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## AIMS AND SCOPE

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ORIGINAL ARTICLE

Basic Study

# Interleukin-34 deficiency aggravates development of colitis and colitis-associated cancer in mice

Zhao-Xiu Liu, Wei-Jie Chen, Yang Wang, Bing-Qian Chen, Yi-Cun Liu, Tiao-Chun Cheng, Lei-Lei Luo, Lin Chen, Lin-Ling Ju, Yuan Liu, Ming Li, Nan Feng, Jian-Guo Shao, Zhao-Lian Bian

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## Abstract

### *BACKGROUND*

Although expression of interleukin (IL)-34 is upregulated in active ulcerative colitis (UC), the molecular function and underlying mechanism are largely unclear.

### *AIM*

To investigate the function of IL-34 in acute colitis, in a wound healing model and

in colitis-associated cancer in IL-34-deficient mice.

***METHODS***

Colitis was induced by administration of dextran sodium sulfate (DSS), and carcinogenesis was induced by azoxymethane (AOM). Whether the impact of IL-34 on colitis was dependent on macrophages was validated by depletion of macrophages in a murine model. The association between IL-34 expression and epithelial proliferation was studied in patients with active UC.

***RESULTS***

IL-34 deficiency aggravated murine colitis in acute colitis and in wound healing phase. The effect of IL-34 on experimental colitis was not dependent on macrophage differentiation and polarization. IL-34-deficient mice developed more tumors than wild-type mice following administration of AOM and DSS. No significant difference was shown in degree of cellular differentiation in tumors between wild-type and IL-34-deficient mice. IL-34 was dramatically increased in the active UC patients as previously reported. More importantly, expression of IL-34 was positively correlated with epithelial cell proliferation in patients with UC.

***CONCLUSION***

IL-34 deficiency exacerbates colonic inflammation and accelerates colitis-associated carcinogenesis in mice. It might be served as a potential therapeutic target in UC.

**Key Words:** Interleukin-34; Ulcerative colitis; Mucosal healing; Colitis-associated cancer; Macrophage; Murine model



**Core Tip:** This study highlights the role of interleukin (IL)-34 in acute experimental colitis and wound healing phase in mice. We found that IL-34 did not drive inflammatory response and tissue destruction in physiological conditions, but protects the host from inflammatory injury and reduces the risk of colitis-associated cancer. IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment of ulcerative colitis (UC) and reducing colitis-associated cancer in UC.

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## INTRODUCTION

Ulcerative colitis (UC) is a chronic progressive recurrent intestinal inflammatory disorder characterized by bloody diarrhea and abdominal pain[1]. UC is an important promoter of colorectal cancer[2]. The exact pathogenesis of UC remains unclear. It is currently assumed that it involves immunological derangement of the gut microbiota in genetically predisposed individuals after exposure to environmental factors[1]. Cytokines exert multiple effects and participate in the pathogenesis of colitis[3]. Identification of distinctive cytokines involved in immunopathogenesis of UC has become a hot spot for the development of biological therapies[4].

Colony-stimulating factor-1 receptor (CSF-1R) signaling regulates intestinal and colon development, gut homeostasis and inflammatory reaction[5]. Interleukin (IL)-34 was discovered in 2008 as a specific ligand for CSF-1R independent of CSF-1[6,7]. Human IL-34 is a homodimeric glycoprotein that consists of 242 amino acids produced by various cell types including epithelial, endothelial and immune cells, and fibroblasts[8]. Secreted IL-34 binds to the extracellular domains of CSF-1R and protein-tyrosine phosphatase, the other receptor for IL-34, which is regulated by syndecan-1, which results in autophosphorylation of its intracellular tyrosine residues and activates several signaling pathways controlling cell biological function[8,9]. IL-34 functions as a pivotal regulator in cell differentiation, proliferation and survival in the mononuclear phagocyte system[10]. Additionally, IL-34 mediates the crosstalk between the innate and adaptive immune systems during inflammation[8,11].

Under normal circumstances, IL-34 is primarily expressed in a tissue-specific manner in keratinocytes and neurons[8]. In pathological status, the expression pattern is changed. Increased IL-34 expression positively associates with disease progression, severity and chronicity in autoimmune diseases such as

rheumatoid arthritis and Sjogren's syndrome[12]. In contrast, IL-34 has demonstrated beneficial effects in other diseases. IL-34 has been identified as a new mediator to induce transplant tolerance by targeting suppressive T regulatory cells[13]. Therefore, the multiple function of IL-34 in different diseases is complex, disputable, and context-dependent[8].

The involvement of IL-34 in inflammatory bowel disease (IBD) has also drawn attention recently. Higher expression of IL-34 in human ileum compared with colon has been revealed[14]. In human and experimental colitis, the mRNA and protein level of IL-34 is markedly increased in inflamed mucosa compared to that in the matched normal mucosa and in healthy controls[14]. Recently, it has been proposed that IL-34 plays a prominent role in intestinal fibrogenesis *via* a p38 mitogen-activated-protein-kinase-dependent mechanism in Crohn's disease[15]. *In vitro,* tumor necrosis factor (TNF)-α significantly upregulates expression of IL-34 in lamina propria mononuclear cells (LPMCs) *via* NF-kB signaling. Intriguingly, LPMCs treated with IL-34 increase expression of proinflammatory cytokines such as IL-6, TNF-α and chemokine CC ligand 20[14]. Accordingly, IL-34 neutralization decreases synthesis of TNF-α and IL-6 in IBD mucosal explants[14]. Therefore, it has been noted that IL-34 sustains gut inflammation *via* regulating positive feedback of proinflammatory cytokine production *in vitro*[8, 14]. On the contrary, IL-34 has previously demonstrated immunosuppressive characteristics that contribute to inflammation improvement by inducing differentiation of monocytes into M2 phenotype [16]. M2 macrophages mediate immunotolerance and promote wound healing in experimental colitis *in vivo*[17,18]. IL-34 is upregulated in colon cancer and sustains the protumorigenic signals by inducing proliferation of cancer cells *via* extracellular signal-regulated kinase (ERK) 1/2 or cancer-associated fibroblasts[19,20]. Whether IL-34 is "friend or foe" in the pathogenesis of UC remains to be explored. In this study, we investigated the potential role of IL-34 in experimental colitis, colitis-associated carcinogenesis and UC.

## MATERIALS AND METHODS

### Animal experiment

IL-34-deficient mice were generated in C57BL/6J mice by using CRISPR/Cas9 technology (Beijing Cas Gene Biotech, Beijing, China). Gene targeting technology was applied to delete exons 3-5 in the *IL-34* gene and led to frameshift mutation of *IL-34* gene. IL-34-deficient and C57BL/6J wild-type mice were bred and maintained in a specific-pathogen-free animal facility in the Laboratory Animal Center of Nantong University. All animal experiments were approved by the Institutional Ethics Committee of Nantong University (Date: 21/12/2015, Number: S20151221-908).

Acute colitis was induced by oral administration of 3% (M/V) dextran sulfate sodium (DSS, MW: 36 000-50 000; MP Biologicals, LLC, California, United States) in drinking water for 7 d. The mice were killed to obtain colon tissues at the indicated time or until day 16 to record the mortality. The murine wound healing model was established by oral administration of 3% DSS for 5 d and then switched to normal water for the following 5 d. The mice were killed on day 8 or 10. Colitis-associated cancer was induced by administration of the carcinogen azoxymethane (AOM; Sigma, Darmstadt, Germany) and repeated administration of DSS. The mice were given a single injection of AOM (10 mg/kg). Seven days later, mice were administrated 1.25% DSS (w/v) in drinking water for seven consecutive days, and fresh drinking water for 14 d. Seven days of DSS and 14 d of fresh water was repeated four times as a cycle [21]. The mice were killed, and the incidence rate of tumors was analyzed. Macrophage depletion from murine colons *in vivo* was performed as described previously[22]. In brief, 200 μL clodronate liposomes (Liposoma Research, Amsterdam, Netherlands) were intraperitoneally injected into mice 2 d prior to onset of experimental colitis and every 2 d during the process.

The percentage of body weight change of each mouse was recorded throughout the duration of DSS administration. Fresh feces from mice were collected for occult blood tests using a fecal occult blood kit (Nanjing Jiancheng Bioengineering, Jiangsu, China). The clinical score index of the murine model consisted of stool consistency and fecal occult blood, as previously described[23].

