ORIGINAL RESEARCH

Protective function of interleukin 27 in colitis-associated cancer via suppression of inflammatory cytokines in intestinal epithelial cells

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ABSTRACT
Numerous studies have demonstrated that inflammation contributes to a variety of cancer formation, among them, colitis-associated cancer (CAC) represents a typical inflammation-related cancer. Interleukin 27 (IL-27) has been demonstrated to play an important role in inflammation-related disease. The effect of IL-27 in intestinal inflammation is controversial and its role in CAC is not elucidated yet. In our present study, we found that IL-27 has protective function in murine model of CAC through suppression of inflammatory cytokines in intestinal epithelial cells (IECs). IL-27Rα (WSX-1) deficiency promotes the CAC development in mice, which is driven by enhanced tumor cell proliferation, more intensive myeloid-derived suppressor cells (MDSC) accumulation in colon lamina propria and higher level of inflammatory cytokines and chemokines in IECs. The levels of IL-6, TNF-α, GM-CSF and CXCL1 triggered in vitro by toll-like receptor ligands are significantly upregulated in IECs from WSX-1 KO mice. Removal of commensal microorganism through antibiotic treatment in mice to eliminate TLR ligands deprives the protective function of IL-27 on CAC tumor growth. Thus, IL-27 suppresses CAC formation through an anti-inflammation mechanism targeting IECs and in turn resists the tumorigenesis. Hence, our study explained how IL-27 exerts its anti-inflammatory function on epithelial cells to fight against chronic-inflammation-associated cancer, which might provide new insights on the potential therapeutic strategies for cancer.

Abbreviations: COX-2, cyclooxygenase-2; CXCL1, chemokine (C-X-C motif) ligand 1; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressive cells

INTRODUCTION
In the last two decades, plenty of studies have demonstrated that excessive and chronic inflammation accelerates various tumorigenesis. Colitis-associated cancer (CAC) is proposed to be one of the representative inflammation-related cancers. Colorectal cancer (CRC) is a major cancer-related killers and remains to be the third most dominant malignancy in the world, especially in western countries. Although sporadic tumorigenesis is responsible for the majority of CRC, colon cancer can also rise from prolonged inflammatory bowel disease (IBD). Patients with ulcerative colitis or Crohn’s disease are at higher risk of colon cancer, and cancer incident is correlated with the degree of inflammation. The pathogenesis of IBD is complicated and not fully understood, besides infiltration of immune cells, the alteration of the status of intestinal epithelial cells (IECs) also contributes to inflammation development.

IECs are the predominant cell type in intestine and colon tissues, which forms the tight junction to compose a surface epithelium barrier and restricts the commensal bacteria in the intestinal lumen. The alteration of tight junction might contribute to intestinal inflammation. Toll-like receptors (TLRs) serve as sensors that recognize pathogen-associated molecular patterns (PAMPs) and usually considered to be expressed at high levels on innate immune cells such as macrophages and DCs. IECs also express TLRs like TLR4 and TLR2 on their basolateral membrane surface. Under homeostatic conditions, IECs express a low extent of TLRs and increase the expression under inflammatory conditions. Limited TLR-related inflammation is beneficial for pathogen clearance, but excessive TLR signaling in IECs promotes intestinal pathologies, accompanied by higher level of inflammatory cytokines and chemokines that mediate the infiltration of immune cells.

During the DSS-induced colitis, epithelium barrier damage amplifies intestinal permeability, following the activation of innate immunity through TLRs in both lamina propria immune cells and IECs. Administration of azoxymethane (AOM) followed by several rounds of dextran sodium sulfate (DSS) is a classical chemically induced mouse model of CAC. In this model, AOM is the carcinogen and DSS-induced colitis greatly accelerate the cancer development. Previously study reported that mice in the absent of TLR4 are protected from AOM/DSS-induced inflammation-associated tumorigenesis. By generating the bone marrow chimera, the same research
group demonstrated that rather than haematopoietic cells, increased TLR4 signaling in IECs is more responsible for higher risk of inflammation-associated neoplasia.16

