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Original Article

Therapeutic potential and deleterious effect of glucocorticoids on azoxymethane/dextran sulfate sodium-induced colorectal cancer in mice

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Abstract: Glucocorticoids (GCs) are widely used in the treatment of various autoimmune and inflammatory diseases, including inflammatory bowel disease (IBD). However, the effect of GCs on the progression of colitis-associated colorectal cancer (CAC) has not been well explored. In this study, we first established a colorectal cancer model induced by azoxymethane and dextran sulfate sodium (AOM/DSS) and a colitis model induced by DSS in mice. Dexamethasone (DEX) was then administered at different periods of time to determine its effect on tumorigenesis and tumor progression. Meanwhile, body weight, stool property and fecal blood of mice were recorded. At the end of this study, the number and load of tumors were evaluated, and the expression of proteins associated with cell proliferation was analyzed. To evaluate the inflammation in colon, we detected the level of pro-inflammatory cytokine TNFα, and the mucosal infiltration of inflammatory cells. Our results revealed that AOM injection followed by three cycles of drinking water containing 1.5% DSS successfully induced multiple tumor formation in mouse colon and rectum. Both early and late DEX intervention suppressed tumor growth in mouse colorectum, and downregulated the expression of PCNA and cyclin D1. Moreover, DEX treatment significantly inhibited TNFα production, mucosal infiltration of inflammatory cells, and the activity of MAPK/JNK pathway, particularly early DEX intervention. However, we also found that DEX treatment deteriorated the general state of mouse manifested by greater loss of body weight and rectal bleeding. In summary, both early and late DEX interventions significantly ameliorate colonic inflammation and inhibit the progression of AOM/DSS-induced colorectal cancer, at least partly due to the inhibition of MAPK/JNK pathway. It is noteworthy that the deleterious effect on the general condition of mouse may limit the duration of GCs treatment.

Keywords: Dexamethasone, colitis-associated colorectal cancer, inflammatory bowel disease, colitis, TNFα

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the world with the third incidence and the second mortality rate [1]. Many factors contribute to the initiation and progression of CRC. Chronic inflammation is one of the most important factors, promoting the progression of different types of cancer and inducing about 20% of cancers [2]. Inflammation bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is the third highest risk factor of CRC [3], causing cancer incidence up to 20% [4]. There are about 2% cases of CRC arising in patients with long-standing IBD, known as colitis-associated cancer (CAC) [4].

The pathogenesis of CAC is complex, affected by many factors including heredity, environmental factors, infection and imbalance of flora. These factors modulate the microenvironment of IBD and induce immune disorders mediated by various immune cells and high level of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6), which are responsible for the initiation and progression of CAC [5, 6]. The

duration and severity of inflammation in intestinal tissue are closely related to the neoplastic transformation of IBD [6]. TNFα, mainly induced by macrophages, can active multiple signal transduction, such as MAPK/JNK and nuclear factor kappa-B (NF-κB) pathways, contributing to cell survival, epithelial cell transformation and tumor progression [7].

Glucocorticoids (GCs) have been used widely in the treatment of autoimmune and inflammatory diseases, due to their immunosuppressive and anti-inflammatory effects [8]. GCs are also used to treat lymphoid cancers and solid cancers associated symptoms or chemotherapy-related adverse reactions [9, 10]. Although GCs' toxicity on various organs are inevitable and emerging immunosuppressive agents have been used in the treatment of IBD, GCs are still used as a mainstay of therapy because they effectively induce rapid remission of IBD [11]. However, the role of GCs in various solid cancers is controversial because of their complex mechanism and extensive functions [9, 12]. In particular, the role of GCs in the initiation and progression of CAC still has not been well elucidated.

In this study, we established an azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced CAC murine model as described previously [13]. This model recapitulates the progression of CAC from normal mucosa to inflammation, hyperplasia and ultimately transforming into adenoma and carcinoma [14]. These mice were then administrated with dexamethasone (DEX), a synthetic analog of GCs, at different periods of the experiment in order to determine the effect of GCs on tumorigenesis and progression of CAC.

Materials and methods

Reagents and antibodies

Azoxymethane (AOM) and sodium dextran sulfate (DSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and MP Biomedical (Carlsbad, CA, USA), respectively. Dexamethasone (DEX) was purchased from YEASEN Biotech Co. Ltd (Shanghai, China). The antibodies used in this study were presented in Table S4.

Animal studies

Male C57BL/6J mice (n = 120, 8-9 weeks) were purchased from the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, China), and housed in a specific-pathogen-free (SPF) condition under a 12 h light/dark cycle, with free access to a standard laboratory diet and water. All mice were adapted to above conditions for one week before the experiment, and 5-6 mice were housed in every cage. All animal experiments were performed in compliance with Institution Guidelines and approved by the Laboratory Animal Center of Xi'an Jiaotong University.

Induction of CAC mouse model and glucocorticoids treatment

We established the CAC mouse model as previously described [13]. Briefly, on Day 1 of the experiment, the mice (n = 36) were injected intraperitoneally with 12 mg/kg of AOM dissolved in water and diluted with normal saline (NS) to a final concentration of 2 mg/ml, and maintained with normal diet and water for one week. The mice were divided into four groups and then given drinking water containing 1%, 1.5%, 2% or 2.5% DSS (wt/vol) for 5 days, followed by 2 weeks of normal drinking water. This cycle was repeated for three times. After that, the mice recovered with regular diet and drinking water till the end of experiment. The control mice (Normal, n = 4) were injected intraperitoneally with 200 μl normal saline (NS), and maintained with regular chow and drinking water till the end.

Next, the mice (n = 61) were induced CAC by AOM and 1.5% DSS. After the first DSS cycle, the mice (n = 41) were divided into three groups: CAC (n = 11), DEX treatment at early stage (DEX-E, n = 18) and DEX treatment at late stage (DEX-L, n = 12). The mice in CAC were injected intraperitoneally with 200 μl normal saline (NS) containing 1% DMSO after the second DSS cycle as the negative control. Meanwhile, DEX (5 mg/kg) dissolved in DMSO and diluted with normal saline (NS) was given intraperitoneally to the mice in DEX-E every other day after the second cycle of DSS and to the mice in DEX-L after the last DSS cycle. The normal mice (n = 4) were injected intraperitoneally with 200 μl normal saline (NS) on the first day as a control for AOM injection. All mice were monitored with body weight, stool consistency, rectal bleeding and survival ratio

twice a week. Disease activity index (DAI) were scored by a semi-quantitative scoring system as previously described (Table S3) [53, 54].

At the end of the experiment (Day 95), all mice were sacrificed by cervical dislocation, and colon tissues and spleens were then collected. Besides, the fasting glucose level was measured before the mice sacrificed. For further analysis, the colons were cut open through longitudinal line, and washed with phosphate buffer saline (PBS, pH 7.4). Tumor number was then recorded and the diameter of each tumor was measured by an experimenter blindly (long diameter = length; short diameter = width; small tumor, medium tumor and large tumor are defined as the length <2 mm, 2 mm ≤ length ≤5 mm, and length >5 mm). Moreover, the area and volume of each tumor was calculated (tumor area = $[(\text{length} + \text{width}) * 0.5]^2$, tumor volume = length * width^2 * 0.52) as previously described [43]. Next, neoplastic tissues were fixed in 4% paraformaldehyde buffer immediately and then processed by dehydration, paraffin embedding and ultimately made into 5 μm sections for histopathological examination and immunohistochemistry (IHC) analysis, while the others were frozen in liquid nitrogen and stored at -80°C until use.

Induction of colitis model and glucocorticoids treatment

According to induce colitis in mice, drinking water with 3% DSS was given to mice for 7 days. To validate the anti-inflammatory effect of GCs, mice (n = 14) were divided into three groups: Normal (n = 3), Colitis (n = 6) and Colitis-DEX (n = 5). The mice in Colitis-DEX were injected intraperitoneally with DEX (5 mg/kg) daily on D2-D7, while the mice in Colitis were injected intraperitoneally with 200 μl normal saline (NS) containing 1% DMSO. During the experiment, mice were weighted every day, and stool consistency and rectal bleeding were recorded every other day. On Day 7, after the fasting glucose level was measured, mice were dissected, then colon and spleen tissues were fixed or stored at -80°C until use.

Histopathological analysis

For histological evaluation of inflammation, paraffin-embedded colon sections were stained with H&E, and the sections were then observed and evaluated blindly by a trained pathologist according to a previously established scoring system (Table S2) [55]. To assess the histopathological tumor incidence and multiplicity in AOM/DSS-induced mice, adenoma with low-grade dysplasia showed simple glandular architecture, branching or elongation of crypts and a low nuclear to cytoplasmic ratio; cell nuclei elongated, slightly crowded, maintained polarity with respect to the basement membrane. Adenoma with high-grade dysplasia was defined as adenomatous changes with complex irregularity of glands, complete loss of goblet cells, marked reduction of interglandular stroma with cribriform structures and back-to-back glands; cell nuclei manifested as high nuclear to cytoplasmic ratio, pseudostratification and loss of polarity. Intra-mucosal carcinoma was applied to high-grade neoplastic lesions that show invasion of neoplastic cells into the lamina propria but not through the muscularis mucosa [56, 57].