### Human tissue

Human tissue specimens were obtained from 40 adult patients with active UC and 20 healthy controls during colonoscopy in Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University between January 2014 and August 2015. Histopathology was evaluated by an experienced pathologist. This study was approved by the Ethics Committee of Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University, and all people signed an informed consent form. All patients met the diagnostic criteria that were in line with the Consensus on Diagnosis and Management of Inflammatory Bowel Disease (2018, Beijing, China). The patients did not receive any therapy before colonoscopy. More details on the patients' characteristics can be found in Supplementary Table 1.

### Histopathology

The entire colon was harvested for measuring the length. The colon was washed in phosphate-buffer saline and fixed in 10% formaldehyde solution for 24 h. Hematoxylin-eosin (H&E) staining was performed on the tissue sections. The inflammatory score was as follows: Presence of occasional inflammatory cells in the lamina propria was scored as 0; increased numbers of inflammatory cells in the lamina propria was scored as 1; confluence of inflammatory cells extending into the submucosa was scored as 2; and transmural extension of the infiltrate was scored as 3. For tissue damage score, no mucosal damage was scored as 0; lymphoepithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage and extension into deeper structures of the bowel wall was scored as 3. The combined histological score was calculated by inflammatory and tissue damage score, and ranged from 0 (no changes) to 6 (extensive infiltration and tissue damage).

### Immunohistochemistry

The colonic tissues were sectioned into 4-μm slices following fixation with 10% formaldehyde and embedded in paraffin. Immunohistochemical staining was conducted as in our previous study[24]. Tissue slices were incubated with 3% $H_2O_2$ for 15 min after deparaffinization. Antigen was retrieved in citrate buffer (pH 6.0; Maxim Biotechnologies, Fuzhou, China) in a microwave for 10 min. Nonimmune 10% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) was used to block nonspecific reactions. The sections were incubated with primary antibodies: rabbit anti human/mouse IL34, 1:100 (Abcam, Cambridge, MA, United States); rabbit anti human/mouse Ki-67 antibody, 1:200 (Abcam); rabbit anti mouse CSF1-R 1:1000 (Abcam); and rabbit anti-mouse CD68, 1:100 (Boster Biological Technology, Wuhan, China) overnight at 4 °C. On the second day, the sections were incubated with horseradish-peroxidase-conjugated secondary antibody (Shanghai Long Island Biotechnology, Shanghai, China) for 30 min at room temperature. 3, 3′-diaminobenzidine (Maxim Biotechnologies) was applied for 30 s. Hematoxylin was applied for contrast staining. Proliferation index of Ki-67 was defined as the percentage of Ki-67-positive cells in crypts (CK4) within the random visual scope. Positive index of CSF1-R was defined as the percentage of CSF1-R-positive cells in colonic mucosa within the random visual scope. All the tissue sections were analyzed by optical microscopy (IX73; Olympus, Tokyo, Japan) by experienced pathologists.

### TUNEL assay

Colonic tissue sections were prepared as described above. Apoptosis was analyzed by fluorescence microscopy according to standard procedure using *in situ* cell death detection (Roche, Basel, Switzerland). Five randomly optical fields were chosen for further analysis.

### RNA isolation and quantitative real-time PCR

Total RNA was extracted from the indicated murine colonic tissues by Trizol Reagent (TaKaRa Bio, Dalian, China). RNA samples were reverse-transcribed into cDNA with a PrimeScript™RT Master Mix kit (TaKaRa Bio). cDNA samples were detected using a SYBR®Premix Ex Taq™II kit (TaKaRa Bio) on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States). PCR was performed at 95 °C for 30 s, and the samples were subjected to 40 cycles of amplification at 95 °C for 5 s and 60 °C for 30 s. Expression of target genes was normalized to β-actin. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers are listed in Supplementary Table 2.

### Statistical analysis

Data are presented as mean ± SD. The difference between more than two groups was analyzed using one-way analysis of variance and comparison of two groups was carried out using a *t*-test. Differences in survival between two groups were analyzed by Kaplan-Meier test. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA, United States). $P < 0.05$ was considered statistically significantly.

## RESULTS

### IL-34 is elevated in colitis and colitis-related cancer

We analyzed IL-34 mRNA expression in gastrointestinal mucosal epithelium in wild-type C57BL/6J mice. Expression of IL-34 was highest in the colon compared with other parts of the digestive tract (Figure 1A). We investigated expression of IL-34 in DSS-induced colitis and AOM-DSS-induced colitis-associated cancer in wild-type mice. Immunohistochemical staining of murine colon tissue revealed that expression of IL-34 was elevated in both acute and chronic colitis, with the highest expression in colitis-associated cancer (Figure 1B and C).

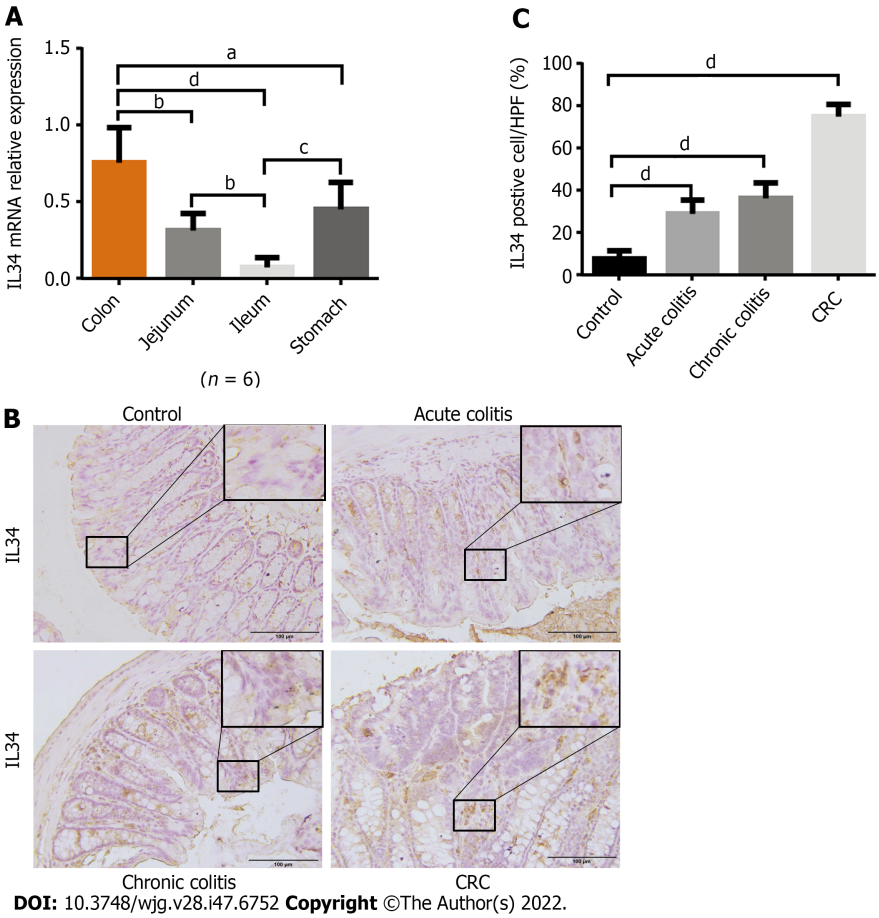


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**Figure 1 Interleukin-34 is elevated in colitis and colitis-related cancers.** A: Interleukin-34 (IL-34) relative mRNA expression in stomach, jejunum, ileum and colon in wild-type mice (C57BL/6J) ($n$ = 6); B and C: The dynamic of IL-34 expression in dextran sodium sulfate (DSS)-induced colitis and azoxymethane-DSS-induced colorectal cancer in wild-type mice ($n$ = 6 per group); representative IL-34 immunohistochemical staining for healthy control, acute colitis, chronic colitis and colorectal cancer mice (B); the percentage of IL-34-positive cells per high-power field were quantified in colonic tissue of four groups of mice ($n$ = 6 per group) (C). AOM: Azoxymethane; CRC: Colorectal cancer; DSS: Dextran sodium sulfate; IL-34: Interleukin-34. Scale bars = 100 μm. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

