dysbiosis and the decrease of SCFAs content [32]. In addition, epidemiological studies have confirmed that high-fiber diets can effectively reduce the incidence rate of colorectal cancer in contrast to high-fat diets, in which the key link for high-fiber diets to exert anti-cancer effects is the SCFAs produced by gut microbiota digestion of dietary fiber [33]. Butyrate, as the most representative SCFAs, can provide energy source for colon cells to exert mucosal anti-inflammatory and anticancer effects, and the anticancer effect of butyrate has been proved by many studies in both vitro and vivo [34, 35]. Although Tian et al have shown that SCFAs mix containing butyrate can effectively protect mice from AOM-DSS induced CAC [36], the effect of butyrate alone on AOM-DSS induced CAC model is unknown.

Current results displayed that the intervention of 2% butyrate could greatly alleviate the CAC induced by AOM-DSS. Through the whole process supervision of CAC mouse model, various tumor manifestations of AOM-DSS group mice, including sharp weight loss, reduced survival rate, higher DAI index and anal prolapse, were in line with expectations compared with the control group. And the use of butyrate had
greatly alleviated these apparent symptoms. Further anatomical and histological analysis also confirmed the remission effect of butyrate on CAC referring to the size and number of tumors and the abnormal hyperplasia shown by HE staining. In addition, the increased expression in Ki67, BCL2 and CD31 was found in AOM-DSS induced CAC mice and butyrate significantly decreased the positive rate of Ki67, BCL2 and CD31, suggesting that this remission effect of butyrate was achieved by inhibiting the proliferation and promoting the apoptosis of colorectal cancer cells. These findings are in consistent with our previous study, in which butyrate administration was protective in DSS-induced colitis [37]. In comprehensive view, butyrate plays an important role in inhibiting the progression from chronic colitis to carcinogenesis.

The formation and growth of tumor are often inseparable from the nourishment of tumor microenvironment (TME). They affect each other and co-evolve in a complementary relationship. TME is generally a chronic inflammatory environment with massive infiltration of inflammatory related cells and inflammatory mediators. The diversity of inflammatory cells and inflammatory related factors, tumor cells and stromal cells constitute a complex regulatory network [38]. The cells and molecules within the TME are in a dynamic change process, reflecting the essence of the evolution of the TME. As the main immunosuppressive cell population present in the TME, MDSCs are generated and recruited to the TME to promote the establishment of an immunosuppressive TME that facilitates tumor escape. In addition, MDSCs also assist tumor invasion, angiogenesis and metastasis [39]. Due to the anti-tumor immune effect, MDSCs have become the major obstacle to many cancer immunotherapies and the immunotherapy targeting MDSCs to overcome immune evasion has attracted a lot of attention [40–42]. The existing view holds that the immunosuppressive function of MDSCs in CRC is mainly achieved by their ability to inhibit T-cell proliferation and to stimulate Treg development [43]. Similarly, accumulating evidence also shows that MDSCs activity plays a vital role in the promotion of CAC through multiple mechanisms and even obstructs CAC immunotherapy [44]. Consistent with these conclusions, our current study found that MDSCs were up-regulated in the colon and spleen of AOM-DSS induced CAC mice, suggesting the role of MDSCs in promoting the development of CAC. Interestingly, the increase of MDSCs in CAC mice was suppressed after butyrate intervention. Therefore, it can be inferred that the inhibitory effect of butyrate on the progression of CAC is achieved by hindering the recruitment of MDSCs in the intestine.

In addition to the recruitment of immunosuppressive cells dominated by MDSCs, tumor inflammatory microenvironment created by the release of various inflammatory mediators also contributes to the development of CAC. And these tumour-derived inflammatory mediators including TNF-α, IL-6 and CCL2 that accumulate in the TME have also been shown to induce the recruitment and expansion of MDSCs [45]. In particular, chemokine CCL2 can promote tumor growth, progression and metastasis by inducing the accumulation of MDSCs and enhancing their immunosuppressive function during colorectal carcinogenesis. And the deletion of CCL2 in mouse model also leads to the reduction of the MDSC level [15, 46]. Our RT-PCR results showed that AOM-DSS induced CAC mice had higher mRNA expression of TNF-α, IL-6 and CCL2 than the NC mice, while butyrate treatment decreased the mRNA expression level of these inflammatory factors of the spleen and colon tissue. It can be considered that butyrate inhibits the accumulation of MDSCs by reducing the release of various proinflammatory and chemokines, thus
ultimately inhibiting the development of CAC. In summary, the final outcome is conceivable that a large number of immunosuppressive cells including MDSCs, Treg cells as well as tumor-associated macrophages (TAM), and inflammatory related factors such as CCL2, TNF-α and IL-6 gather in the TME and jointly lead to a muted immune response that guide tumor immune escape and progression in CAC.