Western blot analysis

The tumors or colonic tissues were homogenized in RIPA buffer containing protease inhibitors, and equal amount of lysates were then subjected to Western blot analysis as previously described [58]. All the experiments were performed in triplicate.

Immunohistochemistry (IHC) analysis

The IHC staining was applied to measure the levels of indicated proteins in colonic tissues. The experimental details were accomplished as previously described [58]. Each stained section was quantified using the Image J, and scored with 0, 1, 2 and 3, which represent negative, weak positive, positive, and strong positive, respectively. An average score was then used to evaluate the relative expression of proteins according to the staining intensity and percentage of positive area. Data shown are representative of independent experiments performed at least in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

The tumors or colonic tissues were sampled and weighed, then homogenized in phosphate buffered saline (PBS). Next, the supernatant was collected and subjected to measure TNFα level using the Mouse TNFα ELISA Kit (Excell

Bio, Shanghai, China) according to the manufacturer's protocol. The values were normalized by tissue weight.

Measurement of fasting blood glucose

At the end of the experiment, after deprivation of food for eight hours, the blood was taken from the tail of mice, and blood glucose was then measured with a glycometer (Sinocare, Changsha, China) according to the manufacturer's instruction.

Statistical analysis

Student's unpaired *t* test was performed to compare the means of data in two groups using GraphPad Prism Software Version 8.0 (GraphPad Software). All data were expressed as mean ± standard deviation (SD), and *P* values <0.05 were considered as a statistically significant difference.

Discussion

It is clear that chronic inflammation is recognized as a promoting factor in tumor initiation and progression, including colorectal cancer [2]. The patients suffering from IBD have an increased risk of developing to colorectal cancer, in which the longstanding of colonic inflammation may play a pathogenic role in malignant transformation [6]. In fact, the etiology and pathology of IBD have not been defined completely. This is ascribed to impaired barrier function and immune disorder resulting from the complex genetic background and various defined or undefined environmental factors [24, 25]. It is well established that blocking cytokines-mediated inflammatory pathways can reduce tumorigenesis of CAC, as supported by several previous studies. For example, blocking TNFα pathway and IL-6 deficiency reduced tumor formation in mice treated by AOM and DSS [18, 26]. TcES treatment inhibited the AOM/DSS-induced tumorigenesis by regulating both STAT3 and NF-κB pathways [16]. The application of GCs in therapy of IBD has not diminished, despite of the toxicity and increased risk of infection due to long-term administration [6, 11]. A growing number of studies have investigated the role of GCs in tumor development and revealed their distinct mechanisms promoting or inhibiting tumor progression in different types of cancer, indicating that their activities were not limited to anti-inflammation [27-31]. Thus, there is a compelling need to determine the effect and underlying mechanism of GCs in the colitis-associated cancer.

In the present study, considering that GCs can rapidly improve IBD symptoms, mice were administered with DEX after the second or the third cycle of DSS drinking. The results indicated that DEX played an anti-inflammatory role in experimental colitis in mice, evidenced by decreased histological score, less releasing of TNFα and less infiltration of mature macrophagocytes. Besides, we also observed that DEX aggravated body weight loss, rectal bleeding and elevated DAI score throughout of the experiment. As supported, a previous study demonstrated that budesonide inhibited the local inflammation, while impaired wound healing and weakened mucosal barrier function, evidenced by decreased expression of claudin-2, PCNA, cyclin D1, cytokeratin 5/8 and claudin-4 in mice, indicating that budesonide has both protective and deleterious effects in the DSS-induced colitis [23]. Similarly, another study analyzed and compared the efficiency of key compounds used in the treatment of IBD, implying that budesonide has limited activity in preventing DSS-induced colitis due to its severe side effects [32]. This will mask its inhibitory effect on local inflammation, thereby leading to unfavorable clinical outcome.

The immune cells and inflammatory mediators in tumor microenvironment play a crucial role in tumor progression [33]. Inflammatory response and tumorigenesis are frequently associated with dysregulation of MAPK pathways including JNK, which is commonly activated by pro-inflammatory cytokines [34], and has been proved to take part in the survival and proliferation of cancer cells [21]. Among them, TNFα plays a critical role in inflammatory response and tumor progression by targeting both immune and tumor cells as well as activating various oncogenic pathways, including MAPK/JNK-AP1 pathway [35]. A previous study has demonstrated that JNK in macrophages plays a critical role in the inflammatory response and the development of hepatocellular carcinoma using myeloid cell-specific JNK-deficient mice [36]. In the present study, we found increased staining score of p-Jnk in stromal tissues of CAC or colitis mice, and demonstrated that DEX administration could alleviate

the inflammation and inhibit tumor growth in the AOM/DSS-induced CAC mice by blocking MAPK/JNK pathway. This was also supported by a previous study that DEX administration decreased the levels of total and phosphorylated JNK [37].

It is the fact that DEX administration from both the second cycle and the third cycle of DSS reduces tumor size in colon, while has no effect on total tumor number, indicating that the first cycle of DSS administration may be critical for AOM/DSS-induced tumorigenesis. Besides, although DEX intervention at early stage showed greater suppression of colonic inflammation, the inhibitory effect on tumor progression was just as prominent as that in DEX intervention at late stage. This was at least partly ascribed to the destruction of tumor immunology due to the long-term immunosuppression by DEX administration. As supported, previous studies have revealed that DEX suppresses the activation of T cell by inhibiting inflammatory cytokines and upregulating PD-1 expression [38, 39]. There is evidence showing that DEX administration can impair intra-tumoral immunity, thereby causing the failure of cancer immunotherapy *in vivo* [40]. These observations indicate that immunosuppression and modulation of tumor microenvironment by GCs treatment may contribute to tumor progression [41].

A recent study showed that DEX accelerated the onset and severity of colitis and colitis-associated cancer mediated by mTOR signaling pathway, in which mice were treated with DEX (2.5 mg/kg) at the beginning of DSS drinking [42]. Besides, another study concluded that corticosterone, given in feeding solution (50 mg/L) one week after DSS administration, inhibited the inflammation but promoted the progression of AOM/DSS-induced colon cancer by activating NF-κB and COX-2 [43]. These diverse outcomes on experimental animals may be explained by different dosages and periods of drug feeding during experiment. The complex function and different outcomes of GCs treatment *in vivo* are explainable because GCs act on almost all types of cells in the body to maintain homeostasis in response to various stimuli [44]. GCs inhibit pro-inflammatory activation of macrophages and also monocytes during initiation of inflammation and enhance macrophage phagocytosis to achieve resolution of inflammation; however, GCs exerts mainly inhibitory effects on T cell-mediated adaptive immunity [45].

Whether in clinical patients or AOM/DSS-induced experimental model, the severity of inflammation plays a crucial role in the progression of colitis-associated cancer [46]. When corticosterone was given to mice one week after the last cycle of DSS drinking, the inflammatory phenotypes, including tissue damage and infiltration of inflammatory cell, have largely alleviated. During this time, the anti-inflammatory of GCs may play insignificant role in tumor progression, while the immunosuppressive activities particularly on T cell-mediated immune responses or anti-apoptosis and pro-survival role on neoplastic cells may become dominant in the process of cancer progression. The role of GCs in other solid tumors was provided by previous studies that exogenous GCs promote cell survival and inhibit cancer cell death pathways by targeting glucocorticoid receptors (GRs) [41]. However, in the present study, we administered DEX just followed DSS drinking, leading to efficient control of inflammation during the acute stage of colitis, which contributed to the inhibition of cancer progression. As supported, a previous study has indicated that the DSS-induced colitis has mostly resolved 4-5 days after DSS removal [47], and the role of GCs through GRs in myeloid cells is crucial to disease resolution of DSS-induced colitis at the later stage of DSS administration [48]. Moreover, the severity of inflammation determined the strengthen of carcinogenesis, and an inducible deletion of GRs in intestinal epithelial cells before but not after DSS-induced acute phase of colitis altered the tumor burden in AOM/DSS induced mice [46].