### *IL-34 deficiency increases susceptibility to acute DSS-induced colitis*

To determine the role of IL-34 in murine acute experimental colitis, IL-34$^{-/-}$ and wild-type mice were fed with 3% DSS in drinking water for 7 d and then killed (Figure 2A). DSS-fed IL-34$^{-/-}$ mice showed significantly greater body weight loss compared to DSS-fed wild-type mice. The average weight loss in DSS-fed IL-34$^{-/-}$ mice was approximately double than that in the wild-type mice (Figure 2B). IL-34$^{-/-}$ mice displayed a significantly higher clinical score compared to wild-type mice (Figure 2C). Strikingly, the mortality of IL-34$^{-/-}$ mice was 100% (10/10), whereas only 20% (2/10) of the wild-type mice died during the experiment, as illustrated by Kaplan-Meier curves (Figure 2D, $P < 0.001$). Following DSS administration, IL-34$^{-/-}$ mice showed remarkably shorter colon compared to wild-type mice (4.83 cm ± 0.13 cm *vs* 6.27 cm ± 0.14 cm, $P < 0.001$), suggesting more severe colitis in IL-34$^{-/-}$ mice (Figure 2E and F). DSS-fed IL-34$^{-/-}$ mice exhibited more severe inflammation with ulceration and necrotic lesions compared to wild-type mice, while there was no difference in the wild-type or IL-34$^{-/-}$ mice fed with normal water. More inflammatory cells infiltrated the lamina propria and submucosa in DSS-fed IL-34$^{-/-}$ mice. More importantly, the normal tissue architecture damage was more severe in DSS-fed IL-34$^{-/-}$ mice compared with wild-type mice (Figure 3A). Semiquantitative score of histopathology confirmed more severe colitis in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type mice (9.17 ± 0.31 *vs* 5.60 ± 1.10, $P < 0.001$) (Figure 3B). The expression of CD68, which serves as a characteristic marker for macrophages, was indistinguishable between IL-34$^{-/-}$ and wild-type mice untreated with DSS (Figure 3C and D). CD68 expression was significantly increased in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type controls (Figure 3C and D), which indicated worsening colitis. IL-1β, IL-23, and macrophage colony-stimulating factor levels were significantly upregulated in DSS-treated IL-34$^{-/-}$ mice compared to wild-type mice treated with DSS (Figure 3E).

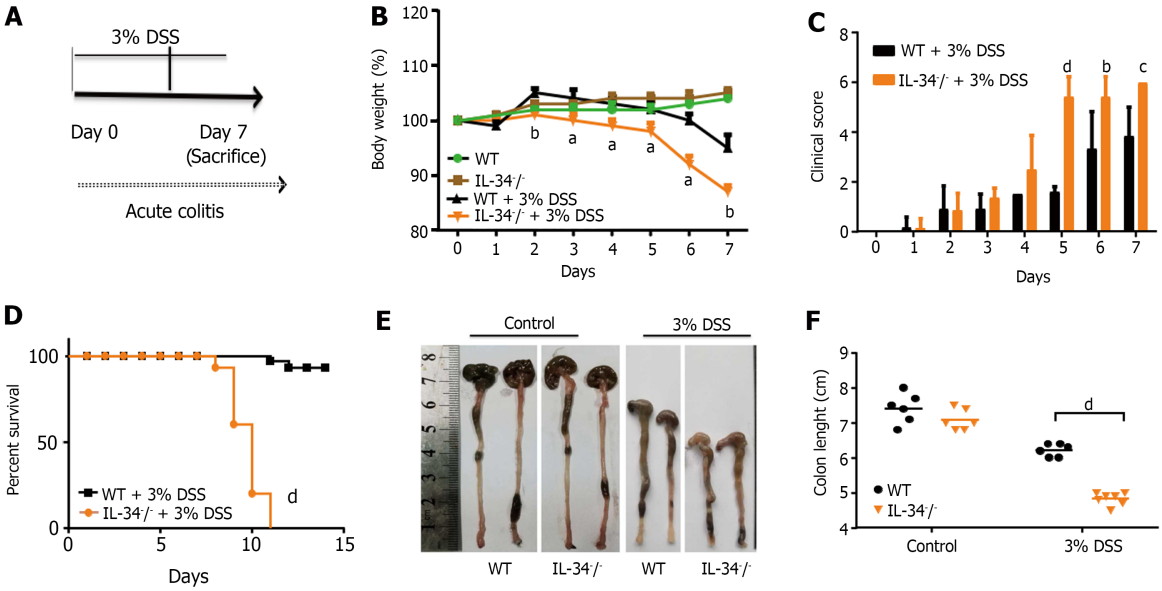


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**Figure 2 Interleukin-34 deficiency aggravates acute colitis induced by dextran sodium sulfate.** A: Schematic of the design of acute dextran sodium sulfate (DSS)-induced colitis model. IL-34$^{-/-}$ and WT mice were fed with a 3% DSS solution in drinking water for 7 d and then sacrificed ($n$ = 6 or 7 per group); B: Body weight was represented as a percentage of starting weight in IL-34$^{-/-}$ and WT mice after administration of 3% DSS ($n$ = 6 or 7 per group); C: The clinical score of IL-34$^{-/-}$ and WT mice treated with 3% DSS ($n$ = 6 or 7 per group). Data depict the mean ± SD; D: Kaplan-Meier survival curves were plotted in IL-34$^{-/-}$ and WT mice administrated with 3% DSS for 15 d ($n$ = 10 per group); E and F: Colon length was measured in IL-34$^{-/-}$ and WT mice fed with 3% DSS for 7 d ($n$ = 6 or 7 per group). DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild-type. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

### *IL-34 deficiency inhibits proliferation and promotes apoptosis of colonic epithelium in DSS-induced colitis*

Prior to DSS administration, the levels of colonic epithelium proliferation and apoptosis were both comparable between wild-type and IL-34$^{-/-}$ mice (Figure 4A-D). A marked reduction in colonic epithelial cells stained positive for Ki-67 was detected in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type mice (Figure 4A and B). A marked increased in colonic epithelial cell apoptosis was noted in DSS-fed IL-34$^{-/-}$ mice compared to the DSS-fed wild-type mice (Figure 4C and D). We detected expression of three receptors of IL-34 in mouse colonic tissue by real-time quantitative PCR. In DSS-fed IL-34$^{-/-}$ mice, we only observed an increase in the expression of CSF1-R, but the other two receptors of IL34, ptprz1, and syndecan-1, did not change significantly (Figure 4E). By immunohistochemical staining of the mouse colonic tissues, we found that CSF1-R was expressed in epithelial and mesenchymal cells. Compared to the control group, the mice in the DSS-fed group showed an increasing positive index of CSF1-R. Expression of CSF1-R did not show a significant difference between IL-34$^{-/-}$ and wild-type mice untreated with DSS ($P > 0.05$, Figure 4F and G). DSS-fed IL-34$^{-/-}$ mice exhibited a higher index of CSF1-R positivity compared to DSS-fed wild-type mice ($P < 0.01$, Figure 4F and G). CSF1-R acts as a receptor for IL-34 and was compensatorily increased when colitis developed and IL-34 was absent. The results were consistent with the results of quantitative real-time PCR of CSF1R.

### *Protective effect of IL-34 against acute DSS-induced colitis is not dependent on macrophages*

Macrophages were increased in the IL-34$^{-/-}$ mice treated with 3% DSS compared with the wild-type mice. We investigated whether the protective effect of IL-34 in colitis was dependent on macrophages. It is known that IL-34 plays a role in macrophage polarization. We studied the potential role of IL-34 in macrophage polarization by examining the selected markers for M1 and M2 macrophages. Most importantly, there was no difference in the ratio of colonic M2/M1 macrophage markers between DSS-treated wild-type and IL-34$^{-/-}$ mice (Figure 5A). It was speculated that the effect of IL-34 on experimental colitis was not attributed to macrophage polarization. In order to confirm that the protective effect of IL-34 in colitis was not dependent on macrophages, we depleted macrophages by intraperitoneal injection of clodronate liposomes (Clo-lips) in IL-34$^{-/-}$ and wild-type mice (Figure 5B). The macrophage cell marker F4/80 was significantly reduced in colonic mucosa of mice treated with Clo-lips, which confirmed that the liposomes effectively depleted macrophages in the colon (Figure 5C). Despite macrophage depletion, DSS-fed IL-34$^{-/-}$ mice still showed significantly higher clinical score and shorter colon length compared with DSS-fed wild-type mice (Figure 5D-F). Similarly, the histopathological manifestation showed that colitis severity in DSS-fed IL-34$^{-/-}$ mice was more pronounced compared to that in DSS-fed wild-type mice (Figure 5G and H). The inflammatory cytokines remained significantly higher in colonic mucosa of DSS-fed IL-34$^{-/-}$ mice compared with DSS-fed wild-type mice (Figure 5I).

