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A common antimicrobial additive increases colonic inflammation and colitis-associated colon tumorigenesis in mice

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Overline: Potential health risks of consumer antimicrobials.

Competing interests: The authors declare no conflict of interest.

Data and material availability: The data sets generated and analyzed during the current study are included in the Supplementary Materials.

Abstract

Triclosan (TCS) is a high-volume chemical used as an antimicrobial ingredient in over 2,000 consumer products, such as toothpaste, cosmetics, kitchenware, and toys. Here, we report that brief exposure to TCS, at relatively low doses, causes low-grade colonic inflammation, increases colitis, and exacerbates colitis-associated colon cancer in mice. Exposure to TCS alters gut microbiota in mice, and its pro-inflammatory effect is attenuated in germ-free mice. In addition, TCS treatment increases activation of Toll-like receptor 4 (TLR4) signaling *in vivo*, and fails to promote colitis in *Tlr4*$^{-/-}$ mice. Together, our results demonstrate that this widely-used antimicrobial ingredient could have adverse effects on colonic inflammation and associated colon tumorigenesis through modulation of the gut microbiota and TLR4 signaling. Altogether, these results highlight the need to reassess the effects of TCS on human health, and potentially update policies regulating the use of this widely-used antimicrobial.

One Sentence Summary:

The commonly used antimicrobial compound triclosan increases colonic inflammation and colon cancer in mice.

INTRODUCTION

Triclosan (TCS) is a high-volume chemical used as an antimicrobial additive in over 2,000 consumer products, such as toothpastes, cosmetics, clothes, kitchenware, and toys (1). Notably, some toothpastes contain 3,000 ppm TCS as the active ingredient. Every year, several million pounds of TCS are used in the US market. Due to the high-volume use of this compound, the entire population is exposed to TCS at almost every stage of life (1). Indeed, the National Health and Nutrition Examination Survey showed that TCS was detected in ~75% of the urine samples of individuals tested in the US (2). The majority of TCS used in commercial applications is ultimately released into the environment. This has led to widespread environmental contamination. For example, TCS is listed among the top-ten pollutants found in US rivers (1). Due to the ubiquitous presence of TCS, there is increasing concern regarding its impact on the environment and human health.

Although previous studies have shown the potentially toxic effects of TCS at relatively high doses, its overall health impact at normal usage concentrations is still poorly understood (3). For this reason, the regulation of TCS use in consumer products was relatively weak until fairly recently. In September 2016, the US Food and Drug Administration (FDA) issued its first regulation of TCS by removing TCS from over-the-counter handwashing products (4). This decision was mainly based on studies that showed that soaps containing TCS gave no additional health benefit compared to plain soaps (5). However, because this ruling only affects over-the-counter handwashing products, TCS remains approved by the FDA and the Environmental Protection Agency (EPA) for use in hundreds of other consumer products, such as toothpaste (4). This means that a better understanding of the effects of TCS on human health could have a significant impact on regulatory policies and public health. To date, the effects of TCS on colonic inflammation and associated colon tumorigenesis are

unknown. Here, we studied the effects and mechanisms of TCS exposure on these colonic diseases in mouse models.

RESULTS

Exposure to TCS increases plasma concentrations of TCS and its metabolite in mice

We treated mice with a diet containing 10 and 80 ppm TCS for 3 weeks (see diet composition in Table S1), and used liquid chromatography tandem mass spectrometry (LC-MS/MS) to measure the plasma concentrations of TCS and TCS glucuronide (a major metabolite of TCS *in vivo*) (3). LC-MS/MS analysis showed that the plasma concentration of total TCS in mice was 246 ± 30 nM (26 ± 2 nM TCS plus 220 ± 28 nM TCS glucuronide) for 10 ppm and 2,422 ± 345 nM (132 ± 6 nM TCS plus 2,290 ± 339 nM TCS glucuronide) for 80 ppm (Fig. S1). These concentrations were similar to those reported in the plasma of TCS-exposed human volunteers (6, 7). Therefore, we treated mice with 10 and 80 ppm TCS in diet in the animal experiments described below.

TCS exposure causes low-grade colonic inflammation in mice

Treatment of mice with 10–80 ppm TCS in diet for 3 weeks enhanced basal inflammation. Compared with vehicle-treated mice, the mice treated with 80 ppm TCS had increased spleen weight ($P < 0.01$, Fig. 1A), higher plasma concentration of pro-inflammatory cytokine IL-6 ($P < 0.05$, Fig. 1B, but not other cytokines, see Fig. S2), enhanced gene expression of *Il-6* in colon ($P < 0.05$, Fig. 1C), and exaggerated crypt damage in colon ($P < 0.05$, Fig. 1D), illustrating enhanced systemic and colonic inflammation.

TCS exposure increases colitis in mice

We studied the effect of TCS on development of inflammatory bowel disease (IBD) using a well-established dextran sodium sulfate (DSS)-induced IBD model in mice (see scheme of animal experiment in Fig. 2A) (8). We found that exposure to TCS (80 ppm in diet) increased severity of DSS-induced colitis in mice. Compared with vehicle-treated DSS mice, the TCS-treated DSS mice had reduced colon length ($P < 0.05$, Fig. 2B), and increased crypt damage in the colon ($P < 0.01$, Fig. 2C), illustrating enhanced colitis. Pro-inflammatory immune cells and cytokines contribute to the pathogenesis of IBD (9). TCS treatment increased infiltration of immune cells, including leukocytes ($CD45^+$, $P < 0.05$), macrophages ($CD45^+$ $F4/80^+$, $P < 0.01$), and neutrophils ($CD45^+$ $Gr1^+$, $P < 0.05$), into the colon (Fig. 2D, see strategies of gating and cell identification in Fig. S3, and representative FACS images in Fig. S4A). In addition, TCS treatment increased plasma concentration of IL-6 ($P < 0.05$, Fig. 2E, the concentrations of other cytokines were not changed, see Fig. S4B), and gene expression of *Il-6* in the colon ($P < 0.05$, Fig. 2F).

We further studied the effect of low-dose TCS (5–10 ppm in diet) on DSS-induced colitis in mice. We found that exposure to 10 ppm TCS exacerbated DSS-induced colitis, as evidenced by enhanced infiltration of immune cells, including leukocytes ($CD45^+$, $P < 0.001$) and macrophages ($CD45^+$ $F4/80^+$, $P < 0.05$), into the colon (Fig. 2G, see representative FACS images in Fig. S4C), and crypt damage in the colon ($P < 0.01$, Fig. 2H). These observations demonstrate a potent effect of TCS on DSS-induced colitis in mice.

To establish the broader range of validity of the observed pro-colitis actions of TCS, we studied its effect on colitis in a second model, *Il-10*$^{-/-}$ mice (see scheme of animal experiment in Fig. 3A). Exposure to TCS enhanced infiltration of leukocytes (CD45$^+$, $P < 0.05$) and macrophages (CD45$^+$ F4/80$^+$, $P < 0.05$) into colon (Fig. 3B, see representative FACS images in Fig. S5), gene expression of *Il-6* in colon ($P < 0.05$, Fig. 3C), and crypt damage in colon ($P < 0.05$, Fig. 3D). These results showed that exposure to TCS increased colitis in *Il-10*$^{-/-}$ mice, which further validates its pro-IBD effect.