Interleukin 27 (IL-27) is a heterodimeric cytokine combined with p28 and Epstein–Barr virus-induced gene (EBI3).17 And its receptor is composed of WSX-1 (IL-27Rα) and gp130. WSX-1 is the unique receptor subunit and gp130 is shared with IL-12.18,19 IL-27 is mostly expressed by activated antigen-presenting cells (APCs). And its receptor is expressed on T cells, NK cells, mast cells, macrophages, neutrophils and epithelial cells.17,19-23 IL-27 has been implicated in the pathogenesis of many chronic inflammation diseases.24 Previous studies showed that the function of IL-27 in IBD is controversial. It has been reported that IL-27/WSX-1 exacerbates DSS-induced colitis,25 while other studies demonstrated that IL-27 attenuates colitis through inhibition of Th17 or upregulation of antibacterial protein in IECs.23,26

The function of IL-27 in CAC has not been reported yet. Here, we first utilized the WSX-1 KO mice to investigate the previously unrecognized protective function of IL-27 in CAC murine model. We provided a theory that IL-27 might serve as an anti-inflammatory mediator to attenuate CAC growth. This protective function is achieved by downregulation of the TLR triggered cytokines in IECs, and in turn decreasing the MDSC accumulation and tumor cell growth within CAC microenvironment.

Results

Both IL-27 and its receptors are upregulated in tissue of AOM/DSS-induced colitis-associated cancer

To determine the expression of IL-27 in inflammation-associated colon cancer, we use the murine model to simulate the CAC tumor development. Wild-type C57BL/6 mice were administrated with AOM and three cycles of DSS in drinking water (Fig. 1A). Then the distal colon tumors from CAC mice were separated and analyzed. The results showed that mRNAs of two subunits of IL-27, EBI3 and p28 are both dramatically upregulated in tumor tissue samples compared to normal tissues controls, and the subunit of IL-27, WSX-1, also showed similar upregulation (Figs. 1B and C). Moreover, as the immunofluorescence result indicated, the protein level of IL-27 is also abundantly detected in the colon samples of CAC mice (Fig. 1D).

To investigate the source of IL-27, the IECs and sorted lamina propria (LP) CD45− immune cells (Fig. S1A) were isolated from colons of CAC mice and healthy control mice. In our observation, lamina propria cells represent the main source of IL-27 while IECs scarcely secrete IL-27 (Fig. 1E). Consistent with the mRNA level, the concentration of IL-27 protein dramatically elevated in the LP from CAC mice compared to the healthy mice controls (Fig. 1E). These results suggest that lamina propria derived-IL-27 and its receptor are both upregulated during tumor development, which implied IL-27 plays an important role in inflammatory colon tumorigenesis.

WSX-1 deficiency results in the increased accumulation of myeloid-derived suppressor cells in colon lamina propria

To investigate the mechanisms involved in the increased tumor burden in WSX-1 KO mice, we separated the colon lamina propria cells in CAC models, and analyzed of the subsets of CD45− immune cells or CD4+ T cells confined in FVD− living cells (Fig. S1A). Among the T cells subsets, WT and WSX-1 KO mice displayed the comparable percentages of Th17 and Treg cells in CD4+ T cells (Fig. 3A) and similar percentage CD4+ T cells and CD8+ T cells among CD45+ cells (Fig. 3B), indicating that WT and WSX-1 KO mice had similar T cell subsets in CAC models. Among innate immune cells subsets, dendritic cells and three subsets of macrophages, including CD11b+F4/80−, CD11b+CX3CR1high and CD11b+CX3CR1int, exhibited similar frequencies in CD45+ cells of colon lamina propria (Fig. 3B). Importantly, myeloid-derived suppressor cells (MDSCs) displayed a significant increase in WSX-1 KO mice (Fig. 3C).

In a previous study, CXCR2+ MDSC accumulation is crucial for CAC model formation and development, CXCR2−/− mice displayed the dramatically reduced tumor formation.27 CXCL1, the ligand of CXCR2, mediates the infiltration of CXCR2+ MDSCs into lamina propria.27 To verify the mechanism of MDSC expansion in WSX-1 KO group, we first examined the CXCR2 expression on MDSCs. According to the FACS results, WT and WSX-1 KO mice had similar level of CXCR2 expression (Fig. 3D). Hence, the elevated MDSC accumulation is not due to the changed expression of its chemoattractant receptor CXCR2. Then, we considered the possibility of the alteration of chemokine or cytokine in WSX-1 KO group that induces the MDSC accumulation.
WSX-1 deficiency leads to higher level of inflammatory cytokines and chemokines in intestinal epithelial cells

