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RESEARCH ARTICLE

Expression of the novel adipokine C1qTNF-related protein 4 (CTRP4) suppresses colitis and colitis-associated colorectal cancer in mice

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Inflammatory bowel disease (IBD) is an important factor in the induction of colon cancer, but its mechanism is unclear. Colitis and colitis-associated colorectal cancer (CAC) models induced using both dextran sulfate sodium (DSS) and the azoxymethane/DSS protocol were established in wild-type (WT) and CTRP4 transgenic (CTRP4-tg) C57BL6/J mice. Body weight, stool consistency and the presence of blood in the stool were analyzed; tumor quantity, size and histological characteristics were analyzed during the development of CAC. The CTRP4-tg mice exhibited significantly reduced colitis and developed far fewer macroscopic tumors; these tumors were smaller in size, and a majority of the colon tumors in these mice were restricted to the superficial mucosa. Tumors of lower grades were observed in the CTRP4-tg mice. Interleukin-6 was markedly downregulated in the CTRP4-tg mice during CAC tumorigenesis. The phosphorylation of ERK, signal transducer and activator of transcription 3 and Akt in the colon and the proliferation of intestinal epithelial cells were decreased in the CTRP4-tg mice. The injection of recombinant CTRP4 protein significantly reduced the colitis symptoms of the WT mice. CTRP4 plays an important role in inflammation and inflammation-associated colon tumorigenesis, and our research may provide a novel method for the treatment of IBD and CAC.
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INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are chronic inflammatory diseases.[1] Disruption of the intestinal mucosal barrier and aberrant infiltration of leukocytes into the lamina propria are the predominant manifestations of these diseases.[1–3] Patients with IBD exhibit a higher risk of colon cancer than does the general population, and approximately 7–8% of patients with ulcerative colitis or Crohn's disease ultimately develop colon cancer within 20 years.[2,4] These observations support the idea that chronic colitis promotes cancer development. In chronic colitis, many of the pro-inflammatory cytokines produced by immune and non-immune cells are believed to be important promoters of tumorigenesis in the colon.[5,6] One of the most important cytokines is interleukin-6 (IL-6), which is primarily produced by inflammatory cells, including activated myeloid cells. Furthermore, some epithelial cells, such as hepatocytes and intestinal epithelial cells (IECs), also produce small amounts of IL-6.[6–8] IL-6 regulates the survival and proliferation of IECs and provides premalignant cells with persistent growth and survival signals that enhance both the initiation and progression of colitis-associated colorectal cancer (CAC).[9,10] The injection of hyper-IL-6 or recombinant IL-6 at either the early or the late stage of azoxymethane (AOM)/dextran sulfate sodium (DSS)-mediated CAC induction enhances tumorigenesis in mice.[6] However, the neutralization or ablation of IL-6 slows tumor growth and reduces the tumor burden during CAC induction.[6,11] IL-6 is also essential for the activation and

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differentiation of T cells, including the pathogenic Th17 cells. Therefore, IL-6 plays a central role in T cell-mediated disorders such as IBD.[12] By acting on T cells, IL-6 induces uncontrollable chronic inflammation that results in the persistent production of cytokines and growth factors that are required for malignant cell survival and growth.[10,13] Additionally, the production of IL-6 and other pro-inflammatory cytokines is increased in patients with IBD, including those who have developed colon cancer.[9,14]

The C1Q/TNF-related protein (CTRP) family, which currently comprises 15 members designated CTRP1-15, was originally identified in 2004 by Harvey Lodish and colleagues.[15,16] The CTRPs perform diverse functions, and recent evidence has shown that CTRP family members play roles in immunity and metabolism.[15] For example, CTRP1 stimulates aldosterone synthesis and activates Akt and pERK1/2-mitogen activated protein kinase in muscle tissues. Additionally, the injection of recombinant CTRP1 reduces the blood glucose levels in mice.[17,18] CTRP3 acts as an anti-inflammatory factor that inhibits lipopolysaccharide (LPS)-mediated inflammation by inhibiting the binding of LPS to toll like receptor 4 (TLR4)/MD-2 on monocytes and adipocytes.[19,20] CTRP9 reduces the blood glucose levels and insulin resistance in mice, and recombinant CTRP9 activates AMP activated kinase, Akt and p42/44 mitogen activated protein kinase in C2C12 myotubes.[21]

CTRP4 is the fourth member of the CTRP family. The structure of CTRP4 includes two globular domains, whereas the other CTRPs contain only one globular domain.[8] Our laboratory first reported the function of CTRP4 in 2011. We have studied its function in HepG2 cells and found that it is produced as a classical secreted protein. Both the overexpression of CTRP4 and the addition of the recombinant human CTRP4 (rhCTRP4) protein increase IL-6 expression and activate the nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling pathways in HepG2 cells.[8] In 2014, another group found that CTRP4 functions in the hypothalamus to modulate food intake and body weight, that refeeding following an overnight fast induces the expression of CTRP4 in the hypothalamus, and that the injection of rCTRP4 centrally suppresses food intake and alters the whole-body balance in mice.[22] Taken together, these results suggest that CTRP4 performs diverse functions, although its roles in inflammation and inflammation-associated cancer remain unknown.

In this study, we employed the widely used DSS-induced acute colitis and AOM/DSS CAC protocols to investigate the roles of CTRP4 in inflammation and inflammation-associated cancer.

MATERIALS AND METHODS

Mice

The wild-type (WT) and CTRP4 transgenic (CTRP4-tg) mice were housed at the Peking University Health Science Center. All mice used in this study were of the C57BL6/J background, and the CTRP4-tg mice were heterozygotes. All of the experiments were carried out in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Peking University.

DSS-induced colitis and CAC models

Colorectal tumorigenesis was induced by administration of the carcinogen AOM and the repeated administration of DSS. Age-matched mice (8–10 weeks old) were intraperitoneally injected with a single dose of AOM (10 mg/kg; #A2853, Sigma, Munich, Germany). After 7 days, 2% DSS (molecular weight of 36–50 kDa; #160110, MP Biomedicals, Santa Ana, CA, USA) was provided in drinking water for 7 days, followed by normal drinking water for 14 days. The DSS treatment was repeated for two additional cycles. Then, the mice were euthanized, and the tumors were analyzed 100 days after the AOM injection.

For the acute colitis and inflammation studies, 3% DSS was administered to the mice for the indicated periods, and the mice were euthanized at the indicated time points. The body weights were measured daily during the DSS treatment. The disease activity index (DAI) was calculated by assigning well-established and validated scores.[23] Briefly, three parameters were used for the calculation: (a) diarrhea (0, normal; 2, loose stools; 4, watery diarrhea); (b) hematochezia (0, no bleeding; 2, slight bleeding; 4, gross bleeding); (c) percentage weight loss (0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, $>20\%$).