Besides, the pharmacological effects of various GCs are similar but not exactly the same [49]. A previous study demonstrated that corticosterone suppressed intestinal T cell function and impaired barrier function after alcohol exposure [50]. Thus, it is rational that corticosterone, as a short-acting GC with weak anti-inflammatory activity, induces inconsistent outcome with DEX, especially when mice were administrated at different doses, time and routes. In another study, mice were treated with DEX (2.5 mg/kg) twice during the DSS

administration. The results showed that DEX promoted tumorigenesis in the AOM/DSS induced model. There are two reasons to explain the outcome differed from our results. On the one hand, DEX administration accompanied by DSS exposure promoted the onset and development of acute ulcerative colitis, providing a more favorable environment for mutation, which contributed to tumor initiation and development. A previous study has indicated that GCs contribute to a pro-inflammatory response at the first time after an inflammatory stimulus or tissue damage, although GCs are clearly anti-inflammatory in situations of ongoing inflammation [51]. In addition, there is evidence showing that the prior exposure of DEX may be unfavorable, even stimulating inflammatory response [52]. On the other hand, the different dosage and frequency of DEX administration may be another reason for the diverse outcomes, since it is well known that high dose and high frequency of GCs may be necessary to obtain their anti-tumor activity [43]. Moreover, the ongoing use of DEX after removal of DSS may be crucial to restoration of inflammation. This was supported by a previous study that the DSS-induced colitis did not resolve in mice lacking GRs in myeloid cells after DSS administration [48]. These observations suggest that various factors contribute to the effect of GCs in the setting of cancer therapy, including but not limited to the form and dosage of GCs, time and duration of drug treatment [9], predisposition of animal, the concentration and duration of DSS administration [32]. Thus, to define proper dosage and time window of GCs application, which is critical to obtain its anti-inflammatory and anti-tumor effect, robust pre-clinical studies and clinical trials are desired to carry out in the near future.

In this study, we systematically explored the different effects of GCs given at different points of time on colitis-associated tumorigenesis and tumor progression. Our results imply that DEX intervention suppresses DSS-induced colitis and the progression of AOM/DSS-induced CAC in mice. However, DEX administration aggravates the body weight loss and rectal bleeding in mice with AOM/DSS treatment. These findings indicate that the strict selection of time and dosage in GCs administration is crucially important whether in preclinical studies or clinical practices, to gain their inhibitory activities and reduce the side effects. Thus, we need to enlarge the number of animals and have more subdivided groups to further clarify the different effects of GCs under different conditions. Besides, prospective clinical trials are also needed to evaluate the effects of GCs in the progression of colitis-associated cancer.

Results

The establishment of AOM/DSS-induced CAC mouse model

Considering that AOM/DSS-induced colorectal cancer is a well-established, powerful and reproducible model in mice to explore the pathogenesis of CAC [15], we thus induced CAC in mice by AOM administration followed by repeated DSS treatment cycles, separated by recovery period of two weeks. The detailed procedure was showed in **Figure 1A**. During the experiment, body weight, rectal bleeding and stool consistency of mice were monitored twice a week. We first treated mice with different concentrations of DSS, and found that CAC mice with 1.5% DSS treatment led to 100% incidence of tumors in colon with a mortality rate of about 40% (Figure S1 and Table S1). Next, we treated with mice according to the above optimized protocol, and found that AOM/DSS-treated mice showed significant loss of body weight compared to control mice at the period of DSS administration (**Figure 1B**). At the end of experiment, the mice were sacrificed and dissected, and the colorectum was opened longitudinally for macroscopic observation. Multiple neoplasms were observed in colon of AOM/DSS-induced mice, particularly in the distal colon and rectum, while there is no lesion in the colon of normal mice (**Figure 1C**). According to pathologic evaluation on H&E-stained slides, most of them were adenoma with high-grade dysplasia or intramucosal carcinoma, and no invasive adenocarcinoma was observed (**Figure 1D**).

Dexamethasone treatment inhibits the progression of colorectal carcinoma in AOM/DSS-induced mice

Previous studies have demonstrated that blocking inflammatory response has protective role in the progression of CAC [16-18]. To determine whether GCs play an inhibitory role in colorectal tumorigenesis by its strong anti-

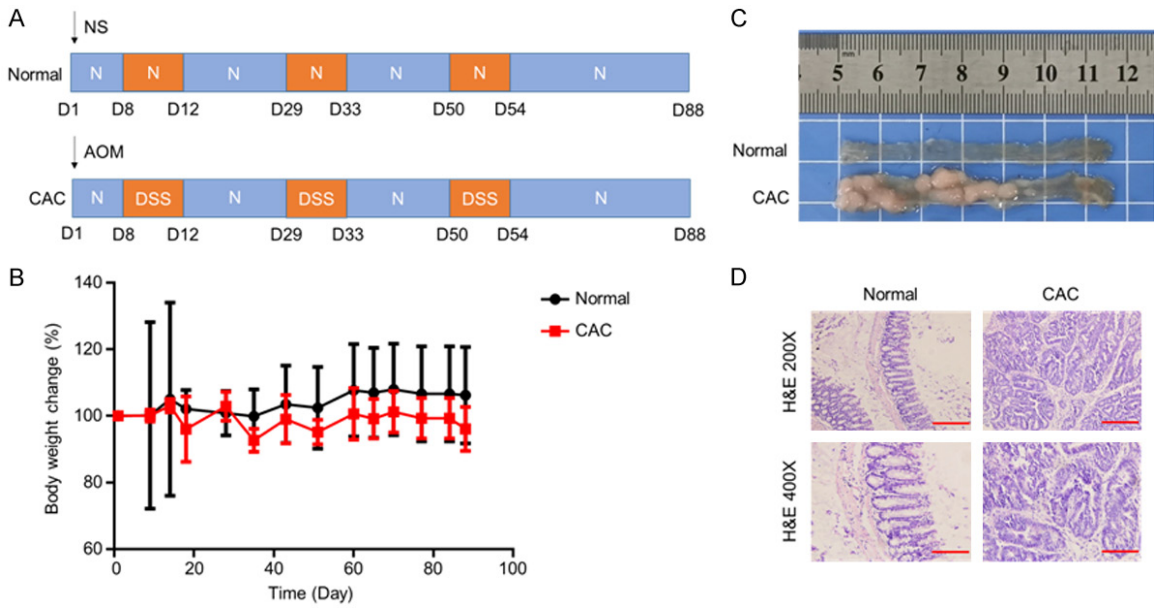

Figure 1. The establishment of CAC mouse model. A. The experimental setup of AOM/DSS-induced colitis associated cancer (CAC). A single dose of AOM (12 mg/kg) was injected to mice followed by three cycles of drinking water with 1.5% DSS for 5 days and two weeks of recovery. The tumors in colon were examined about one month after the last DSS cycle. B. Body weight change in normal mice (n = 4) and AOM/DSS-treated mice (n = 6). C. The representative view of neoplasms in colon of AOM/DSS-treated mice, and the colon of normal mice was presented as the control. D. Histological views of neoplasms in AOM/DSS-treated mice and normal epithelium (H&E staining); 200× amplification in upper panels and 400× amplification in lower panels; the scale represents 100 μm. Data were expressed as mean ± SD; AOM, azoxymethane; DSS, dextran sodium sulphate; NS, normal saline; CAC, colitis associated cancer; H&E, hematoxylin and eosin.

inflammatory effect, CAC was induced in mice and dexamethasone (DEX) was then administrated after the second cycle of DSS (early intervention with DEX, DEX-E) or the third cycle (late intervention with DEX, DEX-L), as shown in **Figure 2A**. Meanwhile, CAC mice were treated with normal saline (NS) as the control (CAC). Macroscopic view of colon from each group was shown in **Figure 2B**. DEX intervention significantly decreased tumor size, evidenced by the reduced number of large tumors (>5 mm) (**Figure 2C**), but not the smaller ones (≤5 mm), and decreased tumor area and tumor volume compared to the control (**Figure 2D** and **2E**). However, there was no significant reduction in the total number of colorectal tumors (**Figure 2F**). According to the assessment of histological tumor incidence and multiplicity in different groups, colorectal lesions in CAC mice were adenomas with 20% low-grade dysplasia, 68.6% high-grade dysplasia, and even 11.4% developed to intramucosal carcinoma with invasion of neoplastic cells into the lamina propria, while more tumors in AOM/DSS induced mice with DEX intervention were classified into low-grade dysplasia (48% in DEX-E and 42.2% in DEX-L) (**Figure 2G** and **Table 1**). These results indicate that DEX intervention inhibits tumor progression. Besides, our data showed that DEX treatment could not significantly reduce the mortality of AOM/DSS-induced mice, probably because of the limited number of animals (**Figure 2H**).

Next, the expression of proliferation markers including cyclin D1 and Pcna was evaluated by immunohistochemistry (IHC) assay. The results showed that their expression was significantly elevated in tumor tissues in CAC mice compared to normal control, while was meaningfully inhibited by DEX in both DEX-E and DEX-L groups (**Figure 3A** and **3B**). These data indicate that DEX administration inhibits the progression of AOM/DSS-induced CAC regardless of early or late intervention, despite the failure to prevent tumor incidence.