It has been well recognized that various cytokine expressions in tumor microenvironment can amplify the accumulation of MDSCs. Several inflammatory cytokines (IL-6, TNF-α, GM-CSF, CXCL11) known to induce MDSC expansion were determined in lamina propria CD45<sup>+</sup> cells from CAC models, but no significant difference was observed between WT and WSX-1 KO group (Fig. S2). In AOM/DSS model, the tumor cells derive from IECs, IECs express a variety of TLRs, which are activated under inflammatory condition. Previous study has demonstrated that TLRs on IECs serve as more important pro-inflammatory factors in AOM/DSS-induced CAC model. So, we examined whether loss of IL-27/WSX-1 signaling changes the production of inflammatory cytokines in IECs.
Figure 2. IL-27/WSX-1 signaling is important for inflammatory colorectal cancer formation and development in mice. (A) Macroscopic view of the representative colons and spleens from WT or WSX-1 KO mice on day 100 of the CAC model. (B–D) Colon tumor load, tumor number and average tumor size from WT (n = 13) and WSX-1 KO (n = 13) mice on day 100 of the CAC model. (E) The size distribution of tumors in WT and WSX-1 KO mice with CAC. (F) K67 staining of the representative mouse colons from WT or WSX-1 KO mice on day 100 of the CAC model. (G) TUNEL staining of the representative mouse colons from WT or WSX-1 KO mice on day 100 of the CAC model. (H) mRNA quantification of IL-6 and TNF-α in the distal colon tumor from CAC models. (I) Macroscopic view of the representative colons from WT or WSX-1 KO mice on day 8 of the DSS colitis model. (J) Statistical analysis of colon length of WT and WSX-1 KO mice on day 8 of DSS colitis model. (K) Percentage of weight loss in DSS-induced colitis model. Body weight was measured every day during DSS administration. Data are mean ± SEM. *p < 0.05; **p < 0.01.
We confirmed that subunits of IL-27 receptors, WSX-1 and gp130 were both detectable on EpCAM⁺ IECs by Flow Cytometry (Fig. 4A). Then, we evaluated the expression of inflammatory cytokines and chemokines in IECs. While IL-6, TNF-α, GM-CSF and CXCL1 had similar expression levels in the IECs between WT and KO naive mice (Figs. 4B and C), however, their expression significantly increased in the IECs of WSX-1 KO mice with CAC when compared to WT mice with CAC (Figs. 4B and C). This observation indicated upregulated inflammatory cytokine expression in the IECs of WSX-1 KO mice.

Figure 3. WSX-1 deficiency results in increased MDSC accumulation in colon lamina propria. (A and B) Statistical results of immune cells subsets by FACS analysis in lamina propria (n > 3). (C) Representative FACS results of MDSCs in lamina propria, and statistical results of MDSCs in lamina propria (n > 3). (D) Representative FACS results of CXCR2 expression on MDSCs, and statistical results of CXCR2 expression on MDSCs (n > 3). Data are mean ± SEM. "p < 0.05; ""p < 0.01.
GM-CSF is essential for MDSC development, and CXCL1 is the main ligand that binds to CXCR2, so GM-CSF and CXCL1 can promote the accumulation of MDSCs. It has been reported that CXCL1 expression is significantly upregulated in the serum of IBD patients. Previous studies indicated IL-6 and TNF-α both promote the proliferation of tumor cells. These cytokines construct the niche that promotes MDSC accumulation and tumor cell proliferation. Other CXCR2 ligands include CXCL2 and CXCL5, had similar expression between KO and WT mice with CAC model (Fig. 4D). Cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) are previously reported to be upregulated in CAC and accelerate the tumor formation, but we did not observe the statistically significant difference of them in WSX-1 KO and WT IECs with CAC model (Fig. 4D). And the level of IL-1β, which has epithelium barrier repairmen function, was also indistinguishable in WSX-1 KO IECs (Fig. 4D). In addition, immunofluorescence analysis suggested that MDSCs aggregated in the CXCL1 positive region, WSX-1 KO CAC tumor tissue had more MDSC accumulation comparing to WT tumor tissue (Fig. 4E). Then, we aimed to further verify whether these inflammatory cytokines derived from CAC IECs contributed to the accumulation of MDSCs by in vitro experiment. We stimulated CT26, a murine colon cancer cell line, with LPS for 24 h and collected the supernatant from culture. Bone marrow-derived MDSCs were stimulated by IL-6 and