The colons were removed from the mice, opened longitudinally, and flushed with cold phosphate-buffered saline (PBS). The lengths of the colons were measured using a digital caliper in a blinded manner.

Histologic analysis

The colons were washed thoroughly in cold PBS, fixed in 10% (wt/vol) formalin, and embedded in paraffin for 24 h. Then, 4-μm-thick sections were stained with hematoxylin and eosin (H&E) using standard procedures. The disease severity was measured in a blinded manner by a professional pathologist from the Department of Pathology at Peking University based on previously described methods.[24] In brief, the measurements were scored as follows: 0, normal tissue; 1, mild inflammation of the mucosa with some infiltrating mononuclear cells; 2, increased levels of inflammation of the mucosa with more infiltrating cells, damage to the crypt glands and the epithelium, and mucin depletion from the goblet cells; 3, extensive cell infiltration into the mucosal and submucosal areas, crypt abscesses with mucin depletion, and epithelial cell disruption; and 4, massive cell infiltration into the tissue and the complete loss of the crypts.

For the tumor grade analysis, briefly, low-grade adenomas were defined as those consisting of a stratified dysplastic epithelium that retained its columnar morphology and displayed nuclei in the basal portion of the epithelium, whereas high-grade adenomas were defined as those that had lost their columnar morphology and displayed nuclei that were primarily located at the surfaces of the crypts.

Culture of the entire length of the colon

The colons were removed, cut longitudinally, thoroughly washed twice in cold PBS, sliced into 5-mm segments, and cultured for 24 h in RPMI 1640 medium containing

2% penicillin and streptomycin. The culture supernatants were collected and frozen at − 80 °C for cytokine determination.

Preparation of the rhCTRP4 protein

rhCTRP4 was expressed and purified in our laboratory as previously described.[8] Briefly, to generate the eukaryotic recombinant protein, CTRP4-His plasmids were transfected into CHO cells, and the CHO cells were cultured in HEKG medium. The supernatant was harvested 72 h after transfection, and the rhCTRP4 protein was purified from the supernatant using nickel affinity chromatography under bacteria-free conditions and was identified using western blot analysis with an anti-His antibody. To generate the prokaryotic recombinant protein, the pET32a-CTRP4 plasmids were transfected into DE3 *Escherichia coli*, the pET32a-CTRP4-positive cells were cultured at 37 °C, and the expression of CTRP4 was induced using isopropyl-β-d-thiogalactoside at a final concentration of 0.4 mM at an optimal temperature. Ten hours later, the cells were collected and re-suspended in lysis buffer (20 mM PIPES, 500 mM NaCl and 20 mM imidazole). Then, the lysate was centrifuged ($13\,000 \times g$), and the supernatant was purified using nickel affinity chromatography. The purified protein was further purified using diethyl-aminoethanol ion-exchange chromatography to completely remove the LPS. The LPS level in the purified protein sample was determined using a tachypleus amebocyte lysate, which was produced in Xiamen, China.

Bone marrow-derived macrophages (BMDMs)

Bone marrow from the WT C57BL6/J mice was isolated and differentiated for 7 days in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, a 1% (v/v) penicillin/streptomycin solution and macrophage colony-stimulating factor (1000 U/ml). For the experiments, the differentiated BMDMs were seeded at 2×10^5 cells/ml.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine levels in the colon or cell culture supernatants were determined at the indicated time points using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Human CTRP4 and mouse CTRP4 ELISA kits were purchased from Hua Mei Biotech Co., Ltd. (Wuhan, China).

Cytometric bead array

The IL-6 levels in the AOM/DSS-treated mouse colons were measured using a Cytometric Bead Array Kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, 50 μl of the colon culture supernatant was mixed with 50 μl of the capture beads and 50 μl of the mouse cytokine PE detection reagent. This mixture was incubated at room temperature for 2 h in the dark and then washed thoroughly. Next, the samples were resuspended in 300 μl wash buffer before measurement using a flow cytometer. The data were analyzed using the CBA software (BD Biosciences). A standard curve was constructed using the bead standard provided in the kit, and the concentrations of IL-6 in the colon culture supernatants were determined by interpolation from the standard curve.

Western blotting

The cells were lysed in RIPA lysis buffer (20 mM HEPES (pH 7.4), 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM $NaVO_3$, 1 mM PMSF and 1% protease inhibitor cocktail), and PhosSTOP (Roche, Basel, Switzerland) was added to the lysis buffer to detect protein phosphorylation. The protein concentrations were measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA); bovine serum albumin was used as a standard. The lysates were separated using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes (Hybond, ECLTM, Amersham Pharmacia, Little Chalfont, UK). After blocking in 5% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature, the membranes were incubated with the appropriate primary antibodies overnight at 4 °C, extensively washed in TBS-T, and then incubated for 1 h at room temperature with the horseradish peroxidase-labeled goat anti-rabbit or anti-mouse IgG antibody. Following three additional washes in TBS-T, the signals were finally visualized using ECL Substrate (Pierce) and imaged using an LAS500 imager (GE, Ontario, CA, USA).

Statistical analysis

The statistical analyses were performed using two-tailed Student's *t*-test or one-way ANOVA followed by the Newman-Keuls *post hoc* test (for survival rate). The data are expressed as the means ± s.e.m.

RESULTS

Transgenic CTRP4 expression in mice suppresses CAC

We generated CTRP4-tg mice, in which the expression of human CTRP4 was driven by a chicken β-actin promoter. Western blot analysis revealed that CTRP4 was successfully expressed in multiple mouse organs, including the kidney and spleen.[25] However, no phenotypic changes were observed in the untreated CTRP4-tg mice as determined by phenotype screening; there were no noticeable body mass differences between the CTRP4-tg mice and WT mice. Furthermore, the subsets and numbers of immune cells in the spleen, lymph node and bone marrow were identical, and no histological changes were observed using H&E staining.[25]

To investigate the role of CTRP4 in CAC, we used an approach in which is AOM is combined with DSS to induce colitis (Figure 1a). Following a single injection of AOM and three cycles of 2% DSS treatment, all of the WT mice developed colon tumors in the distal to middle colon. However, only 70% of the CTRP4-tg mice developed tumors, and the histological analyses suggested that the remaining 30% of these mice demonstrated adenomatous hyperplasia. Additionally, we found that the CTRP4-tg mice developed far fewer macroscopic tumors, and the average tumor load