DEX alleviates colonic inflammation in AOM/DSS-treated mice

Inflammation mediated by multiple immune cells and cytokines, plays an important role in

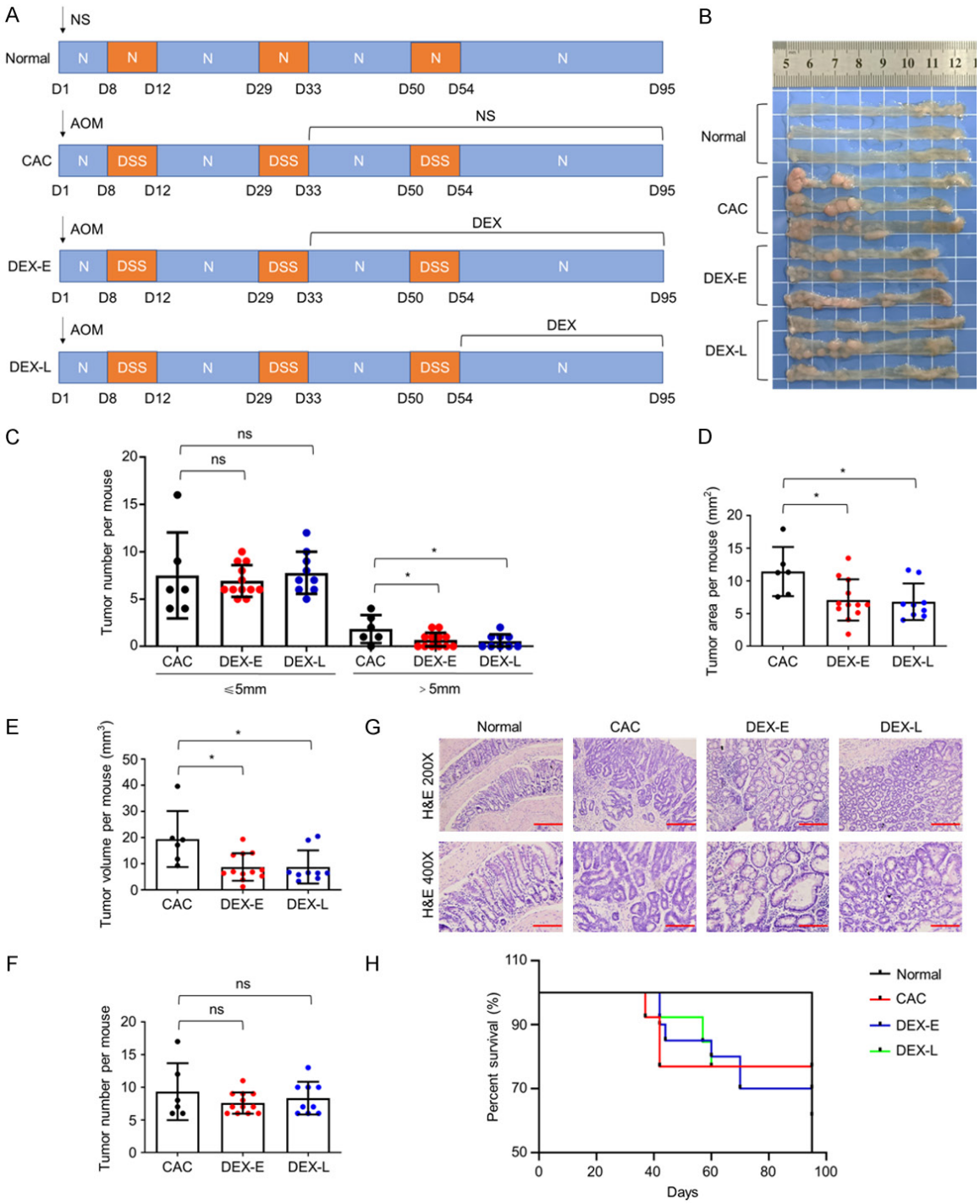

Figure 2. DEX treatment inhibits tumor growth and progression in CAC model. A. AOM/DSS-treated mice were divided into different groups. The normal mice (Normal, n = 4) were injected intraperitoneally with 200 μl normal saline (NS) as a control for AOM injection. The AOM/DSS-induced mice were treated with NS containing 1% DMSO (CAC, n = 11) or subjected to DEX intervention (5 mg/kg, ip) after the second cycle (DEX-E, n = 18) or the third cycle (DEX-L, n = 12) of DSS administration. The tumors in colon were measured on D95. B. The representative longitudinal views of large intestine of mice in different groups (n = 3 per group). C. Comparison of the numbers of small to mediate tumors (length ≤5 mm) and large tumors (length >5 mm) among different groups. D. Comparison of tumor area among different groups. E. Comparison of tumor volume among different groups. F. Comparison of total number of tumors among different groups. G. The histological views of colorectal neoplasms in different groups (H&E staining). Upper panels show 200× amplification; Lower panels show 400× amplification; The scale represents 100 μm. H. Comparison of survival rate of mice among different groups. The number of mice in Normal, CAC, DEX-E and DEX-L groups was 4, 6, 12 and 9, respectively. All data are expressed as mean ± SD. *P<0.05; ns, not significant.

Table 1. Histological tumor incidence and multiplicity in mice treated with AOM/DSS ± DEX

Groups	N	% Animals with macroscopic lesions (incidence)	Number of macroscopic lesions per animal (multiplicity) mean ± SD	% of microscopic lesions classified as low-grade dysplasia	% of microscopic lesions classified as high-grade dysplasia	% of microscopic lesions classified as intra-mucosal carcinoma
CAC	5	100	9.3±4.8	20	68.6	11.4
DEX-E	10	100	7.6±1.6	48	46	6
DEX-L	7	100	8.3±2.5	42.2	53.4	4.4

CAC, AOM/DSS-induced mice were treated with NS; DEX-E, AOM/DSS-induced mice were treated with DEX after the second cycle of DSS; DEX-L, AOM/DSS-induced mice were treated with DEX after the third cycle of DSS.

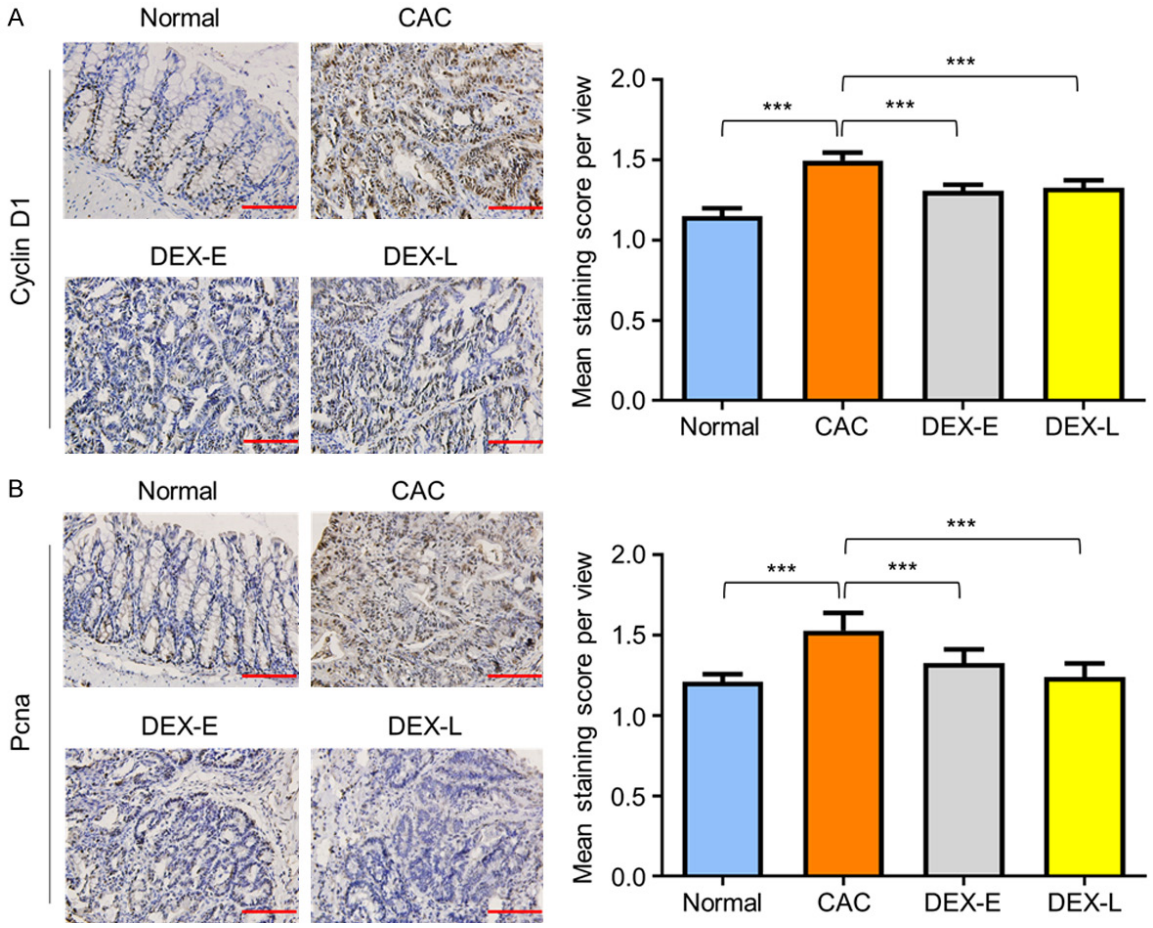

Figure 3. DEX treatment inhibits the expression of cyclin D1 and Pcna. The IHC assay was performed to evaluate the expression of proliferative markers cyclin D1 and Pcna in neoplastic tissues or normal colonic tissues of mice in different groups. Left panels show the representative images (400×) of IHC staining of cyclin D1 (A) and Pcna (B) in colonic epitheliums in the indicated mice (n = 3 per group), and statistical results were presented in right panels. The scale represents 100 μm. All experiments performed at least in triplicate. Data were expressed as mean ± SD. ***P<0.001.