Figure 4. WSX-1 deficiency leads to higher level of inflammatory cytokines and chemokines in IECs. (A) FACS analysis of WSX-1 and gp130 expression gated on EpCAM+ intestinal epithelial cells. (B) Quantification of the mRNAs of IL-6, GM-CSF and TNF-α in the IECs of naive and CAC models (n = 3). (C) Quantification of CXCL1 mRNA in the IECs of CAC models (n = 3). (D) Quantification of mRNAs of iNOS, COX-2, IL-1β, CXCL2, CXCL5 in the IECs from CAC models by qRT-PCR (n = 3). (E) Colon tumor sections from WT or WSX-1 KO CAC mice, stained with anti-CXCL1, anti-Gr-1 or DAPI. Data are mean ± SEM. *p < 0.05; **p < 0.01.
GM-CSF for 5 d\(^3\) (Fig. S3A). Transwell migration assay was utilized to demonstrate the chemo-attractive function of CXCL1. CD11b\(^+\) Gr-1\(^+\) MDSCs (Fig. S3A) were sorted and seeded into the upper chamber, with the 50% supernatant (with or without anti-CXCL1 antibody) in the lower chamber (Fig. S3B). After 12 h, the MDSC number that dropped into the lower chamber was counted, and DAPI was used to stain the MDSCs that cling to the bottom side of polycarbonate membrane. Both the cell number in the lower chamber (Fig. 5A) and the DAPI\(^+\) area (Fig. 5B) were decreased in the presence of anti-CXCL1 antibody, which demonstrated the chemoattractive function of CXCL1. And we also cultured bone marrow-derived MDSCs in the 50% LPS-stimulated CT26 supernatant with or without anti-IL-6 mAb or anti-GM-CSF mAb. We observed MDSCs significantly decreased in the presence with both antibodies (Fig. 5C), which indicates the function of IL-6 and GM-CSF to maintain the phenotype of MDSCs. Consistent with the \textit{in vivo} result, CXCL1, GM-CSF and IL-6 were very important in mediating the accumulation of MDSCs. Hence, these results indicate that defect in IL-27/WSX-1 signaling results in upregulated MDSC accumulation and tumor cell proliferation through inducing the inflammatory cytokines.

\begin{figure}[h]
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\caption{IL-6, GM-CSF and CXCL1 mediated the accumulation of MDSCs \textit{in vitro}. (A) Flow cytometry analysis of cells in the lower chamber of transwell migration assay, the cell number in the Gate is counted. (B) The cells attached to the bottom side of polycarbonate membrane were stained with DAPI and analyzed. (C) Flow cytometry analysis of MDSCs that cultured in 50% LPS-stimulated CT26 supernatant in the presence of anti-IL-6 or anti-GM-CSF or control IgG. Data are mean ± SEM. *p < 0.05; **p < 0.01.}
\end{figure}

\textbf{The alteration of inflammatory cytokines in WSX-1 KO mice is not the consequence of severer epithelium barrier disruption}

Intensive inflammation might be caused by the alteration of PAMP quantity or the inflammation signal itself, the former usually results from the alteration of intestinal epithelium barrier permeability. We evaluated the expression of some classical epithelium tight junction-related genes, such as claudin-1, claudin-2, claudin-3 and occludin,\(^39\)-\(^45\) and observed the similar
expression in both groups (Fig. 6A). In addition to the level of gene expression, the subcellular location of tight junction protein also represents the degree of epithelium barrier permeability, so we also utilized the immunofluorescence to observe the distribution of ZO-1,43 another tight junction protein, in both group. Consistent with the results above, ZO-1 staining in WT and WSX-1 KO colon showed similar expression and distribution in both tumor tissues and tumor adjacent normal tissues (Fig. 6B). And we observed the comparable level of LPS concentration in the serum of portal vein, which indicated the similar extent of exposure to PAMPs between WT and WSX-1 KO CAC mice (Fig. 6C). So these observations indicated it is not the PAMPs quantity alteration but the alteration of IEC intrinsic signaling that leads to the different inflammatory condition in WT and WSX-1 KO mice.