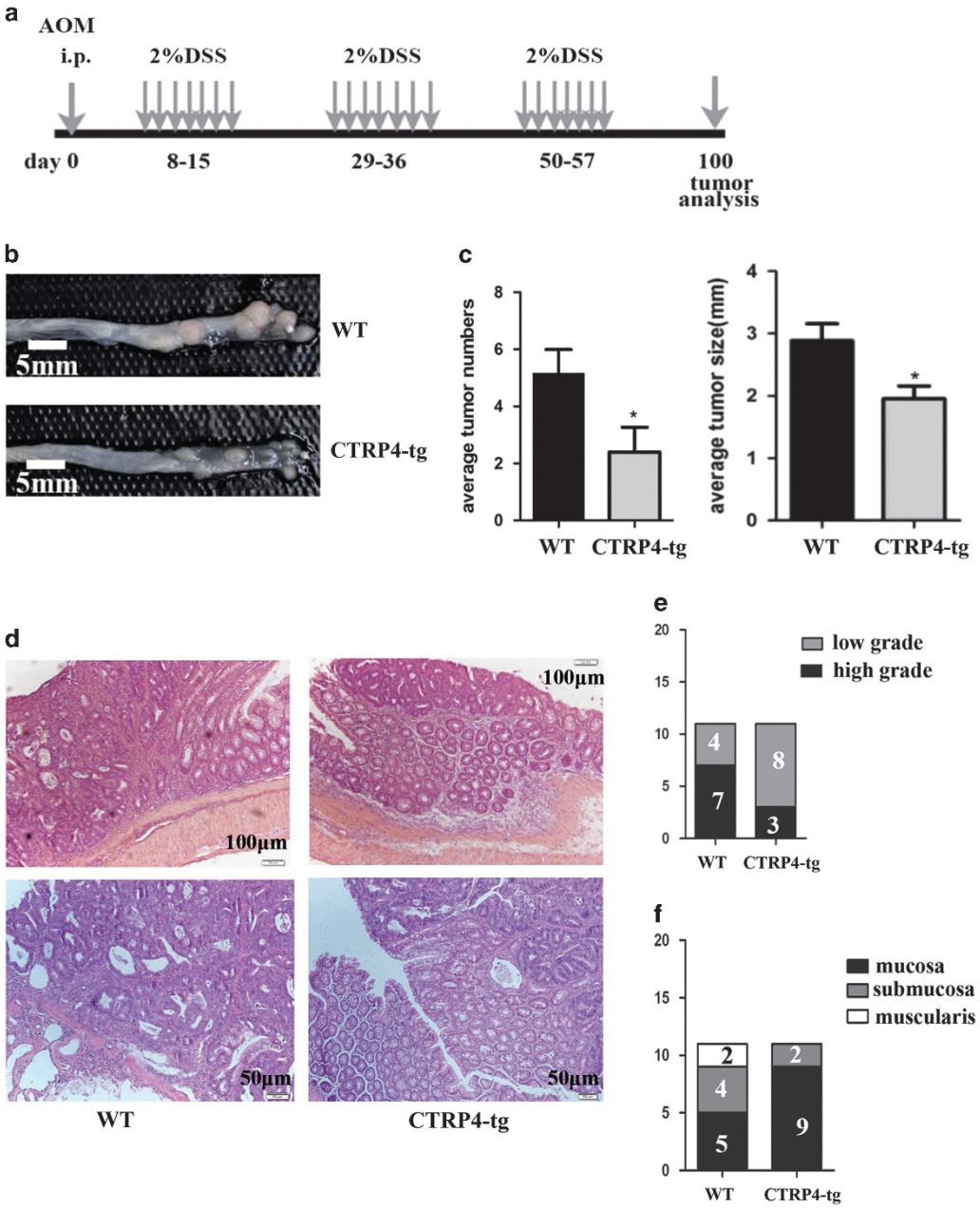

Figure 1 CTRP4 transgenic expression in mice decreases CAC. (**a**) Schematic representation of the AOM/DSS procedures. i.p.: intraperitoneal. (**b**) Macroscopic appearance of colon tumors. (**c**) The number of colon tumors was counted, and the tumor sizes were measured using a digital caliper in a blinded manner. (**d**) Representative H&E-stained images of the colon tumors. (**e**, **f**) The tumors were microscopically analyzed and classified as high- or low-grade as described in Materials and Methods (**e**), and the layers of the colon tissue invaded by the tumor cells were analyzed (**f**). The results are representative of three experiments and are presented as the means ± s.e.m.; $^*P<0.05$; $n=11$.

and size were less than those of the WT mice (Figures 1b and c). Histological analyses revealed that the majority of the colon tumors in the CTRP4-tg mice were restricted to the superficial mucosa and that only a few tumor cells had invaded the submucosa. In contrast, most of the tumor cells had invaded the muscularis of the colon in the WT mice. Furthermore, lower-grade tumors were observed in the CTRP4-tg mice (Figures 1d–f).

The CTRP4-tg mice are less susceptible to DSS-induced acute colitis

DSS-induced colitis is critical for AOM/DSS-induced tumorigenesis. Thus, we hypothesized that CTRP4 suppresses CAC by inhibiting the DSS-induced colitis.

To verify our hypothesis, 3% DSS (v/w) was administered to both the CTRP4-tg mice and the WT mice. After 6 days of DSS administration, the CTRP4-tg mice lost ~5% of their body