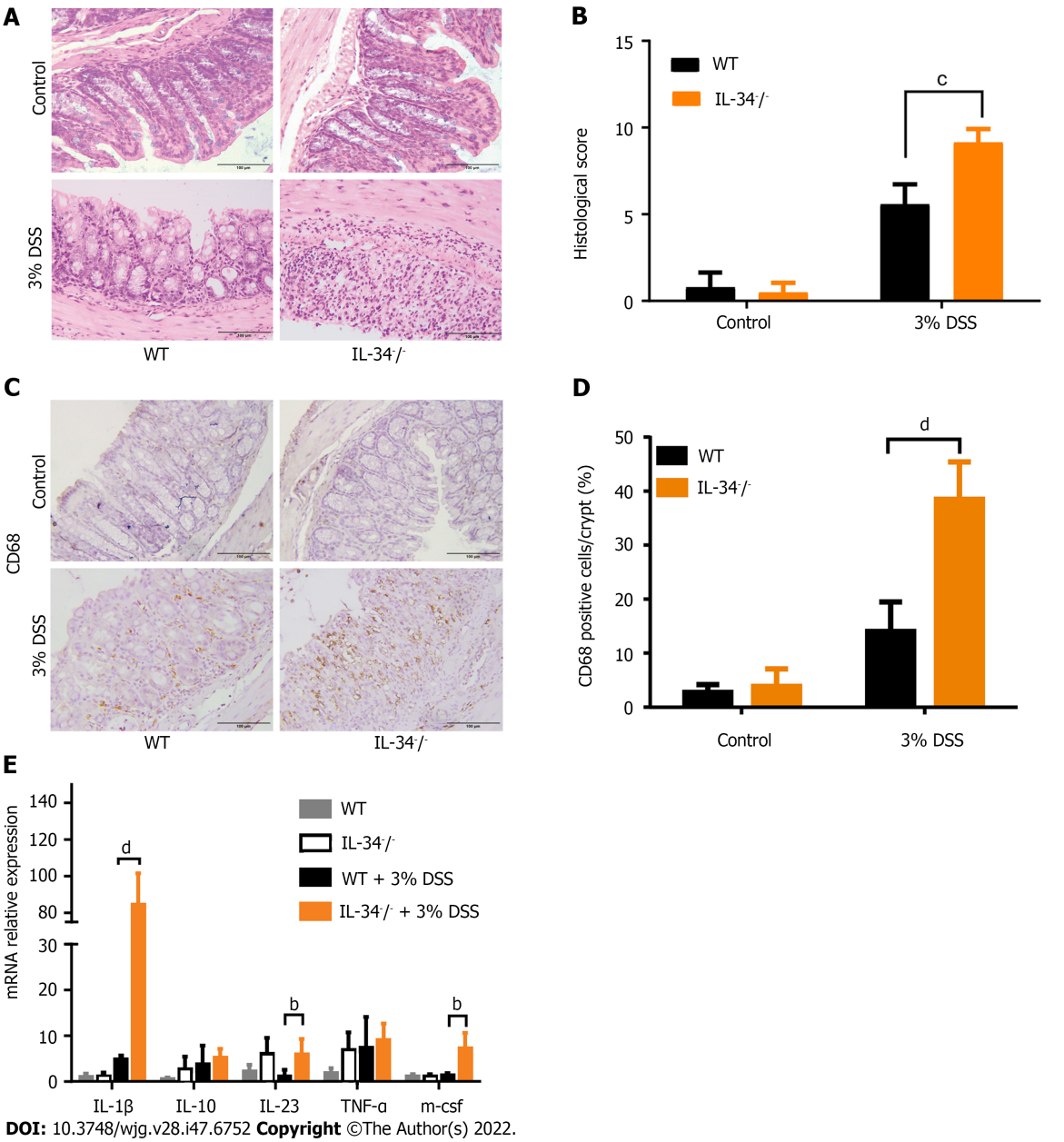


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**Figure 3 Interleukin-34 deficiency aggravates acute colitis and increase proinflammatory cytokines.** A: Representative microscopic pictures of hematoxylin and eosin-stained colon sections of Interleukin-34 (IL-34)$^{-/-}$ and WT mice fed with 3% DSS for 7 d ($n$ = 6 or 7 per group); B: Histological score for IL-34$^{-/-}$ and WT mice fed with 3% dextran sodium sulfate (DSS); C: Representative photomicrographs of macrophage staining in colon sections of IL-34$^{-/-}$ and WT mice treated with DSS; D: Statistical analysis of CD68-positive cells in IL-34$^{-/-}$ and WT mice treated with DSS; E: mRNA expression of proinflammatory cytokines including IL-1β, IL-10, IL-23, TNF-α, and M-CSF in IL-34$^{-/-}$ and WT mice fed with 3% DSS. DSS: Dextran sodium sulfate; IL: Interleukin; M-CSF: Macrophage colony-stimulating factor; TNF-α: Tumor necrosis factor-α; WT: Wild-type. Scale bars = 100 μm. Data depict the mean ± SD. $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

### *IL-34 deficiency delays mucosal healing in a murine model induced by DSS*

IL-34$^{-/-}$ and wild-type mice were fed with 3% DSS for 5 d and then switched to normal water for the following 5 d to establish a mucosal healing model. The mice in both groups were killed on day 8 or 10 (Figure 6A). The body weight loss was significantly greater in IL-34$^{-/-}$ mice compared to the wild-type control mice. The body weight increased quickly on day 8 in wild-type mice, while it did not recover until day 10 in IL-34$^{-/-}$ mice (Figure 6B). The clinical score was higher in IL-34$^{-/-}$ mice compared to wild-type mice from day 5. The clinical score decreased rapidly from day 7 in wild-type mice, while it was falling slowly from day 8 in IL-34$^{-/-}$ mice (Figure 6C). IL-34$^{-/-}$ mice showed remarkably shorter colon length compared to wild-type controls on day 8 (5.25 cm ± 0.32 cm *vs* 6.30 cm ± 0.25 cm, $P < 0.01$) and day 10 (5.03 cm ± 0.49 cm *vs* 7.30 cm ± 0.47 cm, $P < 0.005$) (Figure 6D and E). Histopathological analysis showed that IL-34$^{-/-}$ mice exhibited more severe inflammation and tissue damage compared to wild-type mice on days 8 and 10, respectively (Figure 6F). Semiquantitative scoring of histopathology showed that colitis severity in IL-34$^{-/-}$ mice was significantly higher than that in wild-type mice on day 8 (8.13 ± 0.66 *vs* 3.63 ± 0.58, $P < 0.01$) and day 10 (8.45 ± 061 *vs* 2.60 ± 0.58, $P < 0.01$), respectively (Figure 6G). The