TCS exposure increases colitis-associated colon tumorigenesis in mice

We studied the effect of TCS on colitis-associated colon cancer using an azoxymethane (AOM)/DSS-induced colon cancer model in mice (10) (see scheme of animal experiment in Fig. 4A). Compared with vehicle-treated mice, the mice treated with TCS (80 ppm in diet) had enhanced overall mortality, though the effect was not statistically significant (Fig. 4B). TCS treatment increased average tumor number ($P < 0.05$), average tumor size ($P < 0.05$), and total tumor burden ($P < 0.05$) in mice (Fig. 4C). Consistent with increased colon tumorigenesis, qRT-PCR analysis showed that TCS treatment increased colonic expression of *c-myc*, *axin2*, and *β-catenin* ($P < 0.05$, Fig. S6A), which are genes that promote colon carcinogenesis via the *Wnt* signaling pathway (11). Immunohistochemistry validated that the TCS treatment resulted in an increased expression of β-catenin, as well as proliferation cell nuclear antigen (PCNA), in colon tumors ($P < 0.05$, Fig. 4D). Immune cells and cytokines are important mediators of tumor inflammation (9). TCS treatment increased the infiltration of immune cells including leukocytes (CD45$^+$, $P < 0.01$), macrophages (CD45$^+$ F4/80$^+$, $P < 0.01$), and neutrophils (CD45$^+$ Gr1$^+$, $P < 0.01$) into colon tumors (Fig. 4E, see representative FACS images in Fig. S6B). TCS treatment also increased plasma concentrations of IL-6 and TNF-α (Fig. 4F), and gene expression of *Il-6* and *Tnf-α* in colon tumors (Fig. 4G). Together, these results demonstrate that TCS exposure increased colon tumorigenesis and tumor inflammation.

We further tested the effect of low-dose TCS on AOM/DSS-induced colon cancer in mice. Treatment with TCS (10 ppm in diet) increased tumor number, average tumor size, and total tumor size, though the effects were not statistically significant (Fig. 4H). TCS treatment increased concentration of TNF-α in plasma ($P < 0.05$, Fig. 4I), gene expression of *Tnf-α* in colon ($P < 0.01$, Fig. 4J), and expression of β-catenin and PCNA in colon ($P < 0.05$, Fig. S7). These results demonstrate a potent effect of TCS on colitis-associated colon tumorigenesis.

TCS exposure promotes colonic inflammation through gut microbiota

Gut microbiota contributes to colonic inflammation (12). To explore the action mechanisms of TCS, we studied the impact of TCS exposure on the gut microbiota. After the mice were treated with TCS (80 ppm in diet) for 3 weeks, there was no significant change of total fecal microbial biomass, as analyzed using fecal DNA as a proxy marker (Fig. S8A) (13). 16S ribosomal RNA (rRNA) sequencing showed that TCS treatment decreased α diversity of the microbiota as assessed by PD-whole tree analysis ($P < 0.01$, Fig. 5A) and modulated β diversity of the microbiota as assessed by principal coordinate analysis ($P = 0.002$, Fig. 5B). In addition, TCS exposure altered composition of the microbiota (Fig. 5C-D and Table S2–

3). Notably, TCS treatment caused a ~75% reduction in the abundance of *Bifidobacterium*, which has been shown to have anti-inflammatory effects ($P < 0.05$, Fig. S8B and Table S3) (14).

Since TCS reduced the abundance of *Bifidobacterium in vivo*, we studied whether TCS could directly inhibit the growth of *Bifidobacterium in vitro*. We cultured *Bifidobacterium infantis 272*, which is a gut bacterium with known anti-colitis effect (14), under anaerobic conditions. We found that, compared with vehicle control (DMSO), treatment with 100 nM TCS inhibited ~30% of *B. infantis 272* growth ($P < 0.001$, Fig. S9). This suggests a potential direct effect of TCS on gut bacteria *in vitro*.

To examine the role of the gut microbiota in the actions of TCS, we studied the effects of TCS on basal inflammation in conventionally-raised and germ-free mice. To this end, we maintained conventionally-raised and germ-free mice on an irradiated diet, and treated them with TCS (80 ppm, w/v) or vehicle via drinking water for 3 weeks. Two-way analysis of variance (ANOVA) analysis showed that there was a significant interaction between mouse type (conventionally-raised versus germ-free mice) and treatment (TCS versus vehicle) on inflammation ($P < 0.05$). In the conventionally-raised mice, TCS treatment caused low-grade colonic inflammation. Enhanced colon length reduction (Fig. 6A), expression of *Il-6* in colon (Fig. 6B), concentration of IL-6 in plasma (Fig. 6C), and crypt damage (Fig. 6D) were found. These observations were similar to the results observed via administration of TCS through the diet (Fig. 1), some variations could be caused by differences in administration method of TCS (diet versus drinking water) or in diet (un-irradiated versus irradiated diet). In contrast, TCS treatment had no effect on basal inflammation in germ-free mice (Fig. 6). These results confirm that the gut microbiota contributes to the pro-inflammatory effect of TCS *in vivo*.

TCS exposure promotes colitis through Toll-like receptor 4 (TLR4) signaling

TLRs are important mediators of innate immunity, and are involved in regulating how host cells interact with the gut microbiota (12). Using a TLR4 reporter cell line (HEK-Blue mTLR4), we tested the potential of plasma from the TCS- or vehicle-treated DSS mice to activate TLR4. The reporter assay showed that, compared with the plasma from vehicle-treated DSS mice, the plasma from TCS-treated DSS mice showed enhanced activation of TLR4 ($P < 0.001$, Fig. 7A). Consistent with the reporter assay result, we found that the plasma from TCS-treated DSS mice had a higher concentration of lipopolysaccharide (LPS), which is a well-known ligand of TLR4 (15) ($P < 0.05$, Fig. 7B). In addition, qPCR analysis of *16S rRNA* gene showed that the TCS-treated DSS mice had higher abundance of bacterial DNA in blood ($P > 0.05$) and liver ($P < 0.05$) (Fig. 7C). Together, these results showed that exposure to TCS increased activation of TLR4 *in vivo*, in part through increasing the circulating concentrations of bacterial products.

Previous research has suggested that an increased circulating concentration of bacterial products could be caused by a failure in intestinal barrier function (16). We assessed the effect of TCS on *in vivo* intestinal barrier function, using a FITC-dextran-based intestinal permeability assay. We found that TCS exposure increased leakage of the orally administered FITC-dextran from the gut into the circulation ($P < 0.05$, Fig. 7D). This

suggests that TCS exposure increases intestinal permeability. We next tested whether TCS treatment alters the expressions of colonic proteins critical for maintaining intestinal permeability, including occludin, tight junction protein-1 (ZO-1), trefoil factor-3 (TFF3), and mucin-3 (MUC3) (16). The qRT-PCR analysis showed that only the expression of *Occludin* was reduced by TCS treatment ($P < 0.05$, Fig. 7E). Together, these results show that in the DSS colitis model, TCS treatment disrupted *in vivo* intestinal barrier function, which led to enhanced translocation of bacterial products from the gut to the systemic circulation and activation of TLR4 *in vivo*.

To determine the roles of TLR4 in the pro-colitis effect of TCS, we studied the comparative effects of TCS on DSS-induced colitis in *Tlr4*$^{-/-}$ and wild-type (WT) control mice. Two-way ANOVA analysis showed that there was a significant interaction between mouse type (*Tlr4*$^{-/-}$ versus WT mice) and treatment (TCS versus vehicle) on DSS-induced colitis ($P < 0.05$). Notably, TCS treatment increased immune cells and gene expression of *Il-6* in colon of WT mice, but such effects were not observed in *Tlr4*$^{-/-}$ mice (Fig. 8). These results confirm that the pro-colitis effect of TCS *in vivo* is partially dependent on TLR4.