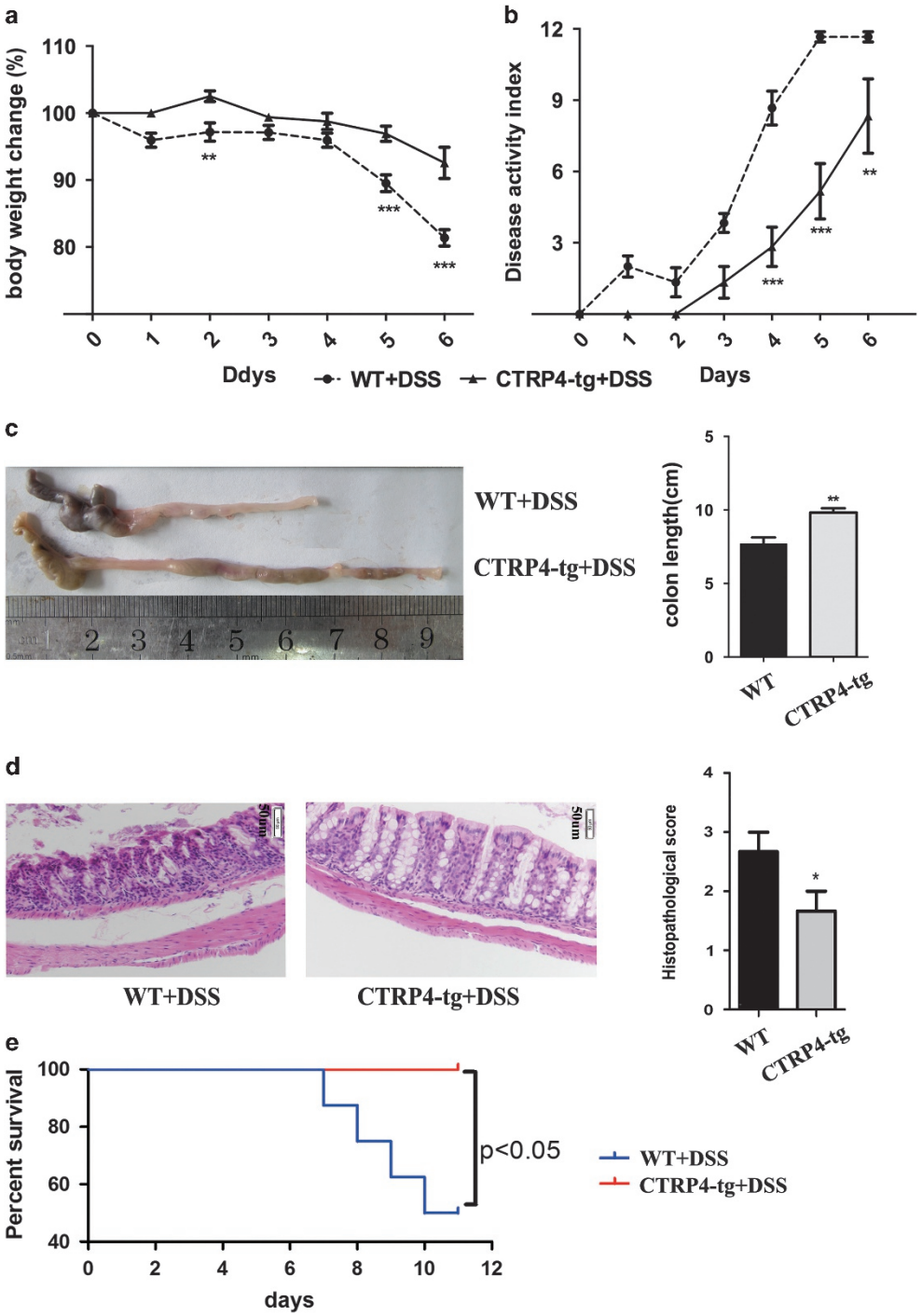

Figure 2 CTRP4 expression protects mice from DSS-induced acute colitis. (**a**) Acute colitis was induced as described in Materials and methods. The body weights were measured every day and are expressed as the percentage of the initial body weight. (**b**) The DAI was calculated using a well-established and validated method. Briefly, three parameters were used for calculation: (**a**) diarrhea (0, normal; 2, loose stools; 4, watery diarrhea); (**b**) hematochezia (0, no bleeding; 2, slight bleeding; 4, gross bleeding); (**c**) percentage weight loss (0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, >20%). (**c**) Colon lengths were measured and analyzed on day 6 following DSS treatment. (**d**) Representative photomicrographs of colon sections stained with H&E were analyzed, and the histological scores were determined as described in Materials and Methods. (**e**) The survival times for the CTRP4-tg and WT mice were recorded. The results are representative of at least four experiments and are presented as the means ± s.e.m.; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. $n=6$.

weight. In contrast, the WT mice lost more than 20% of their body weight, which suggested that the CTRP4-tg mice experienced milder colonic inflammation on the basis that body weight loss is a major clinical indicator of the severity of DSS-induced colitis (Figure 2a). The DAI, which comprises stool consistency, stool occult blood and body weight loss, was

also lower for the CTRP4-tg mice than for the WT mice (Figure 2b). The colon length is another macroscopic indicator of the severity of DSS-induced colitis; shorter colons reflect more severe cases of colitis. As shown in Figure 2c, the CTRP4-tg mice exhibited markedly longer colons than the WT mice. The histological analysis also revealed that the CTRP4-tg mice exhibited less leukocyte infiltration and intestinal injury than the WT mice (Figure 2d).

To further assess the role of CTRP4 in DSS-induced colitis, we determined the mortality rate. After 11 days of DSS treatment, the CTRP4-tg mice exhibited a lower mortality rate than did the WT mice. Specifically, all of the CTRP4-tg mice survived, whereas only 50% of the WT mice survived the effects of the DSS-induced colitis (Figure 2e). Thus, these data suggested that CTRP4 protected the mice from DSS-induced colitis *in vivo*.

Production of pro-inflammatory cytokines and infiltration of macrophages are decreased in the colons of DSS-treated CTRP4-tg mice

Because the CTRP4-tg mice exhibited milder clinical disease and tissue injury resulting from DSS treatment, we used ELISAs to characterize the pro-inflammatory cytokine levels in the colons of the mice before and after DSS treatment. The results showed that prior to DSS treatment, IL-6, IL-1β and tumor necrosis factor-alpha (TNF-α) were produced at very low levels in both the WT and the CTRP4-tg mice. However, after 3 days of DSS treatment, all three of these cytokines were upregulated. Interestingly, we found that the production of IL-6 was suppressed during the early phase of DSS-induced colitis in the CTRP4-tg mice (Figure 3c), but the levels of other two cytokines remained similar between the two groups. These results indicated that CTRP4 might directly inhibit the production of IL-6 in the DSS-induced colitis (Figures 3a and b). However, after 6 days of DSS treatment, the levels of all three cytokines were much lower in the CTRP4-tg mice than in the WT mice (Figures 3a–c).

Because IL-10 is among the most important anti-inflammatory cytokines,[26] we measured its production in the colons and found that its level did not differ between the CTRP4-tg and WT mice during DSS treatment (Figure 3d). This result indicates that the protection from the DSS-induced colitis provided by the transgenic CTRP4 protein was independent of IL-10. IL-22 is believed to serve as an important cytokine that protects the intestinal mucosa.[27,28] The expression levels of IL-23 and IL-22 were analyzed, and the levels of both cytokines were found to be similar in the WT and CTRP4-tg mice during both the early and late phases of DSS treatment (Figures 3e and f), which indicated that IL-22 is not a critical effector in this model.

Because infiltration of immune cells into the colons is another characteristic of IBD,[1] we characterized the infiltrates in the colons of DSS treatment mice using an immunohistochemical assay. We found that the infiltration of macrophages was dramatically reduced in the colons of the CTRP4-tg mice (Figure 3g).