Toll-like receptors (TLRs) mediated MyD88 dependent signals usually represent inflammatory signals, which eventually activate NF-κB and induce the release of various inflammatory mediators and promote the establishment of tumor inflammatory microenvironment. The contribution of TLRs to CAC has also been confirmed that TLRs can promote colon cancer cell proliferation, invasion and metastasis, and conversely inhibition of the expression of TLRs or MyD88 will restrain the growth of colon cancer cells [47]. Paarnio et al found that TLR2 of carcinoma cells was highly expressed while TLR4 expression was lower than that of normal epithelial cells by immunohistochemistry detection in 118 CRC patients [48]. It is also demonstrated that TLR2 stimulation promotes colorectal cancer cell growth via NF-κB signaling pathway and knockout and knockdown of TLR2 can inhibit the proliferation of CAC through animal experiments and cellular experiments, which further proves that TLR2 plays an indispensable role in promoting the progress of CAC [49, 50]. The above conclusions are consistent with our current results. We detected TLR2 in the colon of three groups of mice by immunohistochemistry, Western blot and RT-PCR. It was found that the expression level of TLR2 in AOM-DSS group was significantly up-regulated compared with the NC group, while the administration of butyrate significantly inhibited the expression of TLR2 in colon cancer cells. Similarly, the downstream protein detection of Myd88 and p65 was consistent with the expression of TLR2. These results are highly consistent with the aforementioned results of inflammatory mediators and MDSCs. Therefore, it was concluded that TLR2/MyD88/NF-κB signaling pathway not only played an indispensable role in the progression of CAC, but also was an important mechanism for butyrate to inhibit the progress of CAC.

**Conclusion**

Our data also revealed demonstrated that butyrate had a significant inhibitory effect on the progress of the AOM-DSS induced CAC, and the inhibitory effect of butyrate involved the regulation of MDSCs and inflammatory mediators in TME. Butyrate suppressed the transduction of the TLR2/MyD88/NF-κB signals of colon epithelial cells to reduce the release of inflammatory media such as TNF-α, IL-6 and CCL2, which thereby inhibited the recruitment and aggregation of MDSCs in TME. Once the recruitment of MDSCs in TME was limited, the growth of CAC tumor cells dependent on tumor immune escape was also restrained. Nevertheless, the mechanism by which butyrate inhibit the conversion from IBD to CAC largely remains to be elucidated. Our current research focuses on how butyrate restrains TLR2 signals on colonic epithelial cells of CAC to reduce the aggregation of MDSCs in TME and finally relieve the development of CAC, which emerges a new insight into the mitigation mechanism of butyrate on CAC (Figure 7).

**Abbreviations**

CRC
colorectal cancer
CAC
colitis-associated colorectal cancer
IBD
inflammatory bowel disease
UC
ulcerative colitis
CD
Crohn's disease
SCFAs
short chain fatty acids
Bu
butyrate
TME
tumor microenvironment
MDSCs
myeloid-derived suppressor cells
INOS
inducible No Synthase
Arg1
Arginase-1
IL-6
interleukin-6
TNF-α
tumor necrosis factor-α
CCL2
CC chemokine ligand 2
PRRs
pattern recognition receptors
PAMPs
pathogen-associated molecular patterns
TLRs
Toll like receptors
TLR2
Toll-like receptor 2
Myd88
myeloid differentiation factor 88
NF-κB
nuclear factor kappa-B
HDAC
histone deacetylase
AOM
azoxymethane
DSS
dextran sulfate sodium
RT-PCR
reverse transcription–quantitative polymerase chain reaction
IHC
Immunohistochemistry
HE
hematoxylin and eosin.

Declarations

Authors’ contributions

Tengfei Xiao and Jingjing Kang designed the study; Tengfei Xiao, Jingjing Kang and Yi Chang performed the experiments and prepared the figures; Tengfei Xiao contributed to drafting the manuscript. All authors read and approved the final manuscript.