promoting tumorigenesis and tumor progression [19]. TNFα shows an increased level in tumor microenvironment, while blocking TNFα signaling can decrease mucosal damage, infiltration of inflammatory cells and a significant reduction in tumor formation in AOM/DSS-treated mice [20]. Thus, we evaluated the inflammation degree and TNFα production of neoplasms and colonic tissues in AOM/DSS-treated mice. As shown in **Figure 4A** and **4B**, Western blot and IHC assays demonstrated an increased production of TNFα in tumor tissues of CAC mice compared to normal control, while TNFα production was reduced by DEX treat-

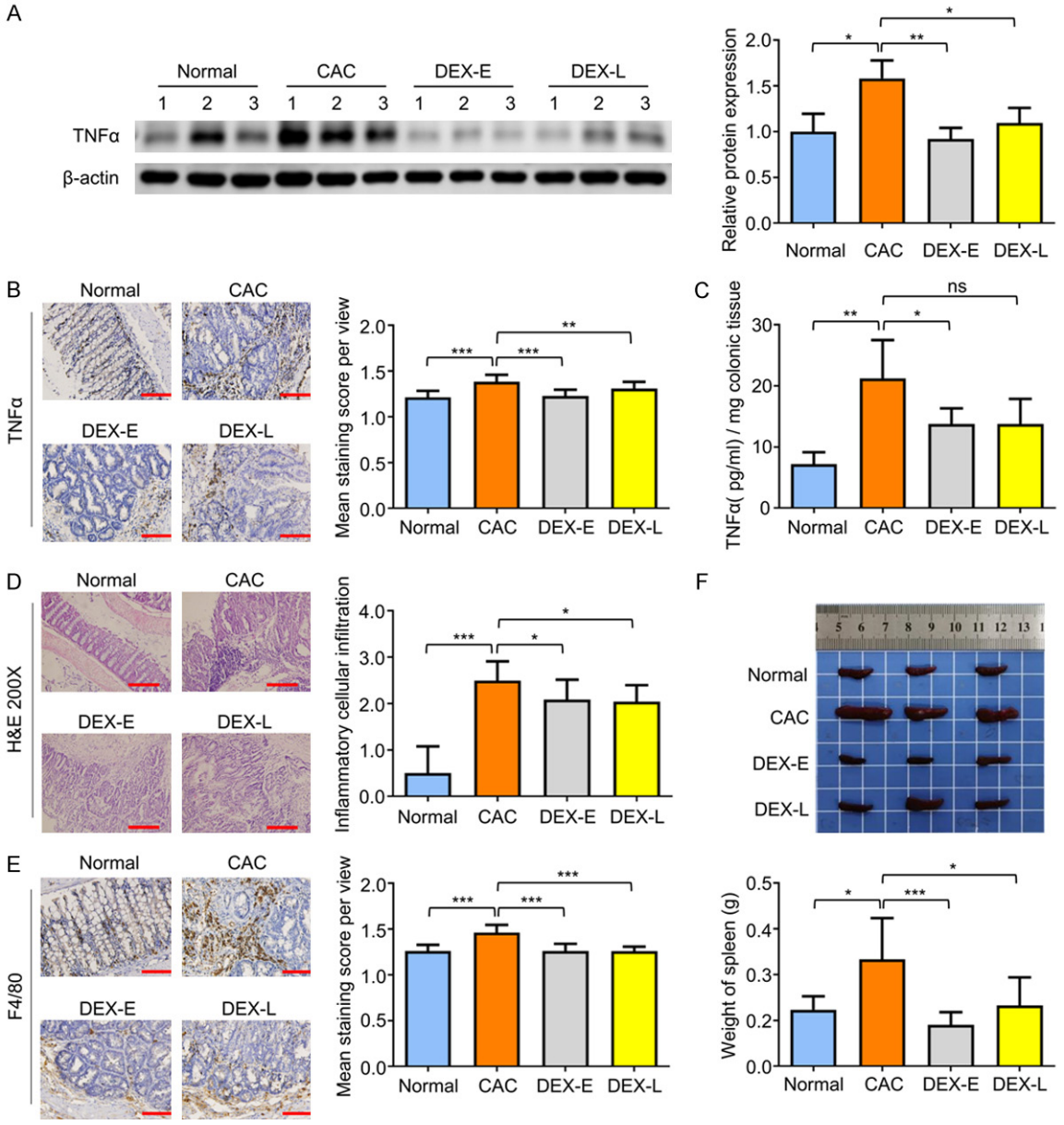

Figure 4. DEX treatment inhibits the colonic inflammation in AOM/DSS-induced mice. DEX (5 mg/kg, ip) was given to AOM/DSS-treated mice at different points of time to determine its anti-inflammatory effect. A. TNFα expression was detected by Western blot analysis (left panel, n = 3 per group) and measured using densitometry. β-actin was used as a loading control. Data shown are representative of independent experiments performed at least in triplicate and statistical results were presented in right panel. B. Left panels show the representative IHC images of TNFα staining in colonic epitheliums of normal mice and neoplastic tissues of the indicated mice (n = 3 per group). Data shown are representative of independent experiments performed at least in triplicate and statistical results were presented in right panel. C. TNFα production in colonic tissues was detected by ELISA and normalized by the weight of tissue specimens in Normal (n = 3), CAC (n = 6), DEX-E (n = 8) and DEX-L (n = 7) mice. D. Left panels show the representative images of normal epithelium and neoplasm area of AOM/DSS-treated mice (H&E staining, 200× amplification), and local infiltration of inflammatory cells was scored by a semi-quantitative method (right panel). E. The infiltration of macrophages in tumors was measured by IHC assay with F4/80 antibody (left panels, n = 3 per group). Data shown are representative of independent experiments performed at least in triplicate and statistical results were presented in right panel. F. The spleen images (upper panel) and statistical results of spleen weight (lower panel) in the indicated mice (n = 3 per group). The scale represents 100 μm. All data were expressed as mean ± SD. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant.

ment. Notably, inhibitory effect was more prominent when DEX was administered at early stage. Besides, we detected local TNFα production in colon tissues by ELISA. The results

showed that DEX intervention decreased TNFα levels in neoplastic tissues, especially in DEX-E group (**Figure 4C**).

Next, we evaluated the infiltration of inflammatory cells in tumor tissues using a semi-quantitative method (Table S2). The results showed that the infiltration of inflammatory cells was significantly increased in tumor tissues of the CAC mice compared to normal control, while reduced in DEX-treated mice compared to the CAC mice regardless of early or late intervention (**Figure 4D**). Meanwhile, the expression of F4/80, a biomarker of mature macrophages, was also analyzed by IHC assay. The results showed that the infiltration of macrophages in tumor microenvironment was increased compared to normal control, while significantly decreased in DEX-treated mice (**Figure 4E**). Besides, an abnormal increase in spleen size and weight was found in CAC mice compared to normal control, while DEX treatment induced clearly immunosuppression, evidenced by reduced size and weight of spleen compared to the CAC mice, particularly in DEX-E group (**Figure 4F**). These data indicate that DEX intervention significantly alleviates colonic inflammation in AOM/DSS-treated mice.

The effect of DEX intervention on the DSS-induced colitis

To further determine anti-inflammatory effect of DEX, we first induced colitis in mice by administrating 3% DSS for 7 days, and DEX (5 mg/kg) was given to mice daily in Colitis-DEX group from the second day (**Figure 5A**). By H&E staining, we found that normal epithelial structure of colon disappeared and was infiltrated with inflammatory cells in DSS-treated mice, while mucosal structure of intestine was retained and the decreased infiltration of inflammatory cells in colon was observed in Colitis-DEX mice (**Figure 5B**). Meanwhile, intestinal inflammation was histologically evaluated on the basis of four parameters: inflammatory infiltration, mucosal injury, crypt damage and lesion area (Table S2). The results demonstrated that inflammatory score of DEX-treated mice was lower than that in colitis mice (**Figure 5C**), indicating that DEX protects against DSS-induced colitis in mice. As supported, DEX-treated mice had lower weight/length ratio compared to the colitis mice (Figure S2). F4/80 staining also demonstrated that macrophage infiltration was significantly elevated in colitis mice compared to normal control, while DEX intervention effectively reverses this effect (**Figure 5D**).