IL-6 is downregulated in CTRP4-tg mice during CAC tumorigenesis

Certain pro-inflammatory cytokines including IL-6 and IL-11 are critical for the proliferation and survival of IECs.[29] Repetitive DSS treatment may result in chronic colitis and overproduction of inflammatory cytokines, which lead to IEC proliferation and transformation. We therefore measured the production of pro-inflammatory cytokines in the colons of mice on day 100 after the injection of AOM and three cycles of DSS treatment using a Cytometric Bead Array Kit (BD Biosciences). We found that the expression of IL-6 was markedly downregulated in the CTRP4-tg mice but that the levels of IL-1β and TNF-α, which were dramatically increased in the WT mice during acute colitis, were not clearly altered during CAC tumorigenesis (Figure 4a). We then analyzed the signals downstream of IL-6 in total colon lysates from the WT and CTRP4-tg mice via western blotting and found that the activation of Akt, ERK and STAT3 was reduced in the CTRP4-tg mice. Additionally, the total STAT3 protein level was slightly decreased in the CTRP4-tg mice during CAC (Figure 4b). Next, we analyzed cell proliferation. We found that the CTRP4-tg mice displayed far fewer cells that were immunopositive for Ki67, a proliferation marker, indicating a lower degree of proliferation in these mice (Figure 4c).

rhCTRP4 suppresses the activation of NF-κB/P65 and STAT3 and the production of pro-inflammatory cytokines in macrophages

The immune cells in the lamina propria of the intestine play important roles during intestinal injury, and macrophages are one of the most important cell types. We investigated whether CTRP4 had any effects on the macrophages. We first pre-treated the BMDMs with 10 or 100 ng/ml eukaryotic rhCTRP4 protein for 2 h and then stimulated them with LPS for 30 min. We found that the LPS-induced STAT3 and NF-κB/P65 activation was downregulated in the pre-treated BMDMs (Figure 5a). Next, we measured the pro-inflammatory cytokine production by the BMDMs using ELISAs. We found that the addition of rhCTRP4 alone did not promote TNF-α and IL-6 production by the macrophages. However, the LPS-stimulated expression of TNF-α and IL-6 was inhibited by the addition of the eukaryotic rhCTRP4 protein, whereas, consistent with our *in vivo* results, the expression of IL-10 was not affected (Figure 5d). Remarkably, the addition of 10 ng/ml rhCTRP4 to the BMDMs resulted in a greater decrease in the pro-inflammatory cytokine expression and NF-κB/P65 and STAT3 activation than that induced by 100 ng/ml rhCTRP4, which indicated that the optimal concentration of CTRP4 on macrophages was only 10 ng/ml. The kinetic features of CTRP4 were similar to those of classical cytokines. Specifically, many cytokines show bell-shaped dose-response curves with optima at low concentrations.[30] This result was also consistent with the results of our previous study, in which we found that 4 ng/ml rhCTRP4 was more effective than 40 ng/ml rhCTRP4 in HepG2 cells.[8]

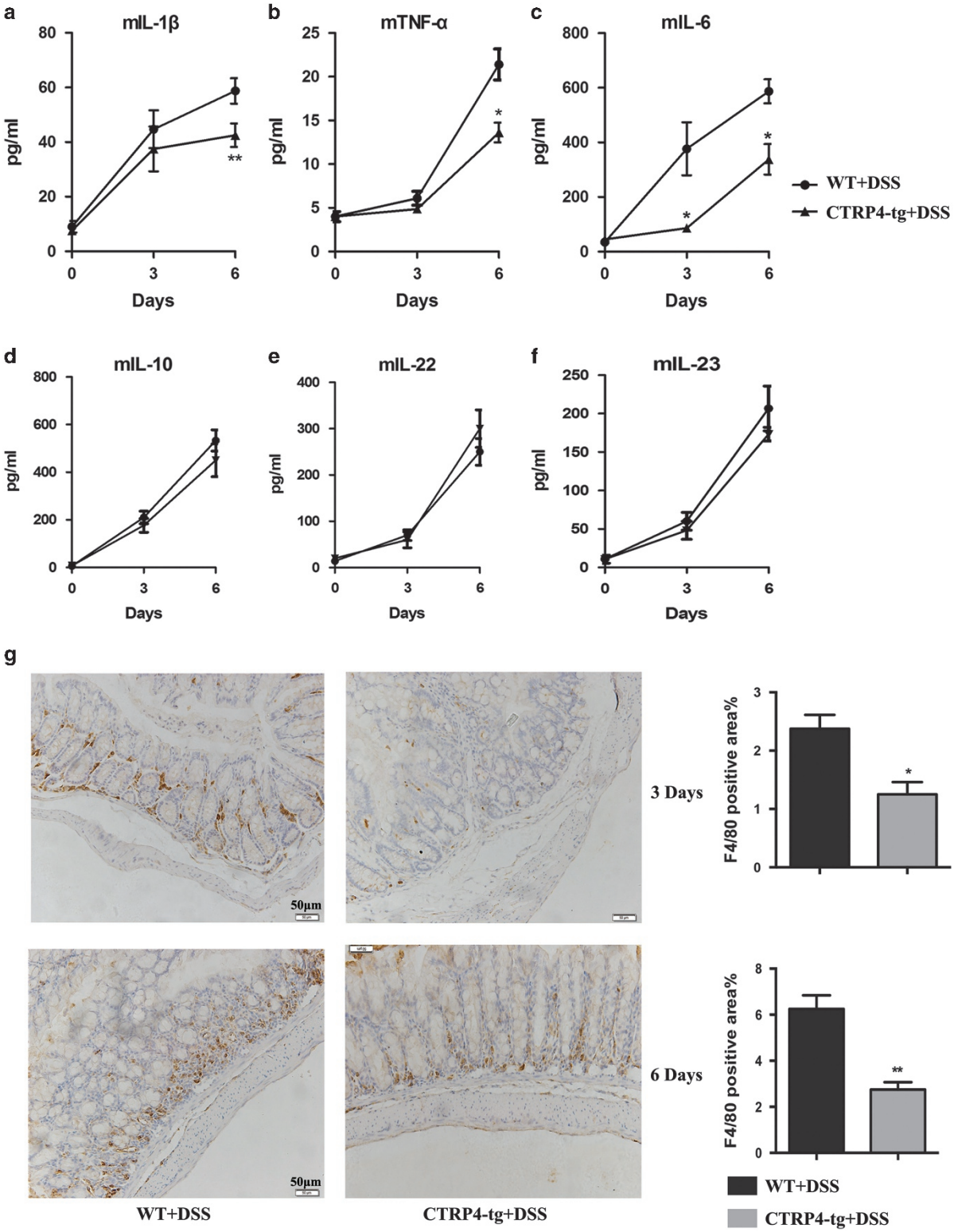

Figure 3 The production of pro-inflammatory cytokines and the infiltration of macrophages in the colons of the CTRP4-tg mice were decreased after DSS treatment. (**a**–**f**) The mice were killed before the DSS treatment or 3 or 6 days after the DSS treatment, and the colons were washed thoroughly in PBS and then incubated in RPMI 1640 for 24 h. The supernatants were collected, and the levels of mIL-1β, mIL-6, mTNF-α, mIL-10, mIL-23 and mIL-22 were measured using ELISAs. The results are representative of at least four experiments and are presented as the means ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$; $n=6$. (**g**) Colon sections were stained with F4/80, and the macrophage infiltration was analyzed and quantified. The data represent the means ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$; $n=6$.