Author details

1Department of Clinical Laboratory, The Sixth Affiliated Hospital of Nantong University, Yancheng Third People’s Hospital, No. 2, Xindu West Road, Yandu District, Yancheng, Jiangsu, 224000, China.

2Department of Clinical Laboratory, Yancheng First Hospital, Affiliated Hospital of Nanjing University Medical School, The First people’s Hospital of Yancheng, No.166, Yulong West Road, Tinghu District, Yancheng, Jiangsu, 224000, China.

Acknowledgements

Not applicable.

Competing interests

No conflicts of interest exist.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

**Ethics approval and consent to participate**

The study was approved by the Ethics Committee of Jiangsu Vocational College of Medicine (Yancheng, China), and all animal experiments strictly followed experimental animal operation specifications.

**Funding**

This study was funded by grants from the Science and Technology Development Project of Yancheng, China (YK2019108) and the Nantong University Clinical Medicine Special Project, China (2019JZ011).

**References**

1. Jin BR, et al., *High-Fat Diet Propelled AOM/DSS-Induced Colitis-Associated Colon Cancer Alleviated by Administration of Aster glehni via STAT3 Signaling Pathway*. Biology, 2020. 9(2).
2. Greten FR, Grivennikov SI. Inflammation and Cancer: Triggers, Mechanisms, and Consequences. Immunity. 2019;51(1):27–41.
3. Feng Y, et al. MicroRNAs, intestinal inflammatory and tumor. Bioorganic & Medicinal Chemistry Letters; 2019.
4. John, et al. The role of aspirin in preventing colorectal cancer. British Medical Bulletin; 2016.
5. Bresalier RS. Aspirin and the prevention of colorectal neoplasia. Gastroenterology. 2003;125(5):1539–41.
6. Dannenberg, et al., *Obesity and Cancer Mechanisms: Tumor Microenvironment and Inflammation*. Journal of Clinical Oncology, 2016.
7. Oya Y, Hayakawa Y, Koike K. Tumor microenvironment in gastric cancers. Cancer Sci. 2020;111(8):2696–707.
8. Karin N, Razon H. The role of CCR5 in directing the mobilization and biological function of CD11b(+)Gr1(+)Ly6C(low) polymorphonuclear myeloid cells in cancer. Cancer Immunol Immunother. 2018;67(12):1949–53.
9. Oh MH, et al. Targeting glutamine metabolism enhances tumor-specific immunity by modulating suppressive myeloid cells. J Clin Invest. 2020;130(7):3865–84.
10. Fresno M, Gironès N. Myeloid-Derived Suppressor Cells in Trypanosoma cruzi Infection. Front Cell Infect Microbiol. 2021;11:737364.
11. Zhang C, et al. The mTOR signal regulates myeloid-derived suppressor cells differentiation and immunosuppressive function in acute kidney injury. Cell Death Dis. 2017;8(3):e2695.
12. Francescone R, Hou V, Grivennikov SI. Cytokines, IBD, and colitis-associated cancer. Inflamm Bowel Dis. 2015;21(2):409–18.
13. Li ZW, et al. GNAI1 and GNAI3 Reduce Colitis-Associated Tumorigenesis in Mice by Blocking IL6 Signaling and Down-regulating Expression of GNAI2. Gastroenterology. 2019;156(8):2297–312.

14. Wang X, et al. Inflammatory cytokines IL-17 and TNF-α up-regulate PD-L1 expression in human prostate and colon cancer cells. Immunol Lett. 2017;184:7–14.

15. Chun E, et al. CCL2 Promotes Colorectal Carcinogenesis by Enhancing Polymorphonuclear Myeloid-Derived Suppressor Cell Population and Function. Cell Rep. 2015;12(2):244–57.

16. Kumar V. Toll-like receptors in the pathogenesis of neuroinflammation. J Neuroimmunol. 2019;332:16–30.

17. Vidya MK, et al. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. Int Rev Immunol. 2018;37(1):20–36.

18. Pimentel-Nunes P, et al. Functional polymorphisms of Toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. Dig Liver Dis. 2013;45(1):63–9.

19. Nihon-Yanagi Y, et al. Tissue expression of Toll-like receptors 2 and 4 in sporadic human colorectal cancer. Cancer Immunol Immunother. 2012;61(1):71–7.