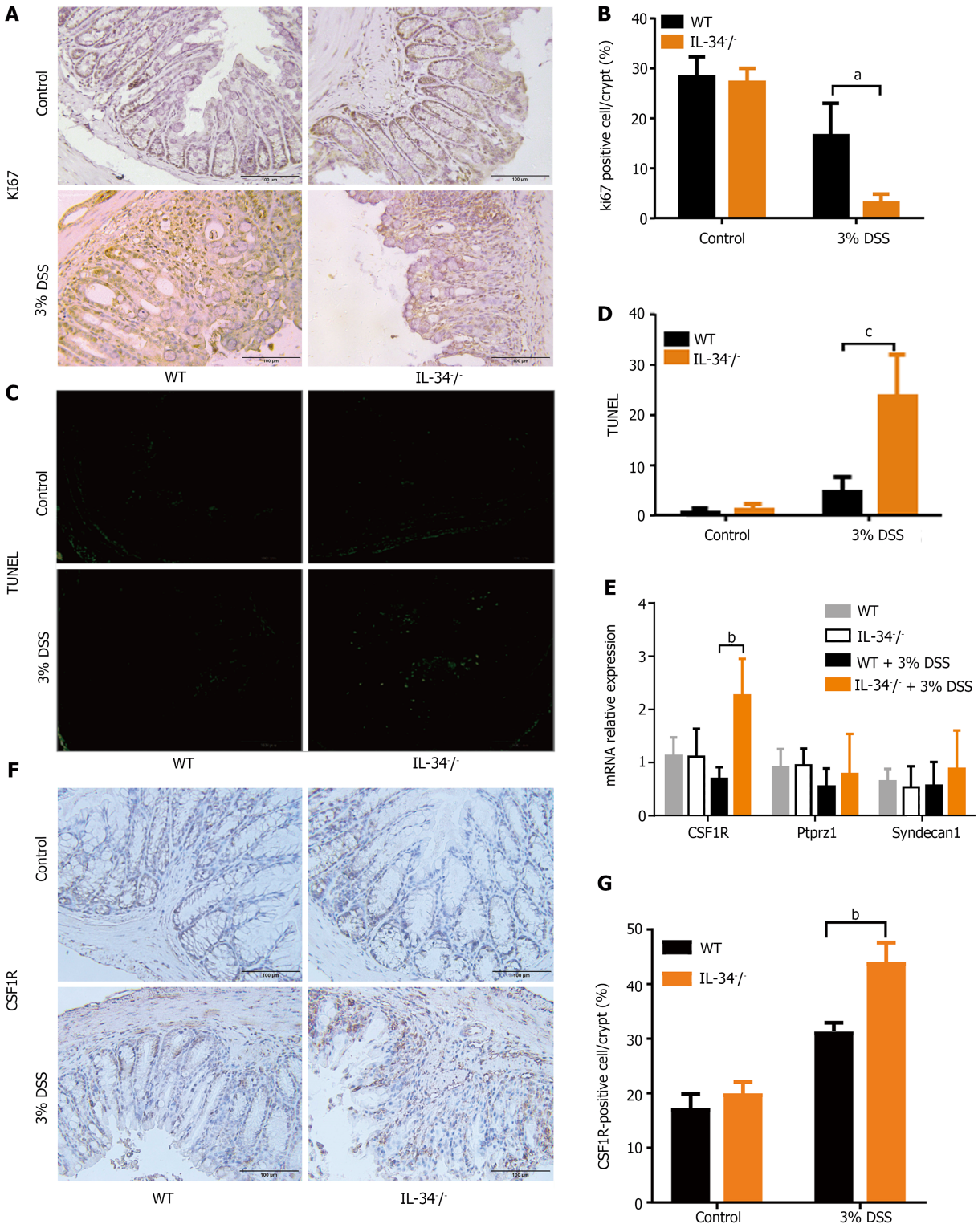


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**Figure 4 Interleukin-34 deficiency inhibits proliferation and promotes apoptosis of colonic epithelium in dextran sodium sulfate-induced colitis.** A: Representative photomicrographs of Ki-67 immunohistochemical staining for colon sections of IL-34$^{-/-}$ and WT mice treated with dextran sodium sulfate (DSS); B: Percentage of proliferation marker Ki-67 per crypt; C and D: Representative microscopic pictures of TUNEL staining (C) and quantification of TUNEL-positive cells per field (D) in IL-34$^{-/-}$ and WT mice treated with DSS as before; E: mRNA expression of three receptors for IL-34 (CSF1R, ptprz1 and syndecan-1) was detected in IL-34$^{-/-}$ and WT mice treated with DSS ($n$ = 6 or 7 per group); F and G: Representative photomicrographs of CSF1R immunohistochemical staining (F) and percentage of CSF1R-positive cells (G) in colon sections of IL-34$^{-/-}$ and WT mice treated with 3% DSS. CSF1R: Colony-stimulating factor-1 receptor; DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild-type. Scale bars = 100 μm. Data depict mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$.

number of colonic epithelial cells positive for Ki-67 was markedly decreased in IL-34$^{-/-}$ mice compared to wild-type mice on days 8 and 10 (Figure 6H and I).

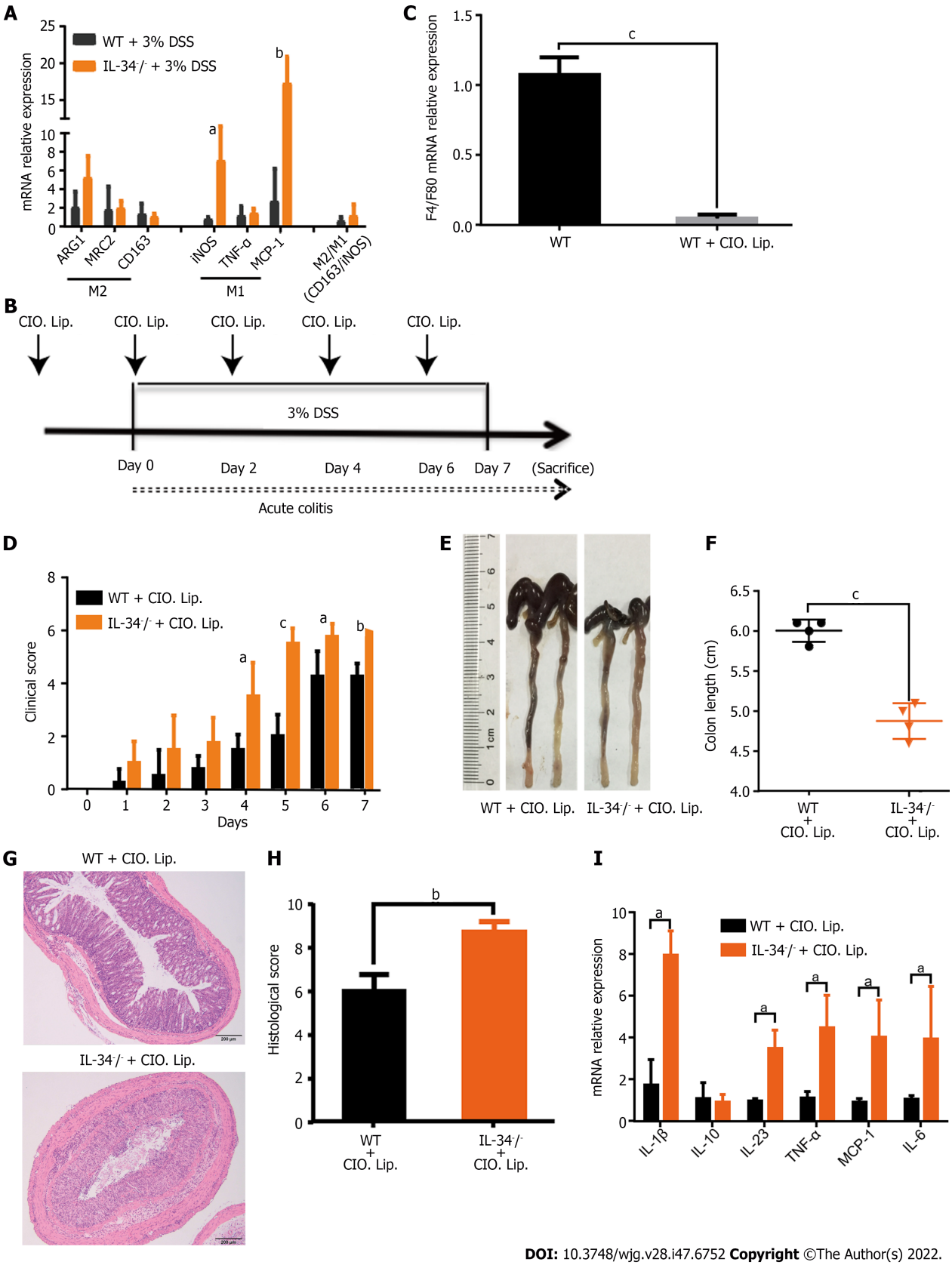


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**Figure 5 Protective effect of interleukin-34 against acute dextran-sodium-sulfate-induced colitis is not dependent on macrophages.** A: mRNA expression levels of M1 macrophage markers (iNOS, TNF-α and MCP-1) and M2 macrophage markers [ARG1, MRC2 (CD163)] in the colonic mucosa of IL-34$^{-/-}$ and WT mice treated with 3% DSS. M2/M1 ratio (CD163/iNOS) was calculated; B: Experimental design. To deplete macrophages, IL-34$^{-/-}$ and WT mice were treated with Clo-lips 2 d prior to 3% dextran sodium sulfate (DSS) administration and continuing once every 2 d until death ($n$ = 4 per group); C: mRNA expression of macrophage cell marker (F4/80) was detected in colonic mucosa of WT mice treated with Clo-lips ($n$ = 4 per group); D: Clinical score of IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips ($n$ = 4 per group); E and F: Colon length of IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips ($n$ = 4 per group); G and H: Representative microscopic pictures (G) and histological scores (H) of IL-34$^{-/-}$ and WT mice fed with 3% DSS and Clo-lips ($n$ = 4 per group); I: mRNA expression of

inflammatory cytokines was detected in IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips ($n$ = 4 per group). ARG1: Arginase; Clo-lip: Clodronate liposome; CSF1R: Colony-stimulating factor-1 receptor; DSS: Dextran sodium sulfate; IL-34: Interleukin-34; iNOS: Inducible nitric oxide synthase; MCP-1: Monocyte chemoattractant protein-1; MRC2: Mannose receptor C type 2; TNF-α: Tumor necrosis factor-α; WT: Wild-type. Scale bars = 200 μm. Data depict mean ± SD. $^{a}P$ < 0.05, $^{b}P$ < 0.01, $^{c}P$ < 0.005.