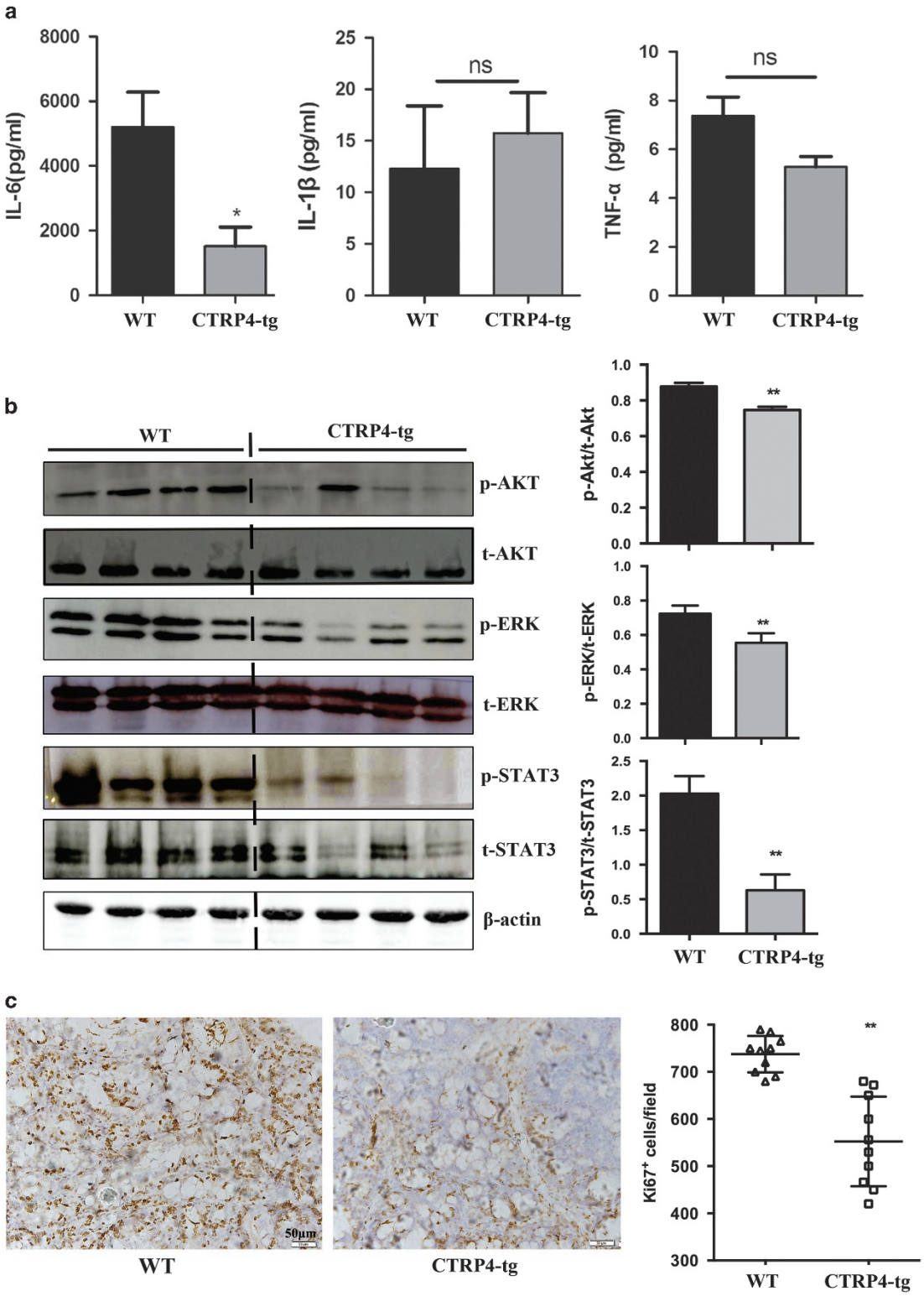

Figure 4 IL-6 is downregulated in the CTRP4-tg mice during CAC tumorigenesis. (**a**) The IL-6, IL-1β and TNF-α levels in the supernatants of the CAC tissues after culture for 24 h were analyzed using a Cytometric Bead Array Kit (BD Biosciences) according to the manufacturer's instructions. The CAC tissues were treated as outlined in Figure 3a. The results are representative of three experiments and are presented as the means ± s.e.m.; $^{**}P<0.01$, $n=5$-6. (**b**) Colon tumor lysates were analyzed via western blotting (one mouse per lane) using the indicated antibodies. The intensities of specific bands were quantified using the Image J software. The results are representative of three experiments and are presented as the means ± s.e.m.; $^{**}P<0.01$, $n=4$. (**c**) Colon tumor sections were stained with a Ki67 antibody, and the Ki67-positive cells were quantified. The data are shown as the means ± s.e.m.; $^{**}P<0.01$, $n=10$.