Next, TNFα level in colonic tissues was measured by ELISA. The results showed that TNFα level was increased in colonic tissues of colitis mice compared to normal control, while DEX treatment reversed this effect (**Figure 5E**). This was also supported by the results of Western blot and IHC assays (**Figure 5F** and **5G**). As expected, we found that spleen weight was increased in colitis mice compared to normal control, although the difference did not reach statistical significance, while DEX treatment effectively reduced spleen weight compared to colitis mice (**Figure 5H**). Besides, we attempted to determine the effect of DEX intervention on the expression of cyclin D1 and Pcna in colitis mice. The results showed that their expression was elevated in colonic tissues of colitis mice compared to normal control but there was no significant difference between them, while DEX treatment clearly down-regulated their expression (**Figure 6A**). This was also supported by the results of IHC assays (**Figure 6B** and **6C**). Collectively, these results indicate that DEX not only plays an anti-inflammatory effect in DSS-induced colitis, but also inhibits the expression of proliferative markers in colonic tissues.

DEX treatment inhibits the activity of MAPK/JNK pathway

The MAPK pathway including JNK, which is activated by inflammatory mediators such as TNFα, is often dysregulated in inflammatory response and tumorigenesis [21]. Given that DEX intervention decreased TNFα production in CAC or colitis mouse model, we thus speculate that DEX treatment inhibits the activity of JNK pathway by decreasing TNFα production. The results showed that the levels of total or phosphorylated Jnk (p-Jnk) were increased in the AOM/DSS-induced mice compared to normal control, while their levels were clearly decreased by DEX, particularly early DEX intervention (**Figure 7A**), which was also supported by the results of IHC assays (**Figure 7B**). Besides, we also demonstrated the inhibitory effect of DEX intervention on the activity of JNK pathway in the DSS-induced colitis by Western blot and IHC assays (**Figure 7C** and

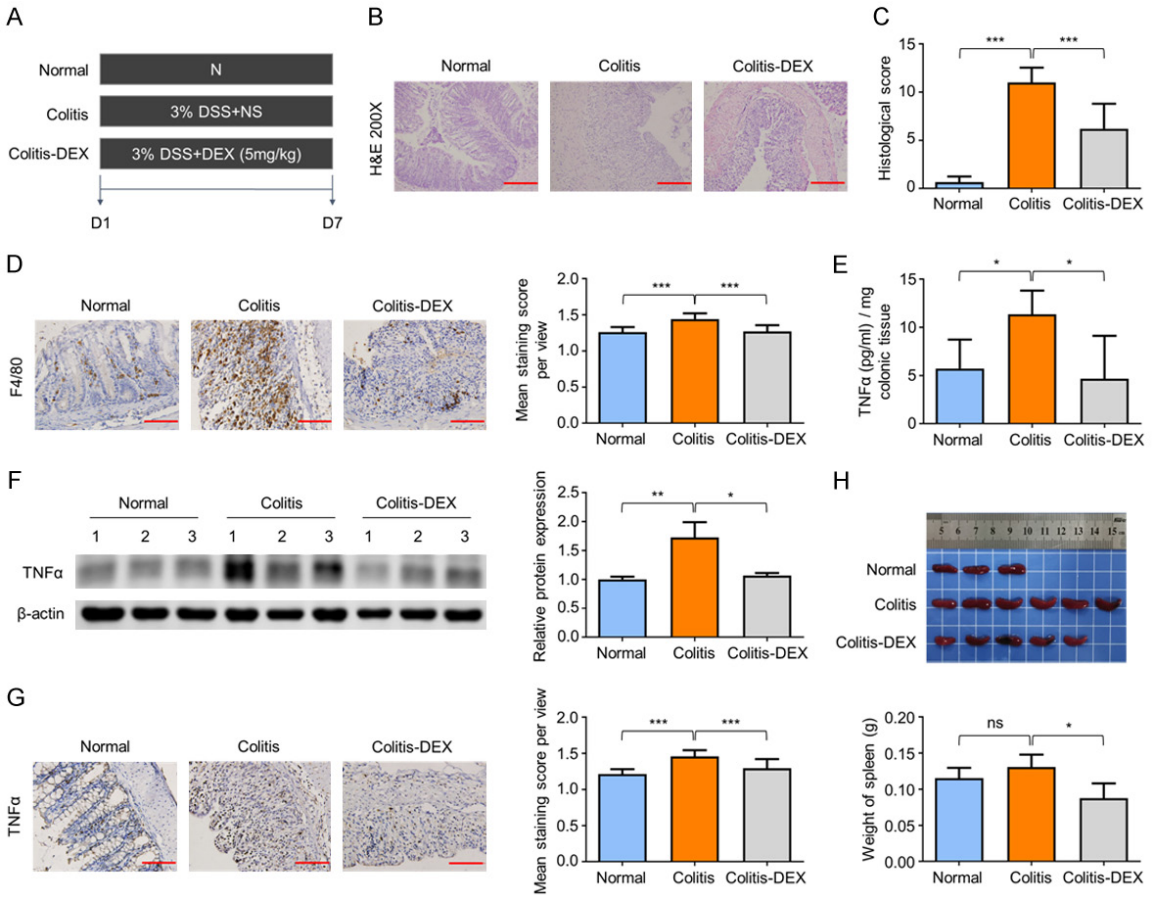

Figure 5. Inhibitory effect of DEX treatment on DSS-induced colitis in mice. A. The scheme shows the process of DSS-induced colitis with DEX (Colitis-DEX, n = 5) or NS (Colitis, n = 6) administration in mice. B. The histological views of colonic epithelium of normal, colitis and colitis-DEX mice (H&E staining, 200× amplification). C. The local inflammation was histologically scored by lymphocytes infiltration, mucosal injury, crypt damage and lesion extent in normal (n = 3), colitis (n = 6) and colitis-DEX (n = 5) mice. D. The infiltration of macrophages in epithelial tissues was measured by IHC staining with F4/80 antibody (left panels, n = 3 per group). Data shown are representative of independent experiments performed at least in triplicate and statistical results were presented in right panel. E. The TNFα production was measured by ELSA and normalized by weight of tissue specimens in normal (n = 3), colitis (n = 4) and colitis-DEX (n = 3) mice. F. TNFα expression was evaluated in colonic tissues of the indicated mice by Western blot analysis (Left panels, n = 3 per group) and measured using densitometry. β-actin was used as a loading control. Data shown are representative of independent experiments performed at least in triplicate and statistical results were presented in right panel. G. Left panels show the representative IHC images of TNFα staining in colonic epitheliums in the indicated mice (n = 3 per group). Statistical results were presented in right panel. H. The spleen images (upper panel) and statistical results of spleen weight (lower panel) in normal (n = 3), colitis (n = 6) and colitis-DEX (n = 5) mice. The scale represents 100 μm. All data were expressed as mean ± SD. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant.

7D). These observations indicate that DEX exerts its anti-inflammatory and anti-tumor properties in the DSS-induced colitis and colitis-associated cancer at least partially by inhibiting the activity of MAPK/JNK pathway.

DEX treatment induces general deterioration of mouse state

The duration of glucocorticoids therapy results in various side effects, such as peptic ulcer, osteoporosis and immunosuppression, limiting their clinical application [22]. A previous study has demonstrated that budesonide aggravates body weight loss, rectal bleeding and impaired epithelium healing in the DSS-induced colitis model [23]. In this study, we recorded body weight, rectal bleeding and stool consistency to monitor the general state of mouse. For AOM/DSS-treated mice, their body weight significantly decreased in the period of DSS administration compared to normal control