20. Lu CC, et al. Upregulation of TLRs and IL-6 as a marker in human colorectal cancer. Int J Mol Sci. 2014;16(1):159–77.

21. Xin C, Zhang H, Liu Z. miR-154 suppresses colorectal cancer cell growth and motility by targeting TLR2. Mol Cell Biochem. 2014;387(1-2):271–7.

22. Lucas C, Barnich N, Nguyen HTT. Microbiota, Inflammation and Colorectal Cancer. Int J Mol Sci, 2017. 18(6).

23. van der Beek CM, et al. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev. 2017;75(4):286–305.

24. Yusuf F, Adewiah S, Fatchiyah F. The Level Short Chain Fatty Acids and HSP 70 in Colorectal Cancer and Non-Colorectal Cancer. Acta Inform Med. 2018;26(3):160–3.

25. Xiao T, et al. Butyrate upregulates the TLR4 expression and the phosphorylation of MAPKs and NF-κB in colon cancer cell in vitro. Oncol Lett. 2018;16(4):4439–47.

26. Wang G, et al. Role of SCFAs in gut microbiome and glycolysis for colorectal cancer therapy. J Cell Physiol. 2019;234(10):17023–49.

27. Snider AJ, et al. Murine Model for Colitis-Associated Cancer of the Colon. Methods Mol Biol. 2016;1438:245–54.

28. Pandurangan AK, et al. Dietary cocoa protects against colitis-associated cancer by activating the Nrf2/Keap1 pathway. Biofactors. 2015;41(1):1–14.

29. Zaki MH, et al. The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. Cancer Cell. 2011;20(5):649–60.

30. Li TT, Ogino S, Qian ZR. Toll-like receptor signaling in colorectal cancer: carcinogenesis to cancer therapy. World J Gastroenterol. 2014;20(47):17699–708.

31. Tsan MF. Toll-like receptors, inflammation and cancer. Semin Cancer Biol. 2006;16(1):32–7.
32. Machiels K, et al. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut. 2014;63(8):1275–83.
33. O'Keefe SJ. Diet, microorganisms and their metabolites, and colon cancer. Nat Rev Gastroenterol Hepatol. 2016;13(12):691–706.
34. Luo S, et al. Sodium butyrate induces autophagy in colorectal cancer cells through LKB1/AMPK signaling. J Physiol Biochem. 2019;75(1):53–63.
35. Ma X, et al. Sodium butyrate modulates gut microbiota and immune response in colorectal cancer liver metastatic mice. Cell Biol Toxicol. 2020;36(5):509–15.
36. Tian Y, et al. Short-chain fatty acids administration is protective in colitis-associated colorectal cancer development. J Nutr Biochem. 2018;57:103–9.
37. Xiao T, et al. Butyrate functions in concert with myeloid-derived suppressor cells recruited by CCR9 to alleviate DSS-induced murine colitis. Int Immunopharmacol. 2021;99:108034.
38. Arneth B, Tumor Microenvironment. Medicina (Kaunas), 2019. 56(1).
39. Dysthe M, Parihar R. Myeloid-Derived Suppressor Cells in the Tumor Microenvironment. Adv Exp Med Biol. 2020;1224:117–40.
40. De Cicco P, Ercolano G, Ianaro A. The New Era of Cancer Immunotherapy: Targeting Myeloid-Derived Suppressor Cells to Overcome Immune Evasion. Front Immunol. 2020;11:1680.
41. Gao X, et al. Immunotherapy Targeting Myeloid-Derived Suppressor Cells (MDSCs) in Tumor Microenvironment. Front Immunol. 2020;11:585214.
42. Law AMK, Valdes-Mora F, Gallego-Ortega D. Myeloid-Derived Suppressor Cells as a Therapeutic Target for Cancer. Cells, 2020. 9(3).
43. Sieminska I, Baran J. Myeloid-Derived Suppressor Cells in Colorectal Cancer. Front Immunol. 2020;11:1526.
44. Wang Y, et al., Role of myeloid-derived suppressor cells in the promotion and immunotherapy of colitis-associated cancer. J Immunother Cancer, 2020. 8(2).
45. Groth C, et al. Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression. Br J Cancer. 2019;120(1):16–25.
46. Li BH, Garstka MA, Li ZF. Chemokines and their receptors promoting the recruitment of myeloid-derived suppressor cells into the tumor. Mol Immunol. 2020;117:201–15.
47. Zhu M, et al. Contribution of drugs acting on the TLRs/MyD88 signaling pathway on colitis-associated cancer. Pharmazie. 2018;73(7):363–8.
48. Paarnio K, et al. Divergent expression of bacterial wall sensing Toll-like receptors 2 and 4 in colorectal cancer. World J Gastroenterol. 2017;23(26):4831–8.
49. Liu YD, et al. Toll-like receptor 2 stimulation promotes colorectal cancer cell growth via PI3K/Akt and NF-κB signaling pathways. Int Immunopharmacol. 2018;59:375–83.
Figures