### *IL-34 deficiency promotes AOM/DSS-induced colitis-associated tumorigenesis*

To investigate the role of IL-34 in colitis-associated tumorigenesis, wild-type and IL-34$^{-/-}$ mice were treated with AOM and DSS (Figure 7A). No wild-type mice died, whereas a mortality rate of 30% was noted in IL-34$^{-/-}$ mice (Figure 7B). More importantly, a marked difference in tumor number was observed between the two groups. IL-34$^{-/-}$ mice developed a greater number of colon tumors than wild-type mice (Figure 7C and D). Representative H&E staining of colitis-associated cancer in wild-type and IL-34$^{-/-}$ mice is shown in Figure 7E.

### *IL-34 expression was elevated in inflamed mucosa and was associated with colonic epithelium proliferation in UC patients*

We investigated the IL-34 expression pattern and its relationship with colonic epithelium proliferation in active UC patients. There was no age or sex difference between the groups (Supplementary Table 1). Consistent with previous studies, we also observed elevated expression of IL-34 in diseased mucosa of UC patients compared with the normal controls as detected by immunohistochemistry. Importantly, IL-34 was suspected to be predominantly expressed in the colonic stromal tissue (Figure 8A and B). Significantly enhanced colonic expression of proliferation index Ki-67 was detected in UC patients with higher IL-34 expression in colonic mucosa (Figure 8C). There was a positive correlation between IL-34 and Ki-67 expression in UC-inflamed mucosa with a correlation coefficient of 0.60 ($P$ < 0.0001) (Figure 8D). No correlation was found between IL-34-positive and TUNEL-positive cells (data not shown).

## DISCUSSION

Mucosal healing has become the goal of therapy because of its association with better prognosis and improved quality of life[25]. However, the underlying molecular mechanism is unclear. Here, we revealed that IL-34 deficiency strongly increased susceptibility to acute DSS-induced colitis and intestinal wounding. Additionally, IL-34 deficiency enhanced AOM/DSS-induced colitis-associated tumorigenesis. Different from the previous reports, our findings suggest the protective role of IL-34 in UC- and colitis-associated cancer and may help to establish a new potential approach for management of UC.

The IL-34 knockout mice used in our study facilitated us to illustrate the role of IL-34 in pathogenesis of UC. Prior to DSS administration, no difference in colon morphological and histological manifestations was observed between IL-34-deficient and wild-type control mice. The results suggest that IL-34 is dispensable for colon homoeostasis under steady state.

In past studies, overexpression of IL-34 stimulated by inflammatory cytokines augmented the production of proinflammatory cytokines *in vitro*, probably raising IL-34 as a pathogenic contributor to sustained colon inflammation[14]. However, the increased expression of IL-34 might be a consequence of inflammation as a protective mechanism rather than a disease cause[16]. Indeed, IL-34 transgenic mice do not display aggravating inflammatory responses in the colon[26]. The precise mechanistic role of IL-34 during colon inflammation has not been clarified yet. Until now, there has been a lack of research on IL-34 involved in UC *in vivo*. A recent study has reported that IL-34 is critical for the suppressive function of CD4$^{+}$ T regulatory cells, and its deficiency leads to increased susceptibility to autoimmunity[27]. We demonstrated for the first time that IL-34 deficiency exacerbates DSS-induced colitis during acute and delayed mucosal healing process and is associated with high colitis-related mortality. We assumed that significantly increased expression of IL-34 in mucosa from patients with active UC might act as an urgent requirement for alleviating inflammation and promoting mucosal healing. Our findings support the protective characteristic of IL-34 during DSS-induced colitis and early healing stage.

Macrophages are critical to mucosal homeostasis in orchestrating innate and adaptive immunity[28]. Depending on the local microenvironment, macrophages differentiated from the peripheral blood monocytes develop towards M1-like or M2-like phenotype macrophages[29]. M1 macrophages secreting proinflammatory cytokines such as TNF-α or IL-12 are considered to amplify the inflammatory response, whereas M2 macrophages producing anti-inflammatory mediators such transforming growth factor-β or IL-10 suppress the inflammatory response[29]. IL-34 has been identified to trigger CSF-1R signaling and induce macrophage differentiation to M2 phenotype *via* activation of the ERK1/2/AKT/AMPK signaling pathway[8], which contributes to mucosal homeostasis maintenance and

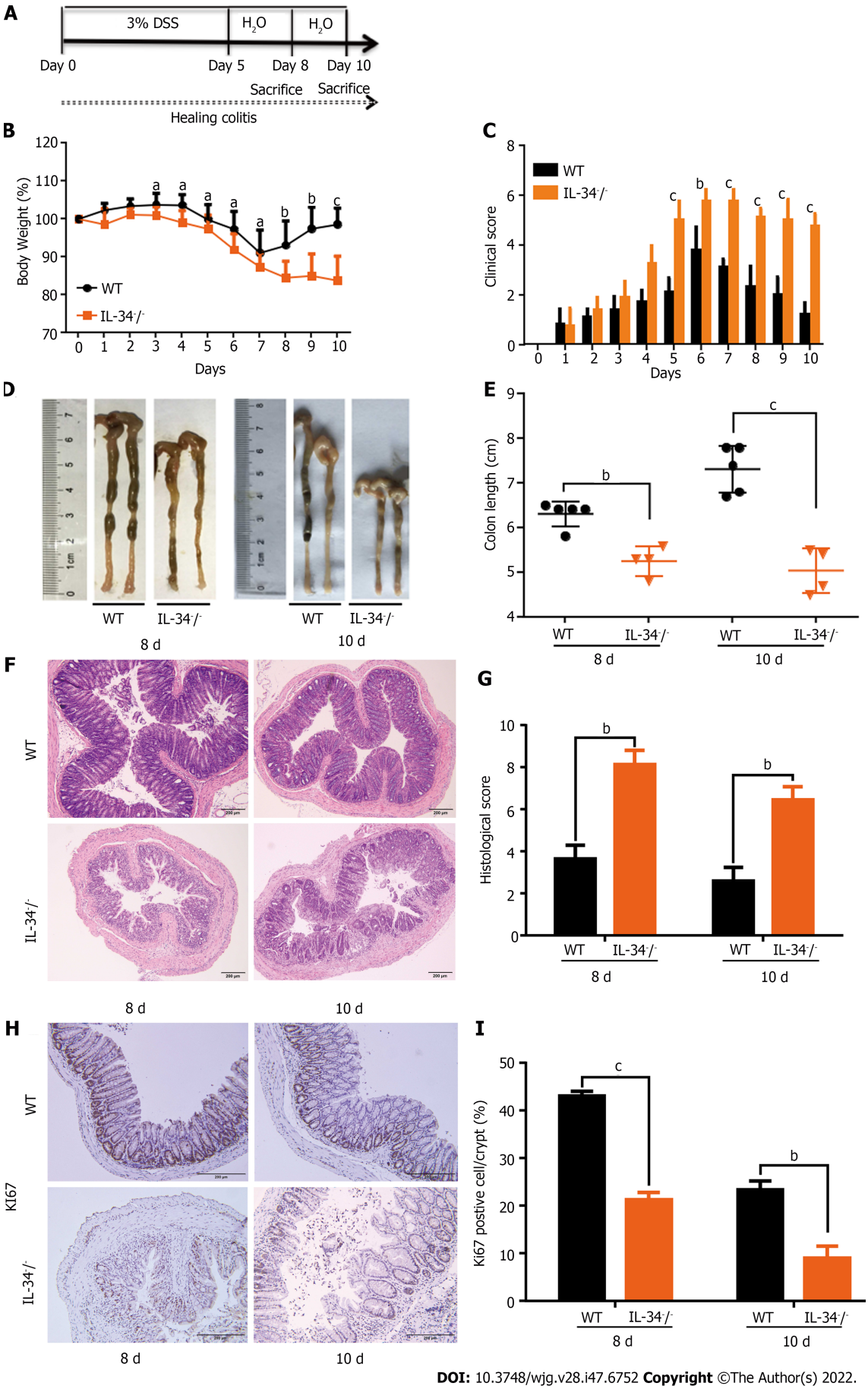


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**Figure 6 Interleukin-34 deficiency delays the mucosal healing in murine model induced by dextran sodium sulfate.** A: Establishing healing

model chart. The Interleukin-34 (IL-34)$^{-/-}$ and WT mice were fed with 3% dextran sodium sulfate (DSS) for 5 d and switched to normal drinking water for the following 5 d. The mice in both groups were killed on day 8 or 10 ($n$ = 4 or 5 per group); B and C: Body weight (B) and clinical score (C) were determined daily in IL-34$^{-/-}$ and WT mice until day 10 ($n$ = 4 or 5 per group); D and E: The colon length was measured in IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively ($n$ = 4 or 5 per group); F and G: Representative microscopic pictures (F) and semiquantitative histological scores (G) of IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively ($n$ = 4 or 5 per group); H and I: Representative photomicrographs of Ki-67 immunohistochemical staining (H) and percentage of Ki-67-positive per crypt (I) in IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively. DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild type. Scale bars = 200 μm. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$.