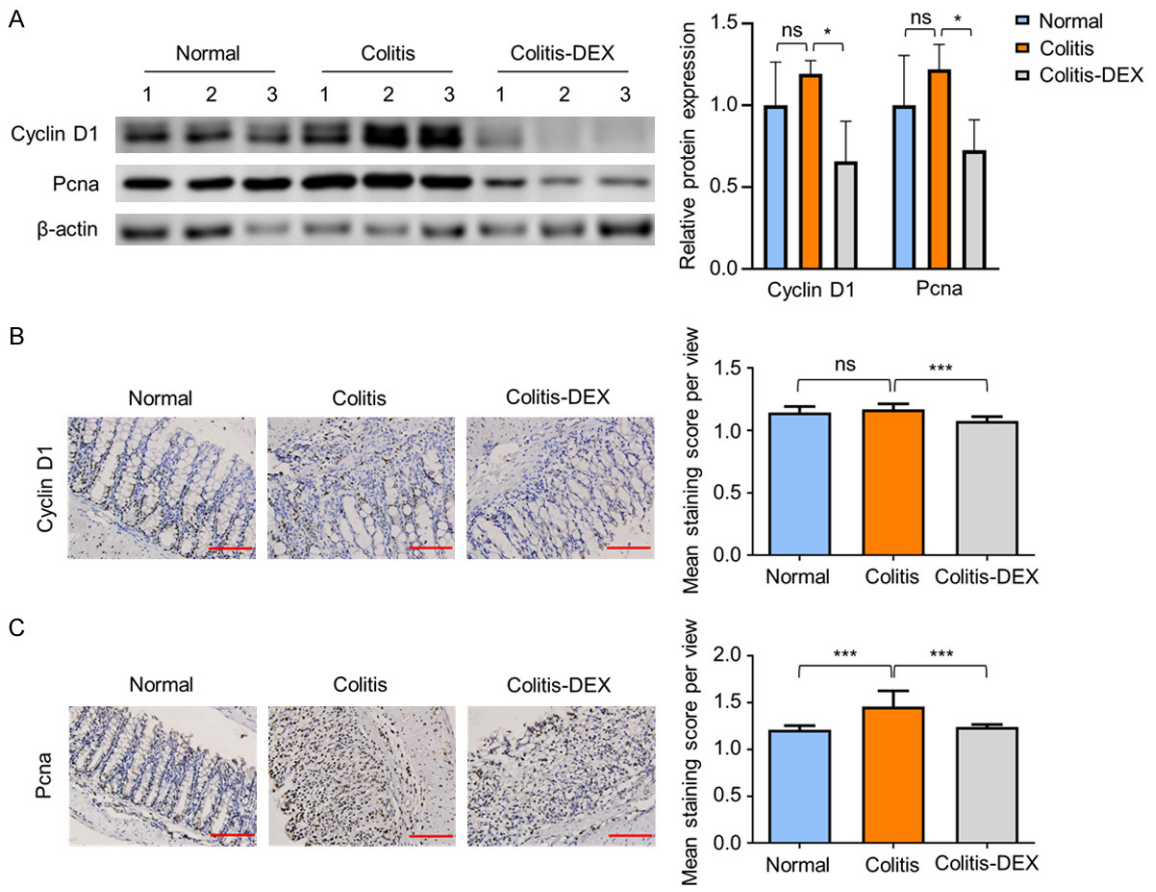

Figure 6. DEX treatment inhibits the expression of cyclin D1 and Pcna in the mice with DSS-induced colitis. (A) The expression of proliferative markers cyclin D1 and Pcna was evaluated in colonic tissues of the indicated mice by Western blot analysis and measured using densitometry (left panel, n = 3 per group). β-actin was used as a loading control. Statistical results were presented in right panel. Left panels show the representative IHC images (400×) of cyclin D1 (B) and Pcna (C) staining in colonic epitheliums in the indicated mice (n = 3 per group). Statistical results were presented in right panel. The scale represents 100 μm. All experiments performed at least in triplicate. Data were expressed as mean ± SD. $^{*}P<0.05$; $^{***}P<0.001$; ns, not significant.

(**Figure 8A**). Moreover, DEX treatment resulted in greater loss of body weight at the end of experiment (**Figure 8B**). Similarly, a sustained and aggravated reduction of body weight was observed in the DEX-treated mice compared to colitis mice (**Figure 8C** and **8D**).

We next evaluated disease severity of DSS administration based on the disease activity index (DAI) scoring system, including loss of body weight, rectal bleeding and stool consistency (Table S3). Throughout the DSS-treated procedure, we found that DEX administration was associated with exacerbated severity of body weight loss, rectal bleeding and diarrhea. As shown in **Figure 8E**, DAI score increased and reached the highest levels in every cycle of DSS administration, and a higher DAI score was observed after the second DSS cycle in the DEX-treated mice. Similar results were also found in the DSS-induced colitis (**Figure 8F**). Besides, we observed that most of mice in the DEX-treated group became weaker, which were manifested by anorexia and reduced activities (data not shown). Thus, we tested the fasting blood-glucose of all mice at the last day of the experiment. Expectedly, DEX intervention decreased the fasting glucose levels in CAC mice regardless of the time of DEX administration (**Figure 8G**). Similarly, DEX induced a lower level of blood glucose in mice suffered from DSS-induced colitis (**Figure 8H**). These data indicate that DEX treatment attenuates the tolerance of mice to DSS challenge and induces general deterioration of mouse statue.

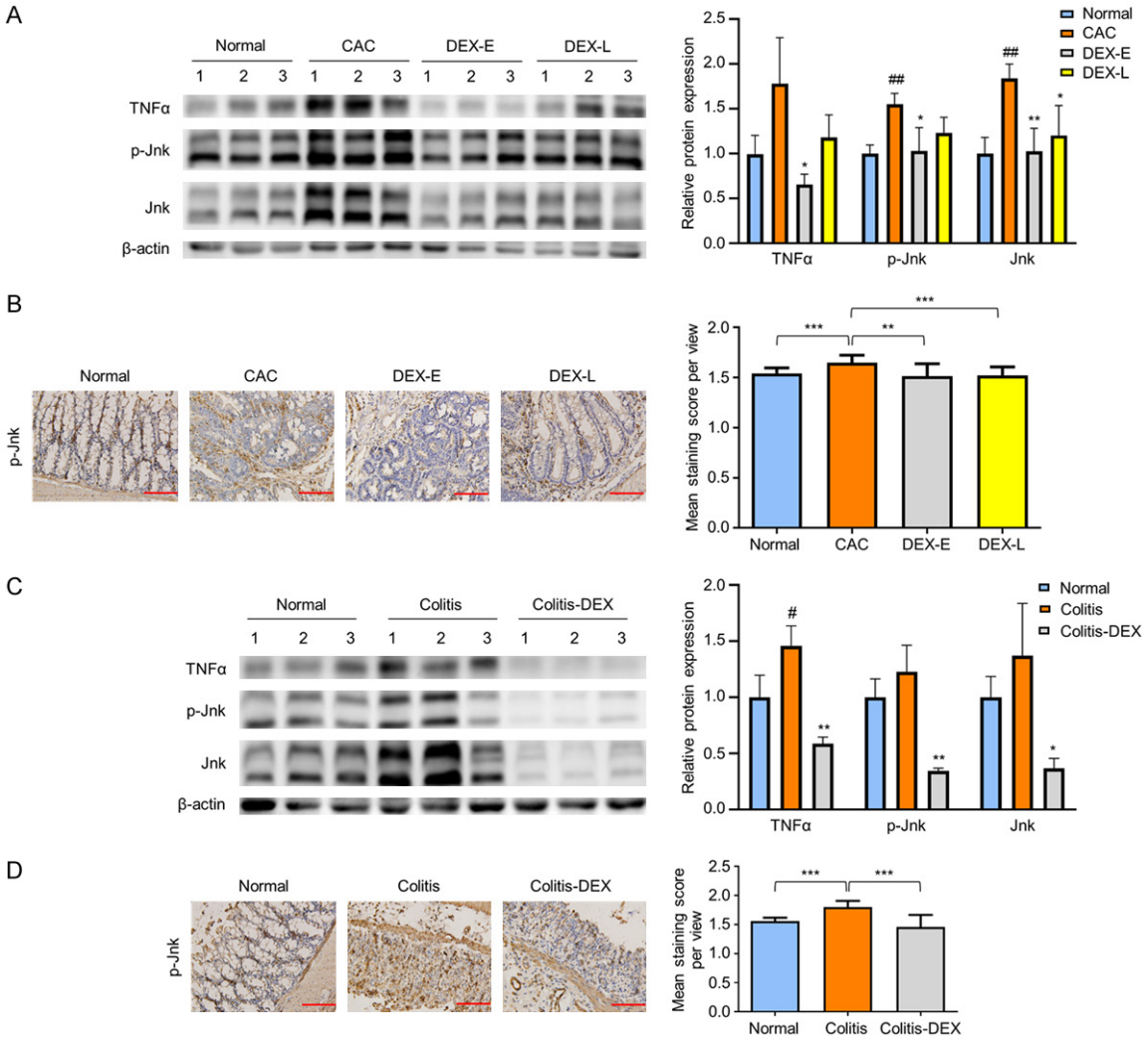

Figure 7. DEX treatment inhibits the activity of MAPK/JNK pathway. A. The levels of Jnk and p-Jnk in tumor tissues of AOM/DSS-treated mice and colonic tissues of normal mice were measured by Western blot analysis and measured using densitometry (left panel, n = 3 per group), β-actin was used as a loading control. Statistical results were presented in right panel. $^{\#\#}P<0.01$ for comparison with Normal; $^{*}P<0.05$; $^{**}P<0.01$ for comparison with CAC. B. Left panels show the representative IHC images of p-Jnk staining in neoplasms or normal epitheliums of the indicated mice (n = 3 per group). Statistical results were presented in right panel. C. The levels of Jnk and p-Jnk were measured in colonic specimens of the indicated mice by Western blot analysis and measured using densitometry (left panel, n = 3 per group), β-action was used as a loading control. Statistical results were presented in right panel. $^{\#}P<0.05$ for comparison with Normal; $^{*}P<0.05$; $^{**}P<0.01$ for comparison with Colitis. D. Left panels show the representative IHC images of p-Jnk staining in colonic epitheliums of the indicated mice (n = 3 per group). Statistical results were presented in right panel. The scale represents 100 μm. All experiments performed at least in triplicate. Data were expressed as mean ± SD. $^{**}P<0.01$; $^{***}P<0.001$.

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Disclosure of conflict of interest

None.