Discussion

TCS is a widely-used antimicrobial ingredient and is incorporated into more than 2,000 consumer products (1). Here our central finding is that short-time exposure to low-dose TCS caused low-grade colonic inflammation, and increased colitis and colitis-associated colon cancer in mice, which suggests a potential adverse effect of this compound on gut health. We found that, after mice were chronically exposed to 10–80 ppm TCS via diet, the concentrations of TCS in mouse plasma were comparable or within several folds of the concentrations reported in the plasma of TCS-exposed human volunteers (6, 7). This supports the idea that the observed pro-colitis and pro-neoplastic effects of TCS in animal experiments could be mimicking responses in human exposure to TCS. In addition, we performed a relatively short-time treatment (several weeks) in our animal experiments; long-term exposure to TCS at lower concentrations might also induce adverse effects on gut health. This is important because humans could be exposed to TCS for many years by using various widely available personal care products. We do acknowledge the challenges associated with using animal models to study human exposure to consumer antimicrobials. The accurate assessment of exposure and absorption of TCS in human populations is largely unknown; and there could be substantial inter-individual variations in exposure and metabolism to TCS (3). In addition, a limitation of this study is that we treated mice with TCS via diet or drinking water, while humans do not regularly consume TCS, though TCS could be orally absorbed when using TCS-containing toothpaste (6, 7). Currently the development of policies for the regulation of TCS is an intensively debated topic (3, 4). Our study suggests an urgent need to further evaluate the impact of TCS exposure on gut health in preparation for the potential establishment of regulatory policies for this widely-used antimicrobial ingredient.

We demonstrated that the gut microbiota contributes to the pro-inflammatory effects of TCS. We showed that a 3-week exposure of TCS (80 ppm in diet) caused low-grade colonic inflammation, and this phenotype was associated with reduced diversity of the gut

microbiota and decreased abundance of beneficial gut bacteria such as *Bifidobacterium*. We further showed that the enhancing effect of TCS on basal inflammation was abolished in germ-free mice, which confirms that gut microbiota is required for the biological effect of TCS. Our results are largely in agreement with recent studies showing that exposure to low-dose TCS modulated the gut microbiota in fathead minnows (17), zebrafish (18), mice (19), and rats (20). Notably, a recent human study showed that routine usage of TCS-containing toothpaste modulated the gut microbiota in humans (21). Inflammation is a hallmark of many metabolic diseases (22). Given our findings that exposure to TCS increases basal inflammation *in vivo*, it would be important to test whether exposure to TCS has any detrimental effects on metabolic diseases.

TLRs are important mediators of innate immunity and regulate how host cells interact with the gut microbiota (12). Our results support a model whereby, during DSS-induced colitis, exposure to TCS disrupted *in vivo* intestinal barrier function, leading to enhanced translocation of TLR4 ligands (such as LPS and other bacterial products) from the gut into the circulation and increased activation of TLR4 signaling *in vivo*. The pro-colitis effect of TCS was abolished in *Tlr4*$^{-/-}$ mice, validating the importance of TLR4 in the biological action of TCS. Previous studies support that TLR4 promotes colitis and colitis-associated colon tumorigenesis (23). Together, these results suggest that TCS increases colitis through activation of TLR4 signaling. Here our data showed that when treated with 2% DSS in drinking water for 6 days, vehicle-treated WT mice and *Tlr4*$^{-/-}$ mice showed a similar degree of colitis. These results are in agreement with a previous study using a similar DSS treatment protocol in WT and *Tlr4*$^{-/-}$ mice (24). Some previous studies showed that, compared to WT mice, *Tlr4*$^{-/-}$ mice had attenuated DSS-induced colitis (25–27), or enhanced colitis (28). These inconsistent results could be in part due to differences in treatment conditions or in mouse strains. High colonic expressions of TLR4 are usually observed in IBD and colon cancer patients (29, 30). Our results suggest that individuals with, or prone to, IBD and colon cancer might be more susceptible to the adverse effects of TCS.

In conclusion, our results suggest that TCS, a widely-used consumer antimicrobial, could cause adverse effects on colonic inflammation and colon cancer through modulation of the gut microbiota and TLR4 signaling. Further studies are urgently needed to better characterize the effects of TCS exposure on gut health in order to establish science-based policies for the regulation of this antimicrobial compound in consumer products. Besides TCS, many other high-volume chemicals are also used as antimicrobial ingredients in various personal care and household products. Compared with TCS, less is known about the health effects of other antimicrobials. Our research suggests the importance of evaluating the potential toxic effects of other antimicrobials on gut microbiota and associated colonic diseases in order to develop safe antimicrobials with minimal adverse impact on human health and the environment.

Methods and Materials

Study design

The aim of the study was to define the effects and mechanisms of TCS on colonic inflammation and colitis-associated colon cancer in mice. First, we measured the plasma concentrations of TCS and its metabolite after the mice were chronically exposed to TCS, in order to determine the optimal dose of TCS in animal experiment to reflect human exposures. Next, we studied the effects of TCS exposure on basal inflammation, DSS-induced colitis, IL-10 knockout-induced colitis, and AOM/DSS-induced colitis-associated colon tumorigenesis in mice. Finally, we explored the roles of gut microbiota and innate immune system in the observed effects of TCS. For each animal experiment, the mice were randomized to receive treatments of TCS or vehicle. The number of mice and the method of statistical analysis are described in the figure legends. Primary data are reported in Table S5.

Animal experiments: All animal experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Amherst (protocol 2014–0007) and the University of Wisconsin-Madison (protocol M005599).

Animal Protocol 1: Effects of TCS on basal inflammation in mice

C57BL/6 male mice (age = 6 weeks) were purchased from Charles River, and maintained on a modified AIN-93G diet (see diet composition in supplemental information Table S1) containing 10–80 ppm TCS (99%, Alfa Aesar) dissolved in polyethylene glycol 400 (PEG 400, EMD Millipore) as vehicle (0.5% in diet, v/w), or vehicle alone, for 3 weeks. At the end of the experiment, the mice were sacrificed, and the blood and colon tissues were collected for analysis of inflammation. The concentrations of TCS and TCS glucuronide in plasma were measured by LC-MS/MS (see LC-MS/MS method in supplemental information).

Animal Protocol 2: Effects of TCS on DSS-induced colitis in mice

C57BL/6 male mice (age = 6 weeks) were maintained on diet containing 5–80 ppm TCS or vehicle throughout the experiment. After 3 weeks on the diet, the mice were treated with 2% DSS (molecular weight in the range of 36–50 kDa, MP Biomedicals) in drinking water for 6–8 days to induce colitis. At the end of the experiment, the mice were sacrificed for analysis of inflammation (see methods in supplemental information).

Animal Protocol 3: Effects of TCS on colitis in *Il-10*$^{-/-}$ mice

Il-10$^{-/-}$ male C57BL/6 mice (B6.129P2-*Il10*^{tm1Cgn}/J, age = 5 weeks) were purchased from the Jackson Laboratory. After acclimation for 1 week in the animal facility, the *Il-10*$^{-/-}$ mice were treated with diet containing TCS (80 ppm in the diet) or vehicle for 12 weeks, then the mice were treated with 200 ppm piroxicam (Sigma-Aldrich) through the diet for 1 week to accelerate development of colitis (31). At the end of the experiment, the mice were sacrificed, and blood and colon tissues were collected for analysis of inflammation.

Animal Protocol 4: Effects of TCS on AOM/DSS-induced colon cancer in mice

C57BL/6 male mice (age = 6 weeks) were maintained on diet containing TCS (10 and 80 ppm in the diet) or vehicle throughout the experiment. After 3 weeks on the diet, the mice were treated with 10 mg/kg AOM (Sigma-Aldrich) via intraperitoneal injection; after 1 week, the mice were given 2% DSS in drinking water for 1 week. At day 50 post the AOM injection, the mice were sacrificed for analysis of colon tumorigenesis (see methods in supplemental information).

