



Toll-like receptor 4 (*TLR4*) deficiency aggravates dextran sulfate sodium (DSS)-induced intestinal injury by down-regulating *IL6*, *CCL2* and *CSF3*

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Background: Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that causes long-lasting inflammation and ulcers in the human digestive tract. The repair role of *TLR4* in the intestinal epithelium is still unknown.

Methods: By comparing to wild-type (WT) mice, Toll-like receptor 4 (*TLR4*)-knockout mice (*TLR4*-KO) were used as dextran sulfate sodium (DSS)-induced colitis models to explore the role of *TLR4* signaling in intestinal injury. High-throughput RNA-Seq, RT-qPCR and ELISA were performed to screen and verify key differences in gut genes between WT and *TLR4*-KO mice. Functional study of core dysregulated factors was performed in intestinal cell lines.

Results: We found that DSS-induced intestinal injury was aggravated by LPS (*TLR4* agonist) and *TLR4*-KO. When compared to WT mice, *IL6*, *CCL2*, *CSF3*, *IL11*, *Ccnb1*, *Cnd1* and *TNF- α* significantly decreased and *Fas* and *FasL* have increased in the gut of *TLR4*-KO mice. *IL6*, *CCL2*, *CSF3*, *Fas* and *FasL* have all increased in CT-26 cells treated with LPS. Combined with the above data and KEGG enrichment, it can be assumed that *TLR4*-KO might aggravate DSS-induced intestinal damage by attenuating cell cycle, cytokine-cytokine receptor interaction, and Toll-like receptor signaling pathway, and enhancing the apoptosis pathway. In the functional study of core dysregulated factors, it was found that LPS, *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8*, *S100A9* and *Mmp3* have improved viability of colon cancer cell lines and decreased apoptosis rate of mouse colon cancer cells when these were treated with DSS. However, Jo-2 (*Fas* agonistic monoclonal antibody) played the opposite role in colon cancer cells treated with DSS.

Conclusions: *TLR4* had a repairing effect on DSS-induced intestinal damage and it up-regulate *IL6*, *CCL2* and *CSF3*. *Fas* and *FasL* enhanced DSS-induced colon injury in mice, but might have little to do with *TLR4* signaling.

Keywords: Toll-like receptor 4 (*TLR4*); dextran sulfate sodium; *IL6*; *CCL2*; *CSF3*

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Introduction

Inflammatory bowel disease (IBD) is categorized into ulcerative colitis (UC) and Crohn's disease (CD). It seriously disturbs the normal function of the digestive tract (1). IBD pathogenesis is still not clear. Some reports suggest that gut microbiota act as initiators of intestinal inflammation (2). TLRs are sensors for pathogen-associated molecular patterns (PAMPs), and play important role in initiating inflammation (3). *TLR4* is the main mediator of responses to lipopolysaccharide (LPS) *in vitro* and *in vivo* (4). *TLR4/NF-κB* overactivation is believed to lead to uncontrolled immune response in the inflamed intestinal mucosa and to accelerate the progression of IBD (5). However, in our experimental system, *TLR4* signaling might actually play a repairing role during the process of IBD, suggesting that the balancing role of *TLR4* between intestinal injury and repair is important (6). Cario and colleagues (7) suggested that in the process of intestinal injury, intestinal stem cells at the base of the intestinal crypts repopulate the depleted surroundings, in a process known as compensatory proliferation. We hypothesized that the protective role of *TLR4* signaling pathway in DSS-induced colitis is related to this compensatory proliferation response.

Our previous research showed that by upregulating *PGE₂* and *GM-CSF*, *TLR4* and its ligands might be contributing to epithelial repair after DSS-induced intestinal injury (6). However, at the time we did not conduct a detailed study on the specific downstream genes associated with *TLR4* signaling and the alleviation DSS-induced colitis. In this study, we increased the number of WT and *TLR4*-knockout (*TLR4-KO*) mice to test the role of *TLR4* in DSS-induced colitis. High-throughput RNA-Seq analysis was used to perform differential screen of key genes' expression in the guts of WT and *TLR4-KO* mice treated with DSS. Furthermore, we performed qRT-PCR and ELISA to confirm the expression of the genes identified by RNA-Seq analysis. Finally, we validated the key molecular functions using intestinal cell lines and, based on the results, built a key genes interactions network map.