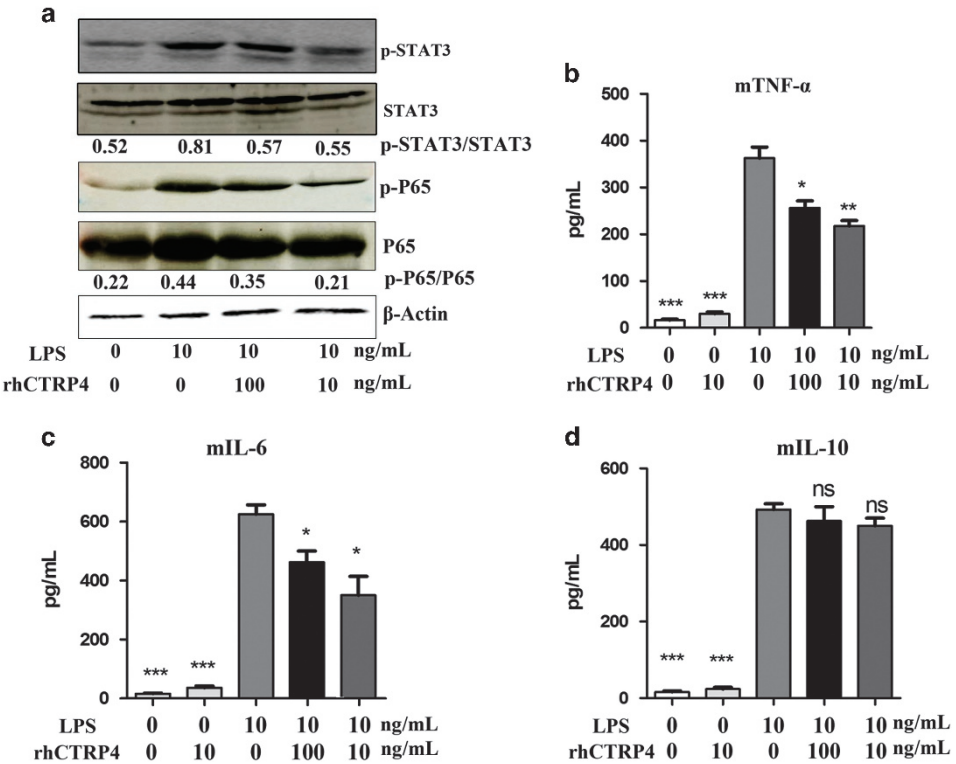

Figure 5 rhCTRP4 inhibits the production of pro-inflammatory cytokines and the activation of NF-κB/P65 and STAT3 in macrophages. (**a**) The BMDMs were pre-stimulated with eukaryotic rhCTRP4 protein for 2 h and then stimulated with LPS for 30 min. The cell lysates were analyzed via western blotting with the indicated antibodies, and the intensities of specific bands were quantified using the Image J software. The figures are representative of three experiments. (**b**–**d**) The BMDMs were treated with LPS, LPS plus rhCTRP4 or rhCTRP4 alone at the indicated doses for 20 h. The supernatants of the cell cultures were collected, and the production of mIL-6, mTNF-α and mIL-10 was measured by ELISA. The results are representative of at least four experiments. The means ± s.e.m. (error bar) were calculated from four independent experiments; $*P<0.05$ and $**P<0.01$.

Ctrp4 is upregulated during DSS-induced colitis, and rhCTRP4 ameliorates DSS-induced colitis in mice

A previous study demonstrated that CTRP4 and IL-6 are mutually upregulated, that is, that IL-6 could stimulate CTRP4 expression and that CTRP4 could also induce IL-6 production in HepG2 cells.[8] We characterized the serum levels of CTRP4 during DSS-induced acute colitis in the WT mice and found that the expression of endogenous CTRP4 was greatly upregulated following the DSS treatment compared with treatment with water (Figure 6a). We then investigated the function of the rhCTRP4 protein *in vivo*. The WT mice were treated with 3% (m/v) DSS as described above, and 10 μg of prokaryotic rhCTRP4 was administered intraperitoneally every day. Compared with the mice that received PBS, the mice that received rhCTRP4 demonstrated less body weight loss and lower DAIs (Figures 6b and c). Furthermore, macroscopic examination of the colons at day 9 revealed longer colons in the mice that received rhCTRP4 (Figure 6d). In addition, the ELISA assays revealed that the production of IL-6 and TNF-α was reduced in the colons of the mice that received the prokaryotic rhCTRP4 but that the IL-1β levels were similar (Figure 6e). Immunoblot analysis of the colon lysates demonstrated that the phosphorylation of STAT3 was downregulated in the rhCTRP4-treated mice (Figure 6f). Finally, the infiltration of macrophages, detected using IHC staining with anti-F4/80 antibody, was also decreased (Figure 6g). Taken together, these data show that treatment with rhCTRP4 protected against the mice from DSS-induced acute colitis *in vivo*.

DISCUSSION

It has been widely recognized that chronic inflammation is associated with many types of cancer.[31,32] AOM/DSS is widely used to investigate inflammation-associated cancers, and DSS is a critical component of the AOM/DSS experimental protocol. DSS induces epithelial damage and inflammation. IECs are important components of the intestinal barrier that separate the intestinal lamina propria components from the enteric microbiota and other luminal components.[33,34] Upon DSS treatment-induced damage to IECs, the immune cells in the lamina are exposed to the enteric microbiota and are subsequently activated. This activation results in the overproduction of pro-inflammatory cytokines, including IL-6 and TNF-α, and in the infiltration of leukocytes into the intestinal mucosa.[34] IL-6 is a critical cytokine in colitis and CAC tumors that mediates the inflammatory response and promotes IEC survival and proliferation.[11,35] The ablation of IL-6 in mice reduces the CAC induced by treatment with DSS/AOM.[6] Our current study confirmed this finding by demonstrating that the