Animal Protocol 5: Effect of TCS on *in vivo* intestinal permeability

C57BL/6 male mice (age = 6 weeks) were maintained on diet containing 80 ppm TCS or vehicle throughout the experiment. After 3 weeks on the diet, the mice were treated with 2% DSS in drinking water for 7 days to induce colitis. Then 600 mg/kg body weight of FITC-dextran (70 kDa, Sigma-Aldrich) was administrated to mice by oral gavage. After 4 hours, the mice were euthanized and blood samples were obtained by cardiac puncture and centrifuged at 2,000 g for 10 minutes at 4°C to prepare the plasma fraction. Fluorescence intensity of the plasma was quantified using a Synergy 2 Multi-mode Detection Microplate (BioTek) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. FITC-dextran concentrations in the plasma were determined against a standard curve.

Animal Protocol 6: Effects of TCS on DSS-induced colitis in *Tlr4*$^{-/-}$ and WT control mice

Tlr4$^{-/-}$ male mice (B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ, age = 6–7 weeks) and control WT C57BL/6 mice (C57BL/6J, age = 6 weeks) were purchased from the Jackson Laboratory. The mice were maintained on diet containing 80 ppm TCS or vehicle throughout the experiment. After 3 weeks on the diet, the mice were treated with 2% DSS in drinking water for 6 days to induce colitis. At the end of the experiment, the mice were sacrificed for analysis of colonic inflammation.

Animal Protocol 7: Effects of TCS on gut microbiota in mice

C57BL/6 male mice (age = 6 weeks) were maintained on a modified AIN-93G diet containing 80 ppm TCS or vehicle for 3 weeks. At the end of the experiment, the fecal samples were collected for 16S rRNA sequencing (see sequencing method in supplemental information).

Animal Protocol 8: Effects of TCS exposure on basal inflammation in conventionally-raised and germ-free mice

Conventionally-raised C57BL/6 mice (housed in a conventional animal facility) and germ-free mice (housed in germ-free isolators) were maintained on an irradiated AIN-93G diet (Envigo), and treated with TCS or vehicle via drinking water (80 ppm TCS, w/v, the drinking water was autoclaved and filtered through a 0.22 μm filter) for 3 weeks. At the end of the experiment, the mice were sacrificed, and the blood and colon tissues were collected for analysis of inflammation.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical comparison of two groups was performed using either Student's *t*-test or Wilcoxon–Mann–Whitney test; and comparison of three groups was analyzed by either one-way parametric ANOVA followed by Tukey's post hoc test (using PROC MIXED) or one-way nonparametric ANOVA (PROC NPAR1WAY) followed by Dunn's post hoc test. Analysis of inflammation in germ-free and *Tlr4*$^{-/-}$ mouse experiments (Fig. 6 and Fig. 8) according to mouse type and treatment was performed by two-way ANOVA, followed by Tukey-Kramer's method, and H&E histology data in these experiments was analyzed by two-way ANOVA Poisson Generalized Linear Model, followed by the Tukey-Kramer's multiple comparison method. The statistical analyses were performed using SAS statistical software, and $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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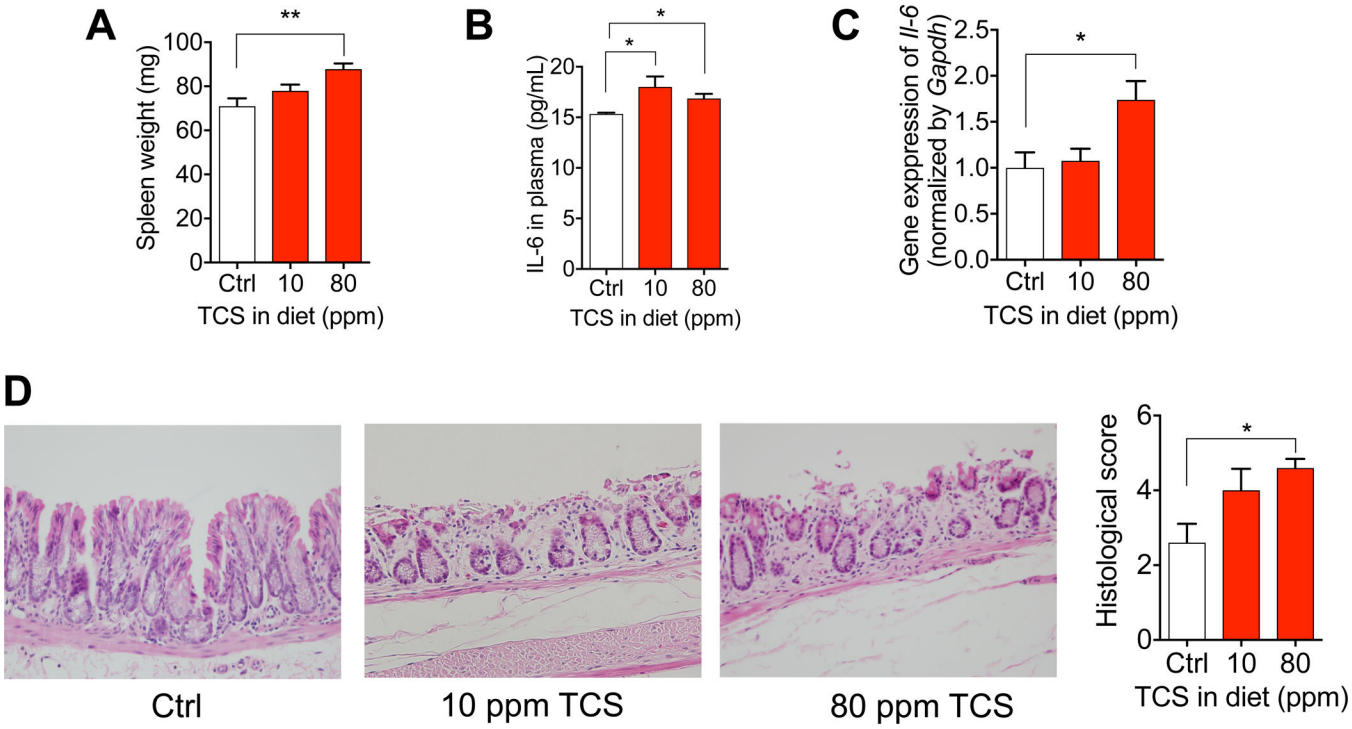

Fig. 1. Exposure to TCS increased basal inflammation in mice.

Mice were treated with 10–80 ppm TCS in diet for 3 weeks. (A) Spleen weight (n = 7 per group). (B) Plasma concentration of IL-6 (n = 6–7 per group). (C) Gene expression of *Il-6* in colon (n = 5–7 per group). (D) H&E staining of colon (magnification 40×, n = 4–5 per group), left; histological score, right. The results are expressed as mean ± SEM, * $P < 0.05$, ** $P < 0.01$, statistical significance was determined using one-way ANOVA.