Methods

Mice

Six-week-old wild-type (WT) mice (C57BL/6) weighing approximately 20 g each were purchased from the Chinese Academy of Science (Shanghai, China). Six-week-old *TLR4-KO* mice (8) (C57BL/6, *TLR4* gene deleted) weighing

approximately 20 g each were provided by the Model Animal Research Center of Nanjing University (Nanjing, China). All mice were littermates and were maintained under specific pathogen-free (SPF) conditions in the Animal Center of Naval Medical University (Shanghai, China). Experimental groups were n=5 in intestinal injury evaluation experiments and n=6 in peripheral blood analysis experiments. Numbers of mice used in experimental groups in the survival studies are shown in the respective figures. RNA-Seq analysis was performed on intestinal samples from four pairs of WT and *TLR4-KO* mice. In verification experiments of quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), the number of mice per group was three. This study was approved by an institutional ethics committee and followed the tenets of the Declaration of Helsinki.

Induction of DSS- induced colitis and LPS treatment

For acute colitis model, mice were given different doses (% g/mL) of DSS (40,000 kDa; ICN Biochemicals, Irvine, CA, USA) in drinking water for seven days. Mice were switched to regular drinking water after that (9). Different doses of DSS were prepared based on the experimental purpose. The specific DSS doses used are indicated in each experimental figure. LPS from *Escherichia coli* was purchased from Sigma (St. Louis, MO, USA) and was re-purified. To explore the toxic effects of exogenous LPS, re-purified LPS was dissolved in pyrogen-free PBS (BaiSai, Shanghai, China). The WT mice were injected with LPS at doses of 0.1, 0.5, 2.5 mg/kg body weight for 3 days, and were then given the different doses (% g/mL) of DSS. Control mice were injected with pyrogen-free PBS. In the experiments performed to study how *TLR4* mediates the expression of *IL-6*, *CCL2*, *CSF3*, *Fas*, and *FasL*, *CT-26* cells were treated with 100 ng/mL of LPS 24 hours before performing the measurements.

Analysis of peripheral blood cells

WT and *TLR4-KO* mice treated with 2.5% DSS were eye-bled on day seven using heparin-coated capillary tubes to take blood while the mice were under anesthesia. The blood collected per mouse was 0.5–1.0 mL. The collected blood was transferred to Eppendorf (EP) tubes with K2-EDTA and inverted multiple times. Peripheral blood index was determined by the standard five-classifications hematological analysis at the Animal Center of Naval Medical University. Peroximeter assay channels and basophil assay channels

were used for leukocyte classification. White blood cells classification was performed according to the characteristics of cytochemically stained cells. A dual angle laser method was used in both red blood cells and platelets analyses.

Fecal blood scoring

Intestinal bleeding samples of WT and *TLR4*-KO mice treated with 2.5% DSS were dissected on day 7. Colonic bleeding was determined via stool bleeding score (8) as follows: 0, no gross blood visible anywhere through the entire colon; 1, gross blood present in <1/3 of the colon; 2, gross blood visible in \geq 1/3 but <2/3 of the colon; 3, gross blood visible in \geq 2/3 of the colon.

Pathological analysis

Colon tissues of WT and *TLR4*-KO mice treated with 2.5% DSS were sampled on day seven of treatment. Colon tissue samples were fixed with 10% neutral formalin, paraffin embedded, sectioned at 2–7 μ m thick and stained with haematoxylin and eosin (H&E) (10). Photomicrographs of the desired sections were obtained using a digital research photographic microscope (Thermo Fisher Corporation, USA). Ki67 kits (Dakewe Biotech Corporation, Shenzhen, China) were applied to assess the proliferative capacity of the intestinal crypt epithelial cells (11).

RNA-Seq and KEGG enrichment analyses

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA Integrity Number (RIN) \geq 7 were subjected to subsequent analysis. Libraries were constructed using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Libraries were then sequenced on the Illumina sequencing platform (HiSeqTM 2500 or Illumina HiSeq X Ten) and 125 bp/150 bp paired-end reads were generated. A cut-off values of \log_2 (fold change) greater than 0.58 and $P < 0.05$ were used to extract the differentially expressed genes. Hierarchical cluster analysis (12) of differentially expressed transcript (DEGs) was performed to explore genes expression patterns. KEGG (13) pathway enrichment analysis of DEGs was performed using enrichR, based on the hypergeometric

distribution. After differentially expressed genes were obtained, pathways with the number of differential genes greater than two were screened. From the total differential KEGG pathways between WT and *TLR4*-KO mice, we selected 15 signaling pathways that were closely related to DSS-induced colitis and performed a KEGG enrichment.

Quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA)

Total RNA from tissue samples was isolated using RNA isolation and purification reagent kit provided by GenePharma Corporation (Shanghai, China). RNA concentration was measured using an ultraviolet spectrophotometer (GeneQuant, Pharmacia, USA), and was subsequently stored at -80°C pending analysis. All the primers were synthesized by Sangon Biotech Corporation (Shanghai, China). cDNA was synthesized using Hifair[®] II 1st Strand cDNA Synthesis Kit (YeaSen Biotech, Shanghai, China). Fluorescence qRT-PCR reactions were performed using SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China) (14). Primers used for qRT-PCR are listed in *Table 1*.

To determine the level of *IL6*, *CCL2* and *CSF3* produced by the gut, each colon was divided into three segments of approximately 1 cm each. Colon segments were washed in phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin (Gibco from Hyclone Corporation, United States). These segments were then cultured in 24-well flat-bottom culture plates in serum-free 1640 medium (Gibco), supplemented with penicillin and streptomycin. After 24 hours, the supernatant fluid was collected and stored at 20°C . Cytokines in the supernatant were measured using ELISA kits provided by Dakewe Biotech Corporation (Shenzhen, China) and were normalized for the number of cytokines per mg of total protein in the supernatant (8).

Cell culture

Mouse intestinal epithelial cell (IEC) line (CT-26) and human IEC line (SW1116) used in this study were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA, Austria) supplemented with 10% fetal calf serum (PAA) at 37°C in a humidified atmosphere of 5% CO_2 in air. All cell lines used in this study were cultured for less than 6 months when the experiments were performed.

Table 1 qRT-PCR primers for the 25 genes evaluated

| Gene symbol | Forward | Reverse |
|---------------|---------------------------|---------------------------|
| <i>TLR4</i> | CCGCTTTCACCTCTGCCTTCAC | ACCACAATAACCTTCCGGCTCTTG |
| <i>TLR2</i> | CTCCCAGATGCTTCGTTGTTCCC | GTTGTGCGCTGCTTCCAGAGTC |
| <i>S100a8</i> | ACAAGGAAATCACCATGCCCT | TGTGAGATGCCACACCCACT |
| <i>S100a9</i> | CCACCATCATCGACACCTTCCATC | AGGTTGCCAACTGTGCTTCCAC |
| <i>IL6</i> | ACTTCCATCCAGTTGCCTTCTTGG | TTAAGCCTCCGACTTGTGAAGTGG |
| <i>IL12b</i> | CATTGAACTGGCGTTGGAAGCAC | GGGCGGGTCTGGTTTGATGATG |
| <i>Ccne1</i> | CAACATCCAGACCCACACCAACAG | GCTCGCTGCTCTGCTTCTTACTG |
| <i>Ccne2</i> | GGCTGCTGCCGCCTTATGTC | GCACCATCCAGTCTACACATTCCG |
| <i>Ccnb1</i> | GCCTGAGCCTGAACCTGAACTTG | CATCTGCGTCTACGCTACTCACTG |
| <i>Ccnb2</i> | TCTACCAGTACCTCAGGCAGCTTG | CACCAGGATGGCACGCATACG |
| <i>Ccl2</i> | CCACTCACCTGCTGCTACTCATTG | CTGCTGCTGGTGATCCTCTTGATG |
| <i>Ccl3</i> | ACCATGACACTCTGCAACCAAGTC | GCGTGGAATCTTCCGGCTGTAG |
| <i>Ccl4</i> | ATGAAGCTCTGCGTGTCTGC | CTGCCGGGAGGTGTAAGAGA |
| <i>Cdk1</i> | CGGCGAGTCTTCCACAGAGACTTG | GATGTCAACCGGAGTGGAGTAACG |
| <i>IL11</i> | GTGCTGACAAGGCTTCGAGTAGAC | CAAGGCCAGGCGAGACATCAAG |
| <i>Ccnd1</i> | TGGATGCTGGAGGTCTGTGAGG | GCAGGCGGCTCTTCTTCAAGG |
| <i>Lgr5</i> | CACTGTCACTGTGAGCTGGATGG | TAGGAAGCAGAGGCGATGTAGGAG |
| <i>Mmp3</i> | CCTGGAACAGTCTTGGCTCATGC | GGTACATCAGAGCTTCCAGCCTTGG |
| <i>Csf3</i> | GCCACCTACAAGCTGTGTCACC | GCTGGCTTAGGCACTGTGTCTG |
| <i>Wnt5a</i> | GCGGCTGCGGAGACAACATC | CTGTCCACGGCCTGCTTCAATTG |
| <i>Cxcl3</i> | CACTGGTCTGCTGCTGCTGCTG | CGTCACCGTCAAGCTCTGGATG |
| <i>Casp3</i> | AGTGGGACTGATGAGGAGATGGC | ATGCTGCAAAGGGACTGGATGAAC |
| <i>Casp6</i> | TGGCGAGGTACGGCAGTTCC | TTGGTCAGCATTGAGGCGAAGC |
| <i>Fas</i> | TGCTTGTGCTGCTCACAGTTAAGAG | TTCACGAACCCGCTCCTCAG |
| <i>FasI</i> | CCACTACCACCGCCATCACAAC | CAACCAGAGCCACCAGAACCATG |