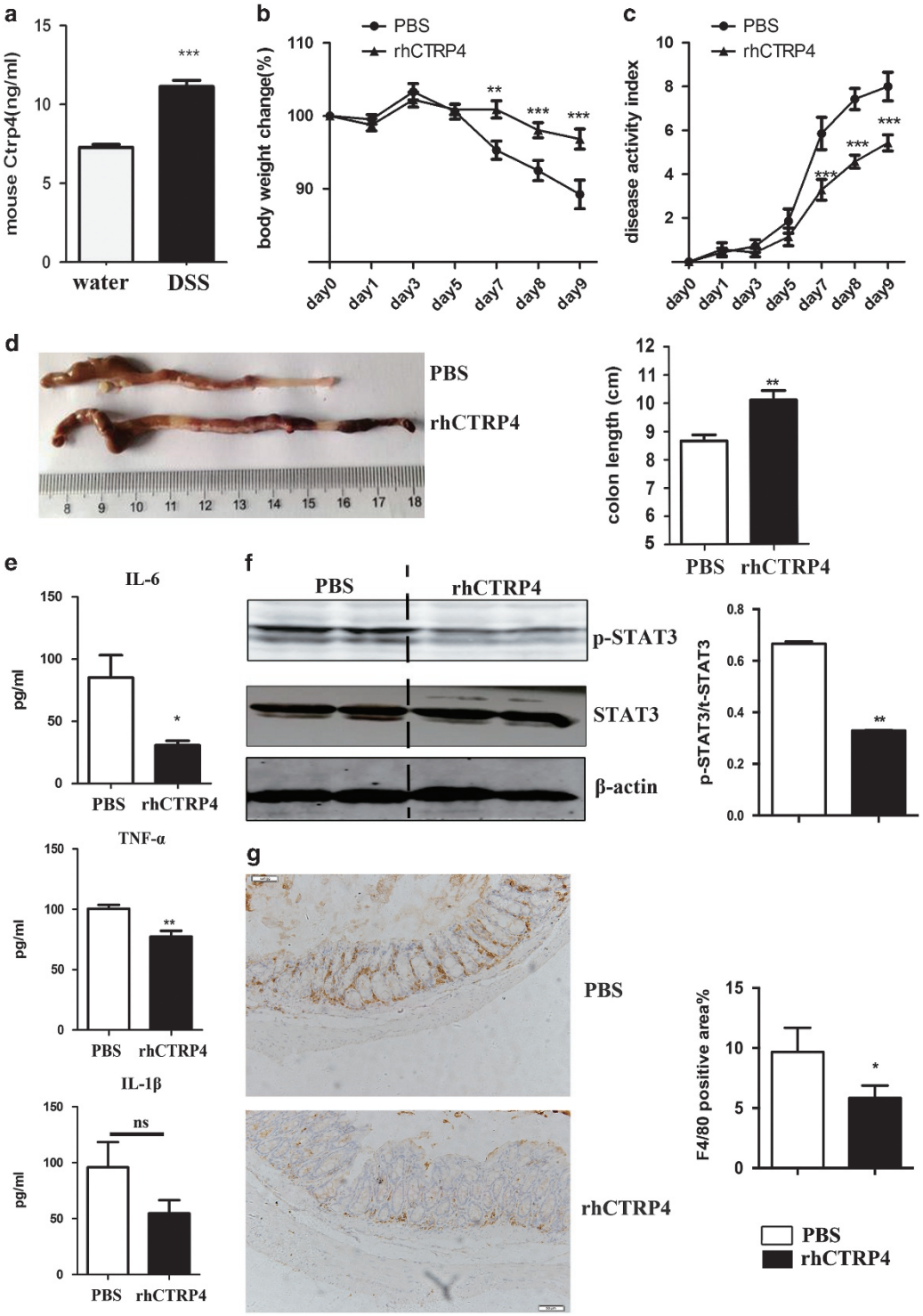

Figure 6 Ctrp4 is upregulated during DSS-induced colitis, and rhCTRP4 ameliorates DSS-induced colitis in mice. (**a**) Two groups of WT mice were treated with either water or 3% DSS, respectively. All mice were killed on day 6, and the endogenous Ctrp4 in the serum was measured by ELISA. (**b**–**e**) The WT mice were administered 3% DSS via their drinking water and separated into two groups. PBS or prokaryotic rhCTRP4 protein was injected intraperitoneally once each day. Weight loss was measured every day and expressed as the percentage of the initial body weight (**b**). The DAI (**c**) and colon length (**d**) were measured and analyzed on day 9. The colons were washed thoroughly in PBS and then incubated in RPMI 1640 for 24 h. The supernatants were collected, and the levels of IL-1β, IL-6 and TNF-α were measured using ELISAs (**e**). (**f**) Colon lysates were analyzed via western blotting (one mouse per lane) with the indicated antibodies. The intensities of specific bands were quantified using Image J software. (**g**) Colon sections were stained with F4/80, and the macrophage infiltration was analyzed and quantified. The results are representative of three experiments and are presented as the means ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $n=6$.

CTRP4-tg mice exhibited milder DSS-induced acute colitis, developed fewer colorectal tumors and expressed lower levels of IL-6 in the colon than did the WT mice. IL-6 acts by binding to its gp-130-associated receptor to activate three signaling pathways, specifically the Shp2-Ras-ERK, JAK1/2-STAT3 and PI3K-Akt-mTOR pathways, through which it promotes IEC survival and proliferation.[36] Of these three signaling pathways, STAT3 is the most important IL-6 effector during CAC induction, and highly phosphorylated STAT3 is observed not only in the mouse model of colitis but also in the mucosa and the lamina propria of human IBD patients.[33,37] The deletion of STAT3 in the IECs results in an enhanced severity of acute colitis in DSS-exposed mice, but this mutation reduces the CAC tumorigenesis by suppressing the IEC proliferation.[6] Consistent with the effects on the production of IL-6 in the colon, the phosphorylation of ERK, STAT3 and Akt in the colon was downregulated in the CTRP4-tg mice. Furthermore, the total STAT3 protein level was slightly decreased, and far fewer Ki67-positive cells were detected in the colon tumors of CTRP4-tg mice. Taken together, these findings indicate that the expression of CTRP4 in mice suppresses IL-6 production, protecting these mice from DSS-induced acute colitis and inhibiting the CAC tumorigenesis induced by treatment with DSS/AOM.

Among the immune cell types, myeloid cells are believed to play an important role in CAC tumorigenesis. These cells often infiltrate the tumor microenvironment and produce large amounts of inflammatory cytokines and chemokines that stimulate the proliferation of premalignant IECs generated during the early stages of CAC tumorigenesis.[38,39] Furthermore, myeloid cells are the primary source of IL-6 during the early stages of CAC tumorigenesis.[37] DSS-induced colitis is also believed to be mediated by myeloid cells on the basis that mice subjected to T- and B-cell ablation do exhibit colitis following treatment with DSS.[40] Inhibiting the production of inflammatory mediators including cytokines such as IL-6 and TNF-α by the myeloid cells prevents IEC proliferation during CAC induction and reduces the tumor frequency and size.[6,41] Our current findings that the infiltration of macrophages into the colons of the CTRP4-tg mice was decreased compared to the WT mice further supported these concepts.

Macrophage polarization has important effects on the progress of inflammatory diseases such as IBD and DSS-induced colitis. In mice, it has been reported that transferring M2 macrophages, which produce less IL-6 and TNF-α upon LPS stimulation, rescues DSS-induced acute colitis whereas transferring M1 macrophages, which produce much more IL-6 and TNF-α upon LPS stimulation, is detrimental.[42] Modulation of the macrophage polarization can greatly affect the severity of inflammatory diseases such as IBD.[43] Many adipokines can modulate the polarization of macrophage. For example, chemerin, which is an adipokine, suppresses macrophage polarization toward M2 and aggravates DSS-induced colitis.[44] In contrast, adiponectin, which is another member of the adipokine family, stimulates the expression of M2 markers in human monocyte-derived macrophages and promotes macrophage polarization toward an anti-inflammatory phenotype.[43] CTRP4 is a member of the adipokine family that is unique in possessing two tandem globular C1q domains. The C1q family comprises over 30 members that are defined by the presence of C1q domain. Both CTRP4 and adiponectin are members of the C1q family.[15] In the current study, we found that CTRP4 suppressed LPS-induced pro-inflammatory cytokine production in macrophages. These macrophages demonstrated markers consistent with the M2 macrophage phenotype, which indicated that CTRP4 may be associated with M2 macrophage polarization.

Our previous study showed that CTRP4 induces IL-6 and activates the STAT3 and NF-κB signaling pathways in HepG2 cells.[8] Although its activities appear to be controversial, CTRP4 is an adipokine whose effects are mediated by receptors on the surfaces of its target cells.[8,22] The functions of cytokines are context-dependent and often vary widely among various target cells and organs. For example, IL-22 is primarily produced by Th17 cells and innate lymphoid cells and acts via IL-22 receptors on the surface of epithelial cells including IECs, liver epithelial cells (LECs) and bronchial epithelial cells. In IECs, IL-22 confers protection via STAT3 during intestinal inflammation.[27,45] However, in LECs, IL-22 is detrimental and can mediate chronic hepatitis and fibrosis, as indicated by the observation that the blockade of IL-22 attenuated the hepatic expression of chemokine (C-X-C motif) ligand 10 and chemokine (C-C motif) ligand 20 (CCL20) and subsequently reduced Th17 recruitment as well as liver inflammation and fibrosis progression.[46]

In summary, our results suggest that CTRP4 plays an important role in colitis and inflammation-associated colon tumorigenesis. CTRP4 may be involved in the regulation of the inflammatory network. Our *in vivo* experiments provide evidence for the efficacy of a novel strategy to treat IBD and CAC, and this study aids in the understanding of the pathogenesis of CAC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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