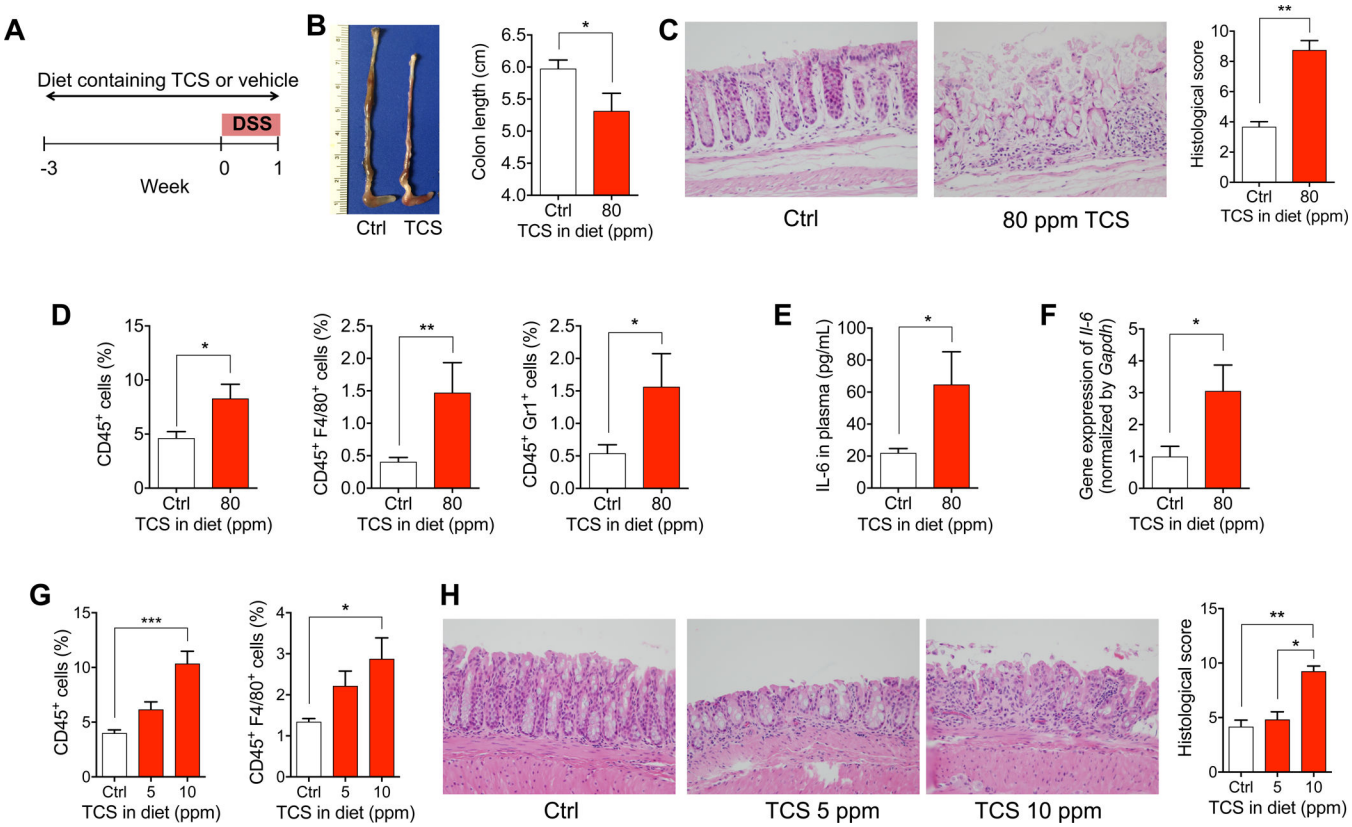

Fig. 2. Exposure to TCS increased DSS-induced colitis in mice.

(A) Scheme of animal experiment. (B) Colon length (n = 6–12 per group). (C) H&E staining of colon, left; histological score, right (magnification 40×, n = 6–7 per group). (D) FACS quantification of immune cells in colon (see representative FACS plots in Fig. S4A, n = 6–11 per group). (E) Concentration of IL-6 in plasma (n = 5–6 per group). (F) Gene expression of *Il-6* in colon (n = 5–8 per group). (G) Effect of low-dose TCS (5–10 ppm in diet) on immune cell infiltration in colon (see representative FACS plots in Fig. S4C, n = 6–8 per group). (H) Effect of low-dose TCS on crypt damage in colon, left; histological score, right (magnification 40×, n = 5–7 per group). The results are expressed as mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, statistical significance of two groups was determined using Student's *t*-test or Wilcoxon–Mann–Whitney test, and comparison of three groups was determined using one-way ANOVA.

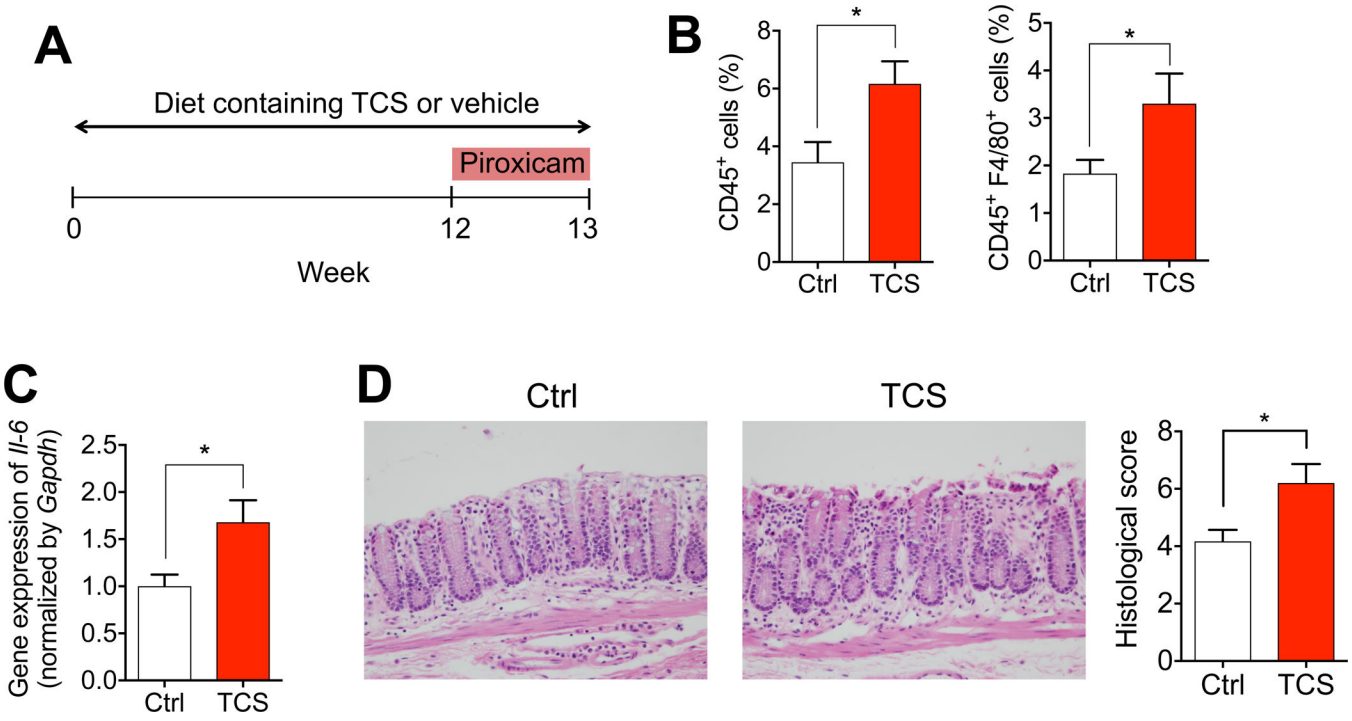

Fig. 3. Exposure to TCS increased colitis in *Il-10*$^{-/-}$ mice.