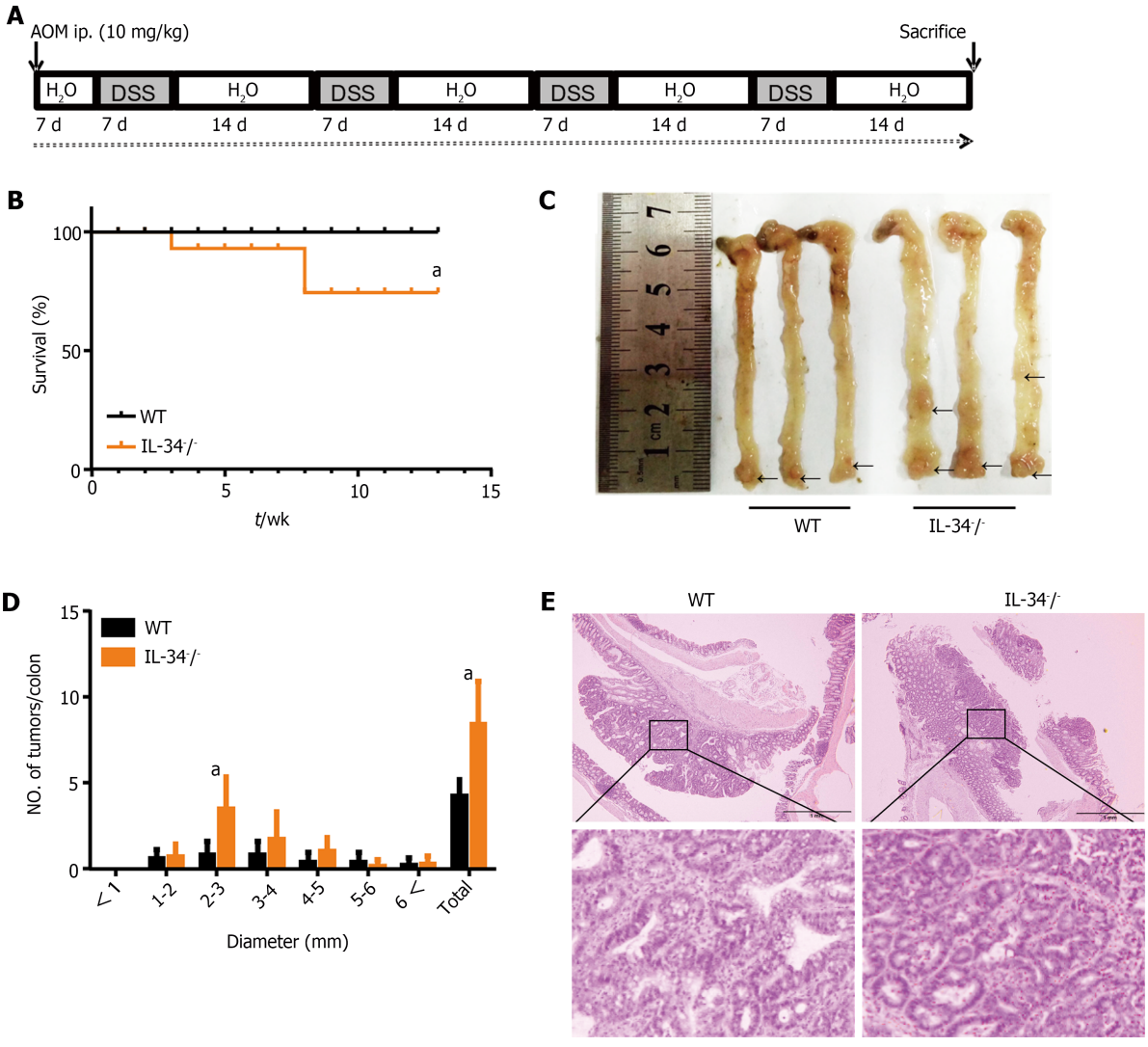


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**Figure 7 Interleukin-34 deficiency promotes azoxymethane /dextran-sodium-sulfate-induced colitis-associated tumorigenesis.** A: Experimental design was shown as diagram. WT and Interleukin-34 (IL-34)$^{-/-}$ mice were given a single injection of azoxymethane (AOM, 10 mg/kg). Seven days later, mice were treated with 1.25% dextran sodium sulfate (DSS) (w/v) in drinking water for seven consecutive days, and given fresh drinking water for 14 d. Seven days of DSS and 14 d of fresh water as a cycle, repeated four times; B: Kaplan-Meier survival curves were plotted in IL-34$^{-/-}$ and WT mice treated with AOM/DSS ($n$ = 10 per group); C and D: Representative photo of colon (C) and tumor number in colon (D) in IL-34$^{-/-}$ and WT mice treated with AOM/DSS ($n$ = 10 per group); E: Representative hematoxylin and eosin-stained colon sections in IL-34$^{-/-}$ and WT mice treated with AOM/DSS ($n$ = 10 per group). AOM: Azoxymethane; DSS: Dextran sodium sulfate; WT: Wild type. Bars: 1 mm (top) and 100 μm (bottom). Data depict mean ± SD. $^{a}P < 0.05$.

tissue remodeling[17]. Regulation of M1 to M2 phenotype switch has been confirmed to promote the proliferative phase of wound healing and ameliorates DSS-induced colitis, indicating a possible role of IL-34 in alleviation of colonic inflammation[17,18]. In our study, IL-34-deficient mice treated with DSS presented with worse colitis with significant accumulation of macrophages compared to DSS-treated wild-type control mice. It is speculated that downregulation of the M1 to M2 macrophage phenotypic switch results in more severe colitis. However, M2/M1 macrophage ratio was not significantly decreased in DSS-treated IL-34-deficient mice. We subsequently depleted the macrophages in the murine colitis model by administration of Clo-lips, which is a well-established method for macrophage