**IL-27 inhibits LPS-induced cytokine expression in IECs in vitro**

The primary IECs were isolated from WT naive mice and simulated by the TLR activation *in vitro*. While treated with LPS, WSX-1 KO IECs expressed higher mRNA level of inflammatory cytokine genes (Fig. 7A). Furthermore, after additional stimulation with IL-27, the mRNA expression inflammatory cytokines were decreased compared to the group that stimulated with LPS alone (Fig. 7B). These *in
**Figure 7.** IL-27 inhibits the LPS-induced cytokine expression in IECs in vitro. (A) WT and WSX-1 KO IECs were treated with different doses of LPS for 6 h. mRNA levels of IL-6, GM-CSF and TNF-α were determined by qRT-PCR. (B) WT IECs were treated with IL-27 (50 ng/mL) and LPS (50 ng/mL) for 6 h. Quantification of the mRNA levels of CXCL1, GM-CSF and TNF-α by qRT-PCR was showed. (C) CT26 was treated by LPS (100 ng/mL) with or without IL-27 (50 ng/mL) for 6 h. Quantification of mRNA levels of IL-6, GM-CSF, TNF-α and CXCL1 was showed. (D) CT26 was stimulated with LPS (100 ng/mL) or IL-27 (50 ng/mL) for indicated time and level of total and phosphorylated p65 was determined by Western Blot. (E) CT26 was stimulated with LPS (100 ng/mL) or IL-27 (50 ng/mL) for indicated time and level of SOCS1, SHP2, total and phosphorylated STAT3 was determined by Western Blot. Data are mean ± SEM. *p < 0.05; **p < 0.01.

**vitro** results also indicated that IL-27 inhibits the TLR-triggered inflammation in IECs. And we observed the suppressive effect of IL-27 in the CT26 cell line (Fig. 7C). Phosphorylation of p65 was detected after LPS stimulation, indicating the activation of TLR signaling pathway. IL-27 stimulation decreased phosphorylation of p65 (Fig. 7D). And the elevated protein level of SOCS1 and SHP2 was observed with IL-27 stimulation (Fig. 7E), SOCS1 and SHP2 were reported to be the suppressor of NF-κB.44 STAT3 is one of the important transcription factors of IL-27/WSX-1 pathway, and sustained STAT3 phosphorylation also suppresses the NF-κB pathway,44 we also observed that in IL-27-treated CT26 group (Fig. 7E). These results implied that the suppressive function of IL-27 might be exerted through upregulation of SOCS1, SHP2 and STAT3 phosphorylation.
Taken together, we verified that IL-27 inhibits the inflammatory cytokines in IECs in vitro and suppressed the TLR signaling pathway through SOCS1, SHP2 and sustained activation of STAT3.

Microbiota removal ameliorates CAC tumor development in WSX-1 KO mice

Given the hypothesis that IL-27 inhibits the TLR triggered inflammation in IECs and in turn leads to the downregulation of inflammation related cancer, we wonder whether ablation of TLR ligands would deprive its protective function for CAC. In order to eliminate the most of TLR ligands, WT and WSX-1 KO mice were treated with antibiotic cocktail in drinking water to ablate the commensal microorganism, then AOM/DSS models were induced. Phosphorylation of NF-κB p65 were determined in IECs and lamina propria immune cells, TLR signaling could not be activated after antibiotic treatment (Fig. 8A). Corresponding with our hypothesis, after TLR ligand ablation, the similar tumor burden was observed between WT and WSX-1 KO mice (Fig. 8B), and comparable quantity of CD11b⁺Gr-1⁺ MDSCs in lamina propria (Fig. 8C), also IECs from WT and WSX-1 KO mice had comparable mRNA expression of inflammatory cytokines (Fig. 8D). These results implied eradication of TLR ligands deprived the protective function of IL-27 in colitis-associated cancer model, which further proved the anti-inflammation function of IL-27 in IECs.

Discussion

In this study, we propose IL-27 as a protective player in CAC development model. IL-27 exerts its effect by targeting IECs to inhibit its TLR-triggered cytokine expression and reduce the inflammation level in CAC tumor microenvironment, and thus leads to a less extent of MDSC accumulation.