(A) Scheme of animal experiment. (B) FACS quantification of immune cells in colon (see representative FACS plots in Fig. S5). (C) Gene expression of *Il-6* in colon. (D) H&E staining of colon (magnification 40×), left; histological score, right. The results are expressed as mean ± SEM, * $P < 0.05$, n = 5–6 per group, statistical significance was determined using Student's *t*-test or Wilcoxon–Mann–Whitney test.

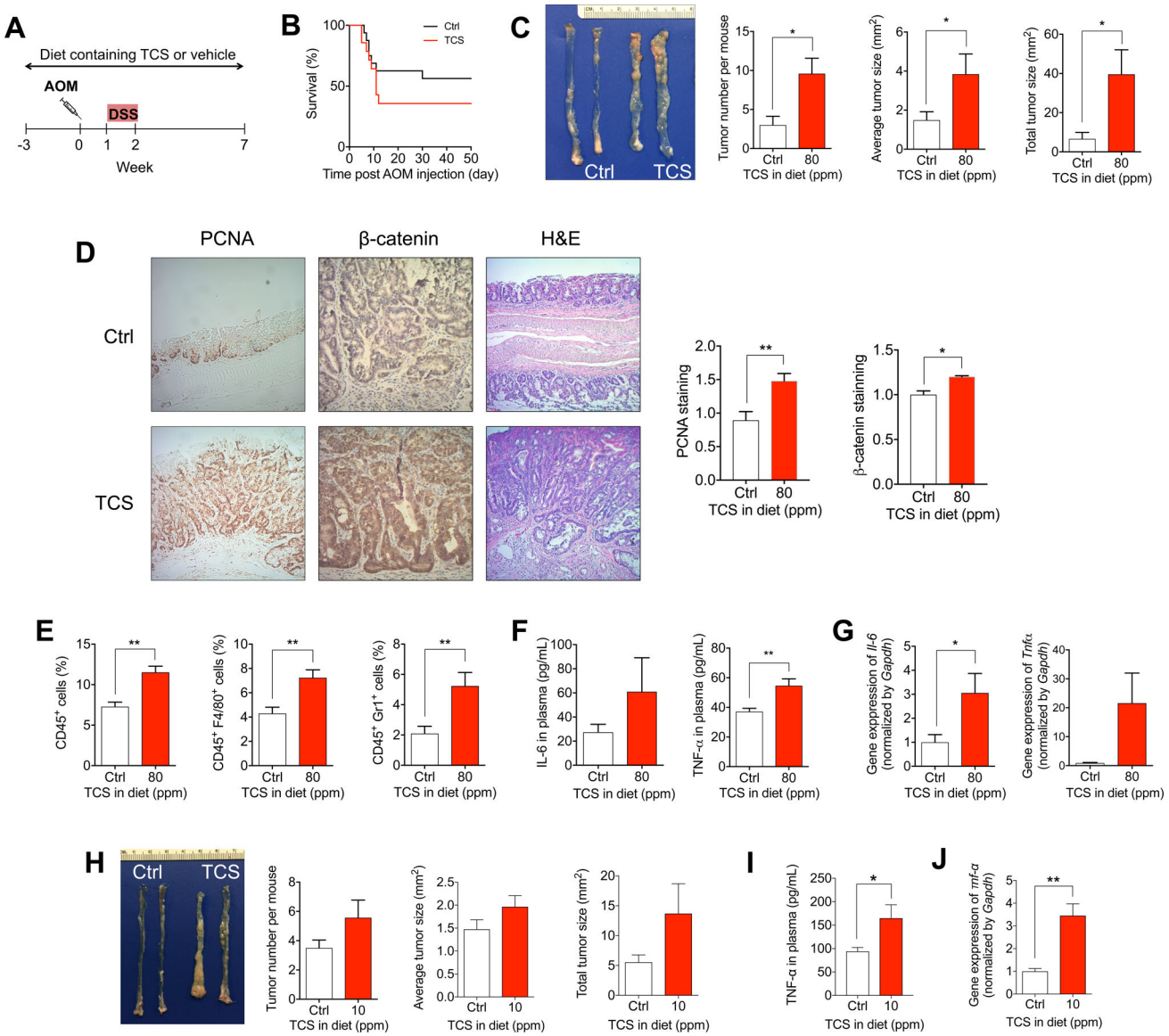

Fig. 4. Exposure to TCS increased AOM/DSS-induced colon tumorigenesis in mice.

(A) Scheme of animal experiment. (B) Survival curve (n = 14–16 per group at day 0). (C) Quantification of colon tumor in mice (n = 5–7 per group). (D) Representative images of immunohistochemical staining of PCNA, β-catenin, and H&E, and quantification of immunohistochemical images (n = 4–5 per group). (E) FACS quantification of immune cells in colon (see representative FACS plots in Fig. S6B, n = 4–7 per group). (F) Concentrations of IL-6 and TNF-α in plasma (n = 5–6 per group). (G) Gene expression of *Il-6* and *Tnf-α* in colon (n = 3–8 per group). (H) Effect of low-dose TCS (10 ppm in diet) on AOM/DSS-induced colon tumorigenesis in mice (n = 12–14 per group). (I) Effect of TCS (10 ppm in diet) on plasma concentration of TNF-α (n = 4 per group). (J) Effect of TCS (10 ppm in diet) on expression of *Tnf-α* in colon (n = 7 per group). The results are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, statistical significance was determined using Student's *t*-test

or Wilcoxon–Mann–Whitney test, and the statistical analysis of survival was determined using Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test.