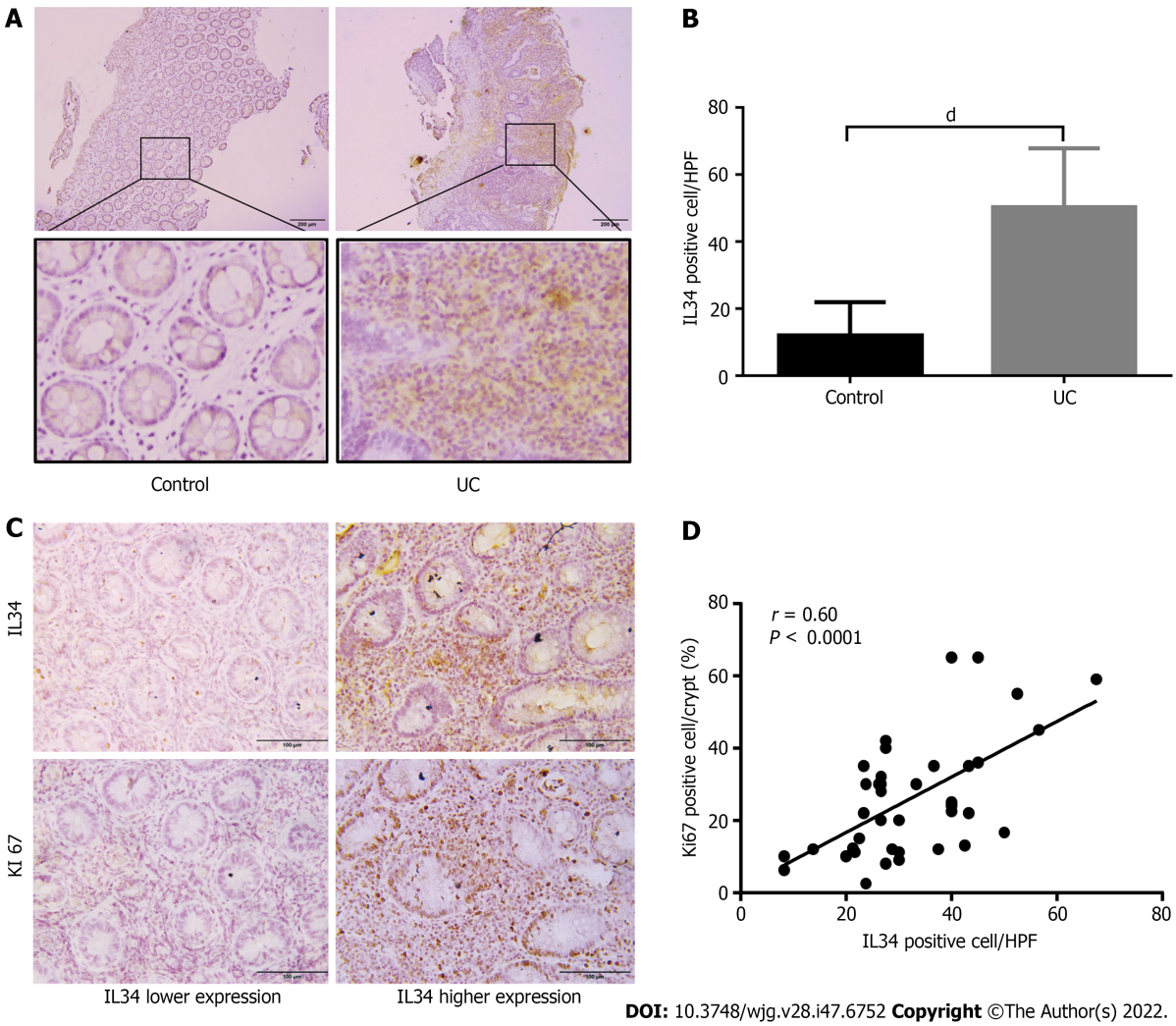


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**Figure 8 Interleukin-34 expression was elevated in inflamed mucosa and associated with colonic epithelium proliferation in active ulcerative colitis.** A: Representative microscopic pictures of interleukin-34 (IL-34) immunohistochemical staining in colonic biopsies of healthy controls or individuals with active ulcerative colitis (UC); B: Percentage of IL-34-positive cells per high-power field were quantified in colonic biopsies of healthy controls ($n$ = 20) and active UC patients ($n$ = 40); C: Representative photomicrographs of immunostaining of Ki67 in colonic biopsies of active UC patients in IL-34 Lower expression and higher expression groups; D: Correlation analysis of colonic IL-34 expression and Ki 67-positive cells in active UC patients. IL-34: Interleukin-34; UC: Ulcerative colitis. Scale bar indicates 100 μm or 200 μm. $^{d}P < 0.001$.

depletion[30], showing that IL-34 deficiency aggravated murine colitis whether or not macrophages were depleted. Our results suggested that the protective effect of IL-34 on experimental colitis was not primarily dependent on macrophage polarization.

Mucosal healing largely relies on controlled colonic epithelial proliferation and apoptosis, which are manipulated by multiple growth factors, gut peptides, cytokines, as well as signaling pathways[25]. Given different DSS-induced injury levels in normal and IL-34-deficient colonic mucosa, we should be cautious to draw the conclusion that IL-34 deficiency inhibits the mucosal healing process *in vivo*. Based upon immunohistochemical staining, IL-34 was predominantly localized in the colon stromal tissue in our study. The specific cellular source of IL-34 needs to be further explored. In the acute colitis model, epithelial cell proliferation was inhibited but epithelial cell apoptosis was induced in IL-34$^{-/-}$ mice. CSF-1R was compensatorily increased in the IL-34$^{-/-}$ mice treated with DSS. Furthermore, it has been proved that IL-34 expression is positively correlated with epithelial proliferation in mucosal biopsies from UC patients. No correlation was shown between IL-34 expression level and apoptosis in UC patients. It is speculated that IL-34 might be produced from colon stromal tissue and promote colonic epithelial cell growth and wound closure by binding to CSF-1R in inflammatory conditions. Further studies are warranted to investigate the potential function of IL-34 in colonic epithelial cells.

Emerging data have demonstrated the multidimensional role of IL-34 in tumor progression and metastasis. The function of IL-34 in cancer may vary among different types of tumors[31]. A recent study on the relationship between IL-34 and gastric cancer proposed that the reduction of IL-34 in gastric cancer was inversely related to the degree of tumor differentiation and was closely related to the poor survival rate of patients[32]. However, the relationship of IL-34 to colorectal cancer remains contro-

versial. It has been recently reported that IL-34 stimulates colorectal adenocarcinoma cell proliferation *via* an ERK1/2-dependent pathway. The connection between chronic colon inflammation and colitis-associated cancer has been documented in UC patients. Repeated mucosal damage and repair in the inflamed microenvironment can result in uncontrolled epithelial cell proliferation and induce cancer events[33]. In a cohort study, Wang *et al*[34] showed that low expression of IL-34 is associated with poor survival in colorectal cancer. Accumulating evidence suggests that chronic inflammation enhances the development of colitis-associated cancer through multiple mechanisms, including oxidative stress, DNA damage, abnormal immune response, and gut microbiome dysbiosis[35]. In our study, tumor burden was markedly enhanced in IL-34$^{-/-}$ mice treated with AOM/DSS compared to wild-type control mice. We assumed that IL-34 deficiency worsened the colonic inflammation response and tissue damage, and thus sustained inflammatory injury increased the risk of colitis-associated cancer.

## CONCLUSION

In summary, our work has highlighted the role of IL-34 during acute experimental colitis and wound healing. We found that IL-34 does not drive the inflammatory response and tissue destruction under physiological conditions, but protects the host from inflammatory injury and reduces the risk of colitis-associated cancer. IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment for UC and reducing colitis-associated cancer in UC.

## ARTICLE HIGHLIGHTS

### *Research background*

The exact pathogenesis of ulcerative colitis (UC) remains unclear. Identification of distinctive cytokines involved in immunopathogenesis of UC has become a hot spot for the development of biological therapies. The expression of interleukin (IL)-34 is upregulated in active UC but the molecular function and underlying mechanism are largely unknown.

### *Research motivation*

Whether IL-34 is a "friend or foe" in the pathogenesis of UC remains be explored. In this study, we investigated the potential role of IL-34 in experimental colitis, colitis-associated carcinogenesis and UC.

### *Research objectives*

To investigate the function of IL-34 in acute colitis, in a wound healing model and in colitis-associated cancer, and the IL-34 expression pattern and its relationship with colonic epithelium proliferation in active UC patients.

### *Research methods*

We conducted a controlled study using IL-34 knockout mice and wild-type mice (C57BL/6J). Colitis was induced by administration of dextran sodium sulfate, and carcinogenesis was induced by azoxymethane. Whether the impact of IL-34 on colitis was dependent on macrophages was validated by depletion of macrophages in a murine model. The association between IL-34 expression and epithelial cell proliferation was determined in patients with active UC.

### *Research results*

IL-34 deficiency *in vivo* exacerbated colitis in mice during acute and wound healing phases and increased tumor susceptibility in the mouse colon. The effect was independent of macrophage differentiation and polarization. IL-34 was markedly increased in patients with active UC and the expression was positively correlated with epithelial cell proliferation in UC.

### *Research conclusions*

IL-34 deficiency exacerbates colonic inflammation and accelerates colitis-associated carcinogenesis in mice.

### *Research perspectives*

IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment of UC and reducing colitis-associated cancer in UC.

## FOOTNOTES

**Author contributions:** Liu ZX performed the main part of the study; Chen WJ and Wang Y performed the main part of the study and wrote original draft; Liu YC, Cheng TC, Luo LL, Chen L, and Ju LL provided technical support and analyzed the data; Chen BQ, Liu Y, Li M, and Feng N contributed to part of the experiments; Shao JG designed the study and guided the manuscript writing; Bian ZL Conceived and designed the study; Liu ZX, Chen WJ, and Wang Y contributed equally to this work; all authors have read and approve the final manuscript.

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**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University.

**Institutional animal care and use committee statement:** All procedures involving animals were reviewed and approved by the Institutional Ethics Committee of Nantong University (No: S20151221-908).

**Informed consent statement:** All patients and healthy people signed an informed consent form.

**Conflict-of-interest statement:** All authors confirm that there are no conflicts of interest.

**Data sharing statement:** No additional data are available.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.



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