IL-27 was first identified as a Th1-promoting cytokine. Previous studies also implicated its anti-inflammatory function. IL-27 is secreted by activated APCs and its receptor is detected on T cells, NK cells, macrophages, monocytes, neutrophils and epithelial cells. In murine model of colitis, the function of IL-27-IL-27R interaction is controversial, and both attenuation and exacerbation of DSS-induced acute intestinal inflammation was reported. In 2005, a study first used the WSX-1 KO mice with DSS-induced colitis and found the severity of colitis was decreased in WSX-1 KO mice in comparison with WT mice in association with reduced inflammatory cytokines such as IL-6, TNF-α and IFN-γ. But another study indicated that IL-27 could induce anti-bacterial gene DMBT1 (deleted in malignant brain tumor 1) through p38 and STAT3 signaling, thus inhibited intestinal bacteria and protected mice from colitis. A study also indicated that IL-27 decreased the frequency of Th17 cells in gut-associated lymphoid tissues and limited colitis. Previous study also indicated oral delivery of IL-27-recombinant bacteria also attenuated T cell-transfer colitis. IL-27 exerts both pro- and anti-inflammatory function, different experimental environments and DSS concentration in these studies might lead to the different conclusions. Cancer frequently rises in the chronical inflamed area. Besides the uncertain effect of IL-27 in colitis, the function of IL-27-IL-27R interaction in CAC remains not studied yet. In our study, we first use the WSX-1 KO mice to reveal the anti-inflammation function of IL-27 in murine CAC model.

Intestinal epithelium is dominantly formed by IECs. The tight junction formed by IECs separates intestinal commensal microbiota from the internal environment, served as the first shield from these pathogens. In addition to the tight junction, TLRs are expressed on the basolateral surface of IECs. When tight junction of epithelium disrupted and PAMPs from commensal microorganism are exposed, these TLRs are crucial for local inflammation and monocyte recruitment to clear the pathogen. These two mechanisms together contribute to the intestinal homeostasis. But excessive TLR activation is often consequent in pathogenesis, previous study indicated that rather than immune cells, the function of TLR signaling pathway in IECs is much more significant in CAC.

Here, we report its anti-inflammatory function which exerts on TLR ligand-induced inflammation in intestinal epithelia cells. In our study, increased tumor incidence and tumor load were observed in WSX-1 deficient mice, along with enhanced tumor cell proliferation and MDSC accumulation. CXCR2⁺ MDSCs is crucial to CAC model because CXCR2−/− mice were observed with significantly reduced tumor formation. We proved loss of WSX-1 upregulates the level of IL-6, TNF-α, GM-CSF and CXCL1 in IECs. IL-6 and TNF-α are not only acknowledged as pro-inflammatory cytokines, but also efficiently promote tumor cell proliferation, which increase tumor formation in CAC mice. CXCL1, the ligand of CXCR2, and GM-CSF are both positive regulator of MDSC accumulation, which in turn also exacerbates the severity of CAC. Here, we demonstrated the WSX-1 deficiency did not influence the expression or distribution of tight junction-related proteins to influence the epithelium permeability, so the higher level of inflammation cytokines in IECs of WSX-1 KO mice is not the consequence of higher level of PAMPs. So the severe inflammation might be the result of direct regulation on TLR-associated signaling pathway. When we stimulated IECs with LPS and IL-27 in vitro, the results proved that IL-27 significantly inhibited the cytokine and chemokine productions of IECs. To further confirm our discovery, we eliminated the PAMPs from microorganism via antibiotic treatment, commensal microorganism removal abrogated the protective function of IL-27. This also implies its function exerts directly on TLR signaling.

Therefore, we present a new theory that how the anti-inflammatory function of IL-27 contributes to the inflammation-related colon cancer. Despite we found the contribution of IECs in our study, due to the animals in our study is knock out system but not cell-specific CRE-Loxp system, we cannot exclude the involvement of other cells. We can only conclude that IL-27 exerts its anti-inflammatory function on IECs to reduce MDSC accumulation and protect against the CAC development, both in vitro and in vivo. The contribution of other types of cells to the increased CAC tumor formation yet needs to be studied in future.

Taken together, our results indicate that IL-27/WSX-1 signaling negatively regulates the inflammation level in local environment, especially targeting IECs, exerts the protective function in CAC. IL-27 is a potential candidate treatment of...
chronic inflammatory diseases, and its function is far beyond veiled. We hope our study might raise interests to uncover its function and shed new light on CAC therapeutic strategies.

**Materials and methods**

**Mice**

WSX-1 KO mice on the C57BL/6 background were purchased from JAX Mice (Stock No: 018078). 8–10-week male WSX-1 KO mice and their wild-type male littermates were used for experiments. All mice were maintained in specific pathogen-free conditions. Experiments and animal care were performed according to protocols approved by the Zhejiang University Institutional Animal Care and Use Committee.