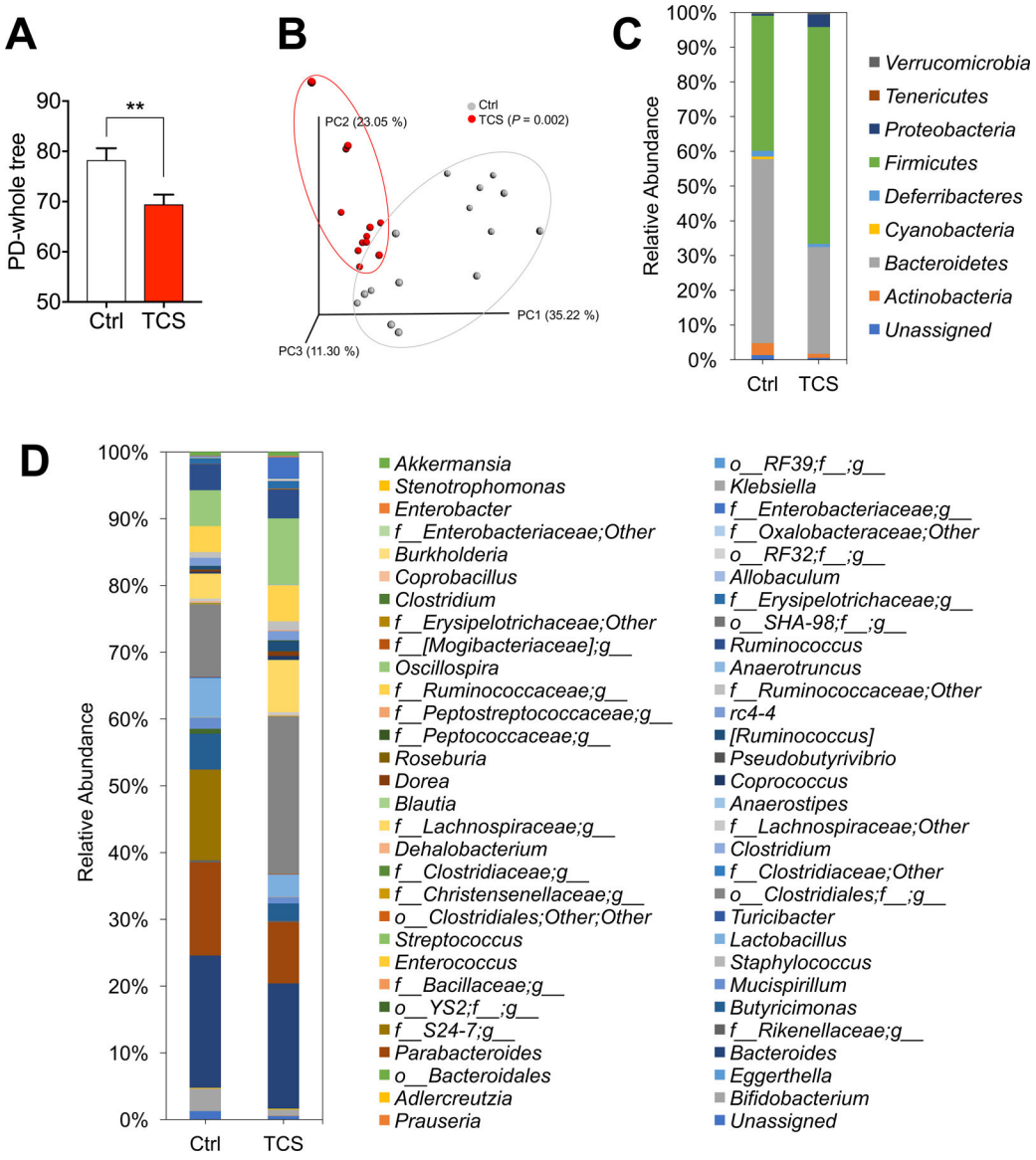

Fig. 5. Exposure to TCS altered gut microbiota in mice.

Mice were treated with 80 ppm TCS in diet for 3 weeks. (A) Effect of TCS on α diversity of fecal microbiota, assessed by PD-whole tree analysis. (B) Effect of TCS on β diversity of fecal microbiota, assessed by principal coordinate analysis. (C) Effect of TCS on composition of the microbiota at phylum levels. (D) Effect of TCS on composition of the microbiota at genus levels. The results are expressed as mean ± SEM, ** $P < 0.01$, n = 12–15 per group, statistical significance was determined using Student's *t*-test or Wilcoxon–Mann–Whitney test.

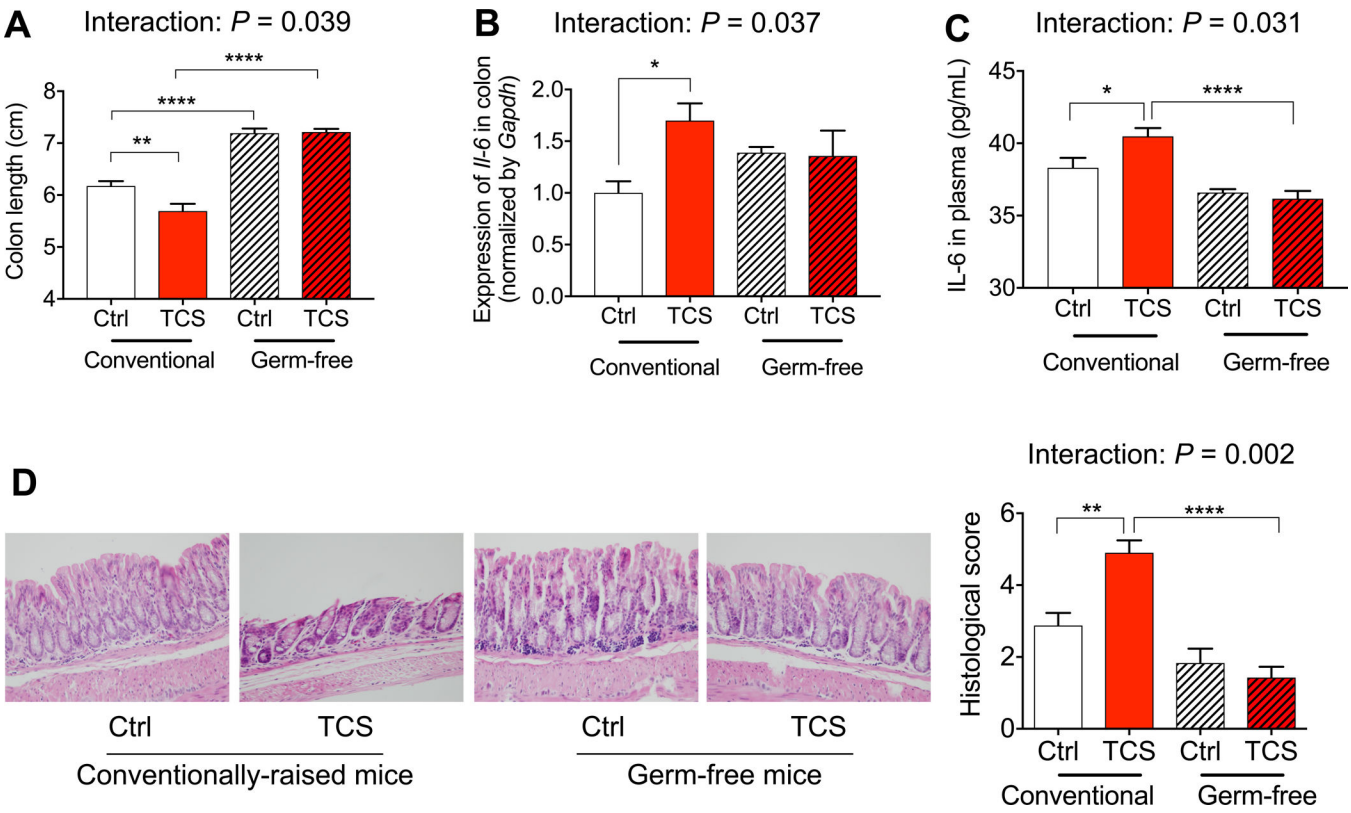

Fig. 6. TCS exposure increased basal inflammation through gut microbiota-dependent mechanisms.

Conventionally-raised and germ-free mice were treated with 80 ppm TCS in drinking water for 3 weeks. (A) Colon length. (B) Expression of *Il-6* in colon. (C) Plasma concentration of IL-6. (D) Colon histology (magnification 40×), left; histological score, right. The results are expressed as mean ± SEM, n = 5–12 per group. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, the statistical significance of the interaction effect between mouse type (conventionally-raised versus germ-free mice) and treatment (TCS versus vehicle) on inflammation was determined by two-way ANOVA.