Figure 1

Butyrate administration alleviates AOM-DSS induced symptoms in CAC mice. A. Protocol for the induction of the CAC model. B. The rectal prolapse of mice at the 10\textsuperscript{th} week. C. Survival curve analysis. D. The changes in body weight E. The DAI of three groups mice (n=10). Data are represented as the mean ± standard. *P < 0.05, **P < 0.01 and ***P < 0.001. NS, not significant;
Figure 2

Butyrate protects mouse intestine from AOM-DSS induced CAC. A. Macroscopic morphology of colon tumors. B. Colon length statistical analysis. C. Tumor counts statistical analysis. D. Tumor size statistical analysis. E. Macroscopic morphology of spleen. F. Spleen weight statistical analysis. G. H&E staining of spleen and colon (microscope ×40). H. Inflammation score in colon. I. Hyperplasia score in colon (n=3). Data are represented as the mean ± standard. *P < 0.05, **P < 0.01 and ***P < 0.001. NS, not significant;
Bu, butyrate; AOM, azoxymethane; DSS, Dextran sulfate sodium NC, negative control; H&E, Hematoxylin and eosin.

Figure 3

Butyrate inhibited the proliferation and promoted the apoptosis of CAC cells. ICH detection and statistical analysis of positive rate for (A)Ki67, (B)BCL2 and (C)CD31 (microscope: left ×40, right ×100) (n=3). Data
are represented as the mean ± standard. *P < 0.05, **P < 0.01 and ***P < 0.001. NS, not significant; Bu, butyrate; AOM, azoxymethane; DSS, Dextran sulfate sodium NC, negative control; IHC, Immunohistochemistry.

Figure 4
Butyrate reduced the proportion of MDSCs in AOM-DSS–induced CAC mice. A. Flow cytometry analysis of MDSCs (CD11b+Gr-1+) in the colon and spleen. B-C. Statistical analysis of MDSCs (CD11b+Gr-1+) and CD11b+Gr-1- cells in the cell population (n = 3). Data are represented as the mean ± standard. *P < 0.05, **P < 0.01 and ***P < 0.001. NS, not significant; Bu, butyrate; AOM, azoxymethane; DSS, Dextran sulfate sodium NC, negative control; MDSCs, myeloid-derived suppressor cells.

Figure 5
Butyrate inhibited the mRNA expression levels of proinflammatory mediators in CAC mice. RT-qPCR analysis of (A) TNF–α, (B) IL–6 and (C) CCL2 in both colon(left) and spleen(right) tissues (n=3). Data are represented as the mean ± standard. *P < 0.05, **P < 0.01 and ***P < 0.001. NS, not significant; Bu, butyrate; AOM, azoxymethane; DSS, Dextran sulfate sodium; tumor necrosis factor–α, TNF–α; interleukin-6, IL-6; NC, negative control; RT-PCR, reverse transcription–quantitative polymerase chain reaction
Butyrate inhibited the activation of TLR2 signaling pathway in AOM-DSS induced CAC. A. ICH detection of TLR2. B. TLR2 positive rate. C. RT-qPCR analysis of TLR2. D. Western blot analysis for TLR2, Myd88 and NF-κB (P65). E-G. Densitometric analyses of the protein bands (n = 3). Data are represented as the mean ± standard. *P < 0.05, **P < 0.01 and ***P < 0.001. NS, not significant; Bu, butyrate; AOM, azoxymethane; DSS, Dextran sulfate sodium NC, negative control; TLR2, Toll-like receptor 2; Myd88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa-B; RT-PCR, reverse transcription–quantitative polymerase chain reaction; IHC, Immunohistochemistry.

**Figure 7**

Schematic diagram of inhibition of butyrate on AOM-DSS induced mouse CAC.