**Induction of colitis-associated colon cancer**

8–10-week old wild type and WSX-1 KO mice littermates were intraperitoneally (i.p) injected with AOM (Sigma) dissolved in PBS at the dose of 10 mg/kg body weight. After a week, mice were given 2% DSS in drinking water for 5 d.
and then followed by water for 2 weeks. The cycles were repeated twice. Then mice were sacrificed on the day 100 after AOM injection. Colons were removed and analyzed. After flushed with PBS, macroscopic colon tumors were counted and diameters were measured by caliper as previously described.

**Isolation of intestine epithelia cells and lamina propria**

Whole colons were removed, opened longitudinally and washed with PBS. Then, colons were cut in to 3 mm pieces and incubated in 10 mM EDTA, 1 mM DTT solution in PBS at 37°C for 45 min. Then incubation was shacked vigorously for 30 s. Supernatants from incubation were collected and centrifuged at 400 g for 5 min at 4°C. Pelleted cells were suspended and filtered. Filtered cells mainly consist of epithelial cells (85%~95%). To obtain lamina propria leukocytes, colon pieces after EDTA incubation were collected and further cut into smaller pieces (1 mm) and digested by collagenase IV (300 U/mL, Worthington) in 5% FBS PBS solution at 37°C for 1 h. After digestion, the cell suspension was filtered to get single-cell suspension containing lamina propria leukocytes. To quantify the IECs and lamina propria leukocytes, single-cell suspension were stained with CD45 and EpCAM and analyzed by FACS Calibur (BD Bioscience).

**Immunohistochemistry**

For the determination of cells proliferation, paraffin-embedded colon tumor sections were stained with antibodies against Ki67 (Ebioscience). For the determination of cell apoptosis, TUNEL assay was performed with Cell Death Kit (Roche).

**Immunofluorescence**

Colon tissues were maintained in OCT at −80°C overnight then sliced into sections. To determine IL-27 expression, epithelium permeability and CXCL1, anti-IL-27 (Santa Cruz), anti-ZO-1 (Abcam), anti-cytokeratin (Abcam), biotin-anti-Gr-1(MACS), streptavidin-PE (Ebioscience), anti-CXCL1 (Protein-tech) and DAPI were used.

**Commensal depletion**

At first, mice were given a cocktail of 1 mg/mL of neomycin, 0.5 mg/mL vancomycin, 1 mg/mL metronidazole and 1 mg/mL of ampicillin in drinking water for 4 weeks. Fresh antibiotics were changed every week. After 4 weeks, drinking water was further added with 1 mg/mL of streptomycin, 170 μg/mL of gentamicin, 125 μg/mL of ciprofloxacin, and 1 mg/mL of bacitracin as previously described. Depletion was assessed by collecting feces which serially diluted with PBS and then plated on 10% sheep blood trypticase soy agar for 48 h at 37°C. After mice were treated with the antibiotics for 5 weeks, colon cancer was induced as described in the section of induction of colitis-associated colon cancer.

**Transwell migration**

BM-MDSCs (1 × 10^5) were seeded in the upper Transwell chambers (5 μm, BD Biosciences) with 50% LPS-stimulated CT26 supernatant (with or without neutralizing antibody) as a chemoattractant in the lower chamber. After 12 h inoculation at 37°C, 5% CO2, the cells dropped into lower chamber were counted by flow cytometry and cells that cling to the bottom side of polycarbonate membrane was stained by DAPI.

**RNA isolation and real-time quantitative-PCR**

Total RNA was extracted using Trizol Reagent (Takara, Japan), real-time PCR was conducted on CFX-Touch real-time PCR machine (Bio-Rad, Hercules, CA, USA) using SYBR Green reagent (Roche, Switzerland). Q-PCR primer sequences were listed in Table S1.

**Western blot**

Total cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes, followed by immunblotted with indicated antibodies.

**Statistical analysis**

Statistical analysis was performed using Student’s t-test, analysis of variance (ANOVA) using SPSS, with a p < 0.05 considered statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

Q. W. and B. C. designed the research, S. L., L. L., Y. X., J. H., Y. X., P. X., L. C., T. P., Y. L. and X. C. performed the experiments and analyzed the data. B. C. and Q. W. wrote the paper.

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