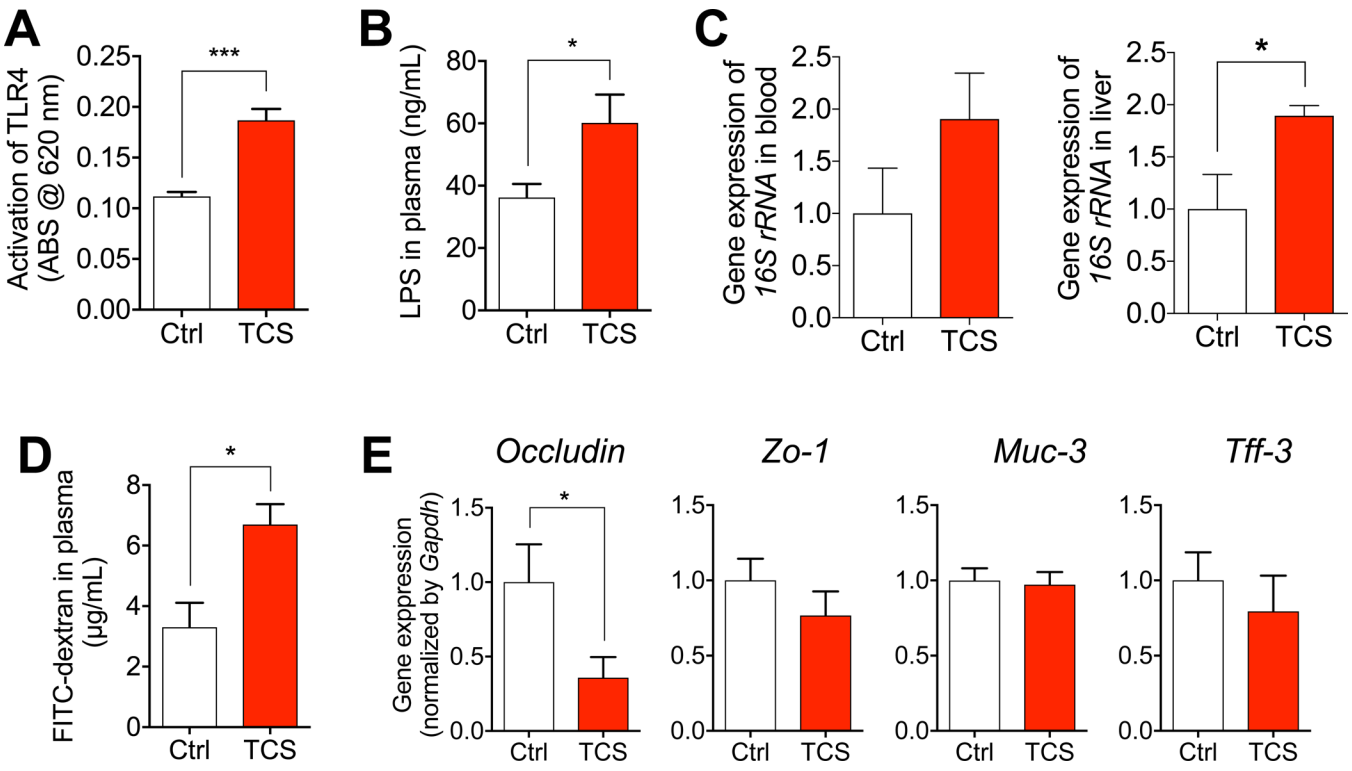

Fig. 7. Exposure to TCS activated TLR4 signaling in the DSS-induced colitis model.

Mice were treated with 80 ppm TCS in diet for 3 weeks, then stimulated with DSS to induce colitis. (A) Effect of the plasma from the TCS- or vehicle-treated DSS mice on activation of TLR4, using a TLR4 reporter cell line (n = 6–9 per group). (B) Plasma concentration of LPS from TCS- or vehicle-treated DSS mice (n = 6 per group). (C) qRT-PCR analysis of *16S rRNA* gene in the blood and liver from TCS- or vehicle-treated DSS mice (n = 4–6 per group). (D) Plasma concentration of FITC-dextran (administered via oral gavage at 4 h before euthanasia) from TCS- or vehicle-treated DSS mice (n = 4–5 per group). (E) qRT-PCR analysis of *Occludin*, *Zo-1*, *Muc-3*, and *Tff-3* in colon of TCS- or vehicle-treated DSS mice (n = 6–11 per group). The results are expressed as mean ± SEM, * $P < 0.05$, *** $P < 0.001$, statistical significance was determined using Student's *t*-test or Wilcoxon–Mann–Whitney test.

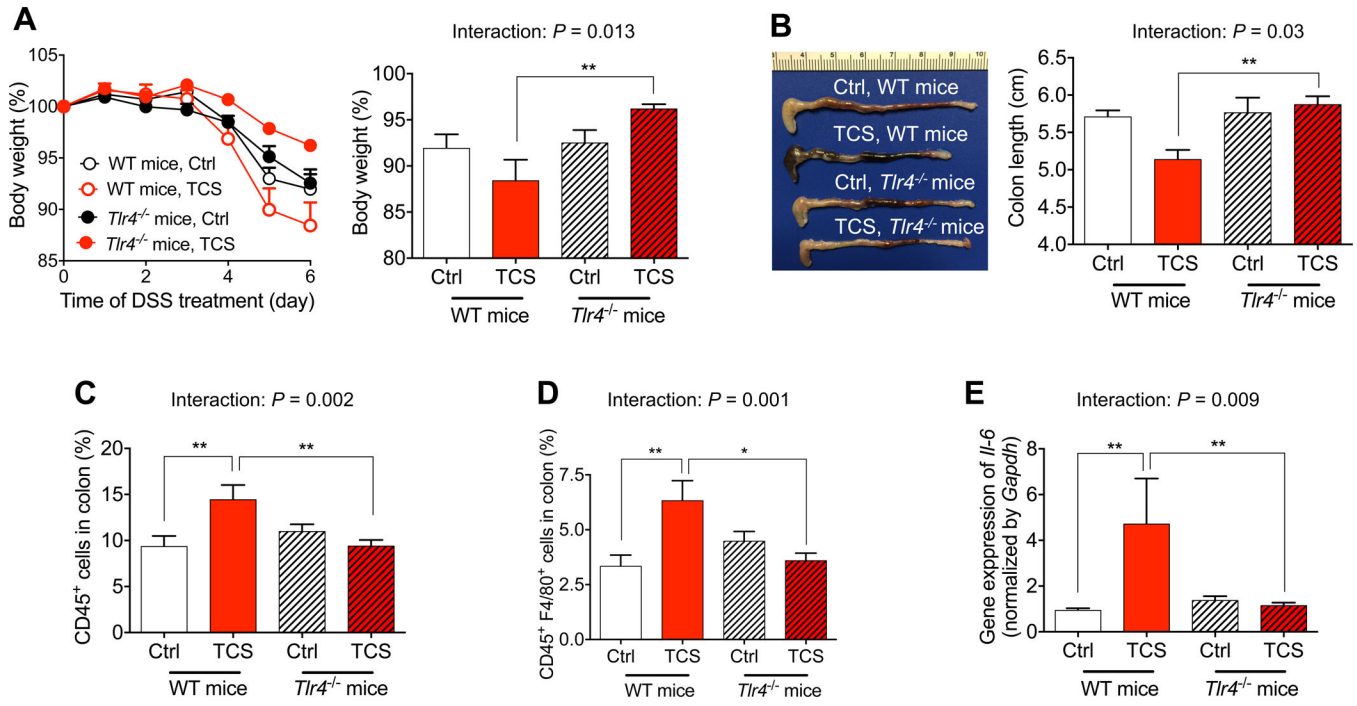

Fig. 8. TCS enhanced DSS-induced colitis through a TLR4-dependent mechanism.

WT and *Tlr4*$^{-/-}$ mice were treated with 80 ppm TCS in diet for 3 weeks, then stimulated with DSS to induce colitis. (A) Body weight. Time-course of mouse body weight after DSS treatment, left; quantification of mouse body weight on the day of euthanasia (day 6 post the initiation of DSS treatment), right. (B) Colon length. (C) FACS quantification of CD45^{+} cells in colon. (D) FACS quantification of CD45^{+} F4/80^{+} cells in colon. (E) Gene expression of *Il-6* in colon. The results are expressed as mean ± SEM, n = 6–10 per group, * $P < 0.05$, ** $P < 0.01$. The statistical significance of the interaction effect between mouse type (*Tlr4*$^{-/-}$ versus WT mice) and treatment (TCS versus vehicle) on colitis was determined by two-way ANOVA.