Abbreviations

GCs, Glucocorticoids; IBD, inflammatory bowel disease; CAC, colitis-associated cancer; AOM, azoxymethane; DSS, dextran sodium sulphate;

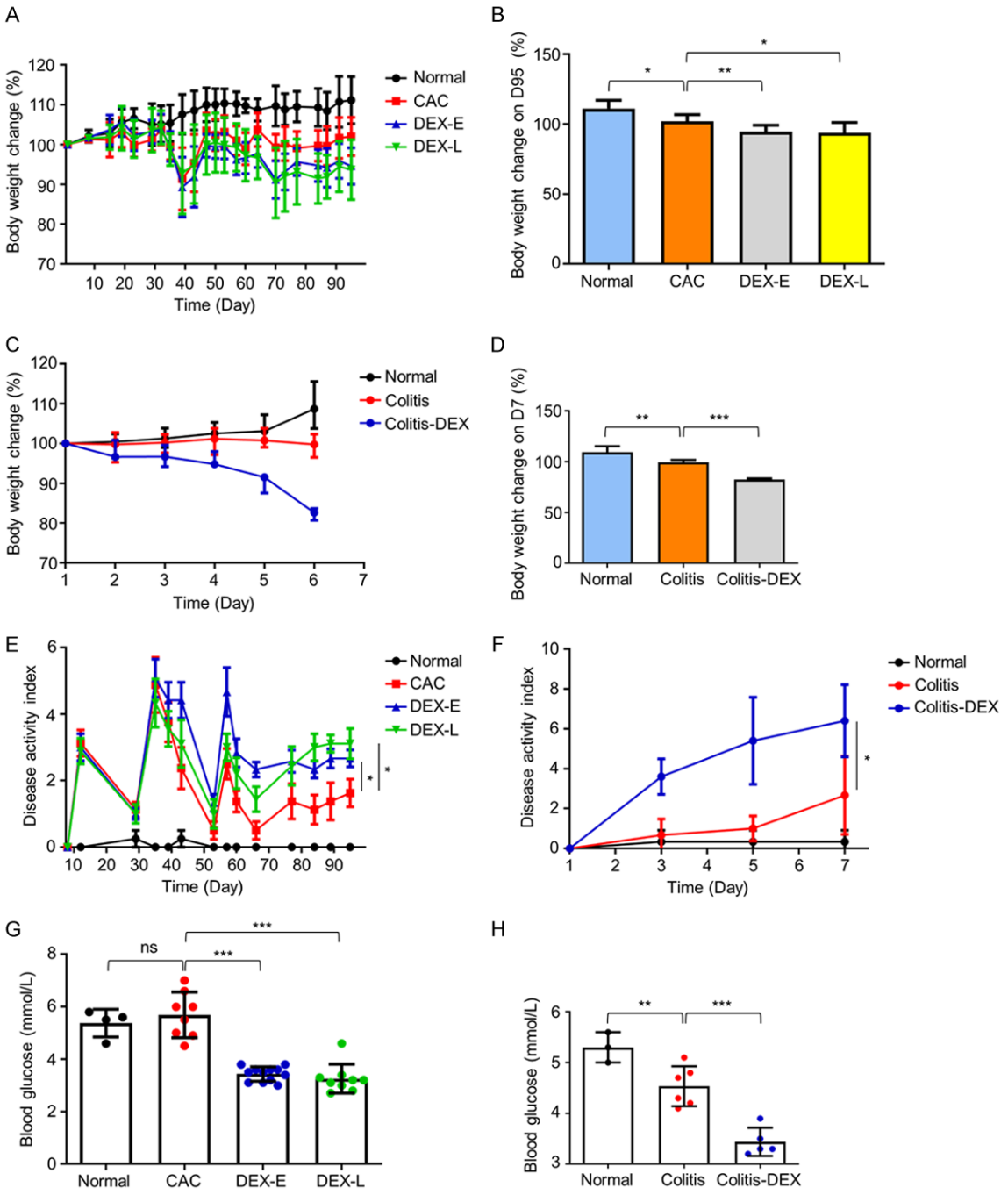

Figure 8. DEX administration deteriorates the general state of mice. A. The effect of DEX treatment (5 mg/kg) on body weight of Normal (n = 4), CAC (n = 6), DEX-E (n = 12) and DEX-L (n = 9) mice. B. Body weight change of Normal (n = 4), CAC (n = 6), DEX-E (n = 12) and DEX-L (n = 9) mice on D95. C. The effect of DEX treatment (5 mg/kg) on body weight of Normal (n = 3), Colitis (n = 6) and Colitis-DEX (n = 5) mice. D. Body weight change of Normal (n = 3), Colitis (n = 6) and Colitis-DEX (n = 5) mice on D7. E. Disease activity index (DAI) of Normal (n = 4), CAC (n = 6), DEX-E (n = 12) and DEX-L (n = 9) mice, scored by loss of body weight, rectal bleeding and stool consistency. F. Disease activity index (DAI) of Normal (n = 3), Colitis (n = 6) and Colitis-DEX (n = 5) mice. G. The fasting glucose of Normal (n = 4), CAC (n = 6), DEX-E (n = 12) and DEX-L (n = 9) mice at the end of the experiment. H. The fasting glucose of Normal (n = 3), Colitis (n = 6) and Colitis-DEX (n = 5) mice on D7 of experiment. Data were expressed as mean ± SD. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant.

DEX, Dexamethasone; PCNA, proliferating cell nuclear antigen; TNFα, tumor necrosis factor α; MAPK, Mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; CRC, colorectal

cancer; CD, Crohn's disease; UC, ulcerative colitis; DEX-E, DEX treatment at early stage; DEX-L, DEX treatment at late stage; NS, normal saline; DMSO, dimethylsulfoxide; DAI, Disease activity index; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ELISA, Enzyme-linked immunosorbent assay; STAT3, signal transducer and activator of transcription 3; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; GRs, glucocorticoid receptors.

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Table S1. Macroscopic lesion incidence and multiplicity in mice induced by AOM with different concentrations of DSS

Groups	N	Mortality (%)	% Animals with macroscopic lesions (incidence)	Number of macroscopic lesions per animal (multiplicity) mean ± SD
AOM + 1% DSS	6	0	66.7	9±3.9
AOM + 1.5% DSS	10	40	100	11.7±2.5
AOM + 2% DSS	10	70	100	15.7±4.5
AOM + 2.5% DSS	10	100	-	-

Table S2. The histological scoring system

Score	Inflammatory infiltration	Mucosal injury	Crypt damage	Lesion area (%)
0	Absent	Absent	Absent	absent
1	Signs	Mucous layer	1/4	1-25
2	Mild	Submucosa	1/3	26-50
3	Moderate	Muscularis	2/3	51-75
4	Severe	Muscularis and serosa	>80%	>75

Table S3. The scoring system of disease activity index (DAI)

Score	Weight loss (%)	Rectal bleeding	Stool consistency
0	0	No visible	Normal
1	1-5		Soft with well-formed pellets
2	5-10	Occasional blood traces visible	Soft without pellets
3	10-20		
4	>20	Gross bleeding	diarrhea

Table S4. The antibodies used in this study

Antibodies	Catalog#	Source
anti-CCND1	ab134175	Abcam
anti-PCNA	sc-56	Santa Cruz
anti-TNFα	sc-52746	Santa Cruz
anti-JNK	sc-7345	Santa Cruz
anti-p-JNK	sc-6254	Santa Cruz
anti-β-Actin	sc-1616	Santa Cruz
anti-F4/80	sc-52664	Santa Cruz

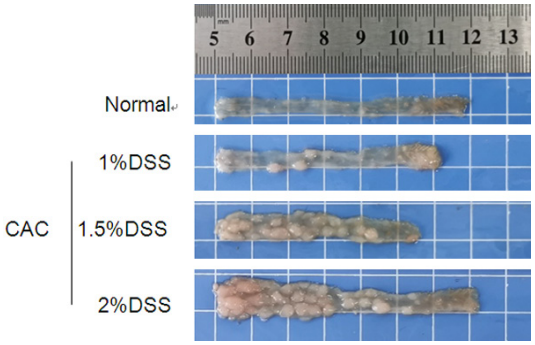

Figure S1. The representative view of neoplasms in colon of mice induced by AOM with different concentrations of DSS.

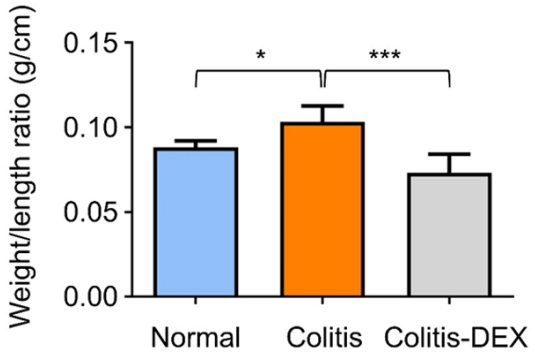

Figure S2. Colonic weight/length ratio of Normal (n = 3), Colitis (n = 6) and Colitis-DEX (n = 5) mice. The data are expressed as mean ± SD. $^{*}P<0.05$; $^{***}P<0.001$.