MTT assay

Relative cell viability was analyzed using tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,2,5-diphenyltetrazolium bromide (MTT) (Dojindo Corporation, Shanghai, China). CT-26 cells were treated with 100 ng/mL of LPS (Sigma Corporation, USA), *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8/A9*, *MMP3*, Jo-2 (PeproTech Corporation, USA) for 24 h. Cells were then treated with 0.5% DSS. After 24 h, 20 μ L of MTT substrate were added to each well. The SW1116 cells were treated with 100 ng/mL of LPS (Sigma Corporation, USA), *IL6*, *IL11*, *CCL2*, *S100A8*, Jo-2 (PeproTech Corporation, USA) for 24 h. Cells were then treated with 0.5% DSS. After 24 h, 20 μ L of

MTT substrate were added to each well. The plates were returned to the incubator for an additional 4 h at 37 °C and humidified atmosphere of 5% CO₂ in air. After the incubation stage, the medium was removed, the cells were solubilized in 150 μ L of dimethylsulfoxide and colorimetric analysis was then performed (wavelength: 490 nm). The plates were analyzed on day 3 (15).

Apoptosis analysis

Cell apoptosis was analyzed using apoptosis detection kit (16) (TransGen Biotech, Beijing, China). CT-26 cells were treated with 100 ng/mL LPS (Sigma Corporation,

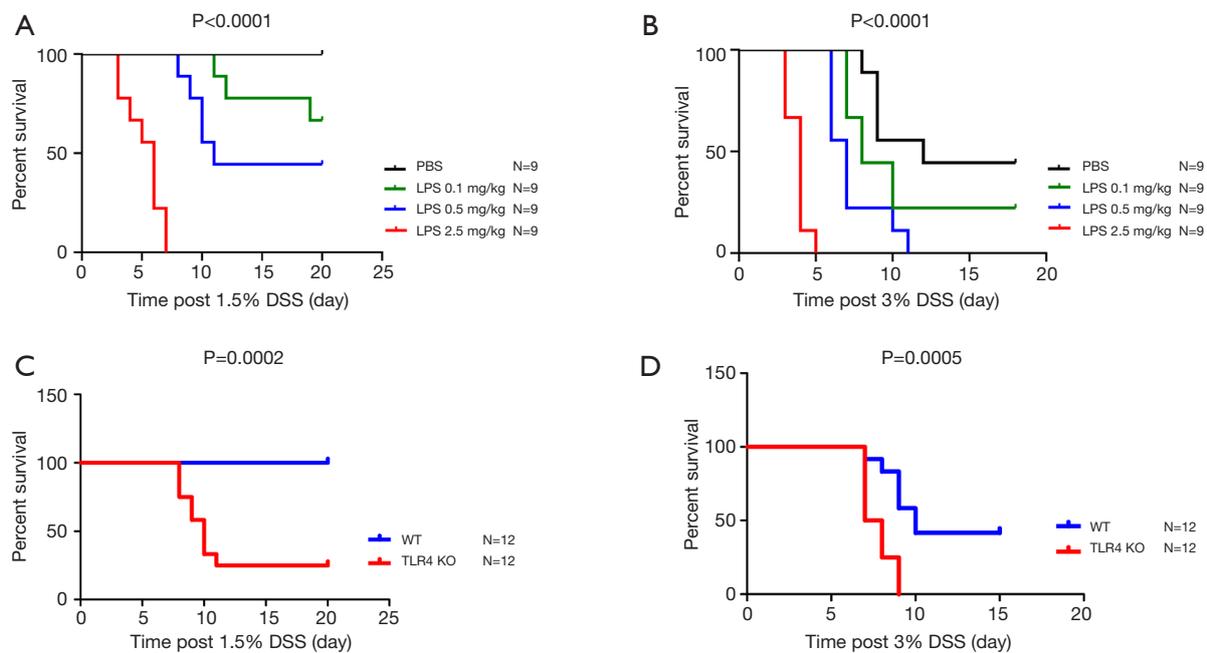


Figure 1 Exogenous LPS exacerbated dextran sulfate sodium (DSS)-induced colitis. Moreover, *TLR4*-KO mice displayed higher mortality compared to WT mice after receiving different doses of DSS. Survival rates were recorded for WT mice injected with PBS or different doses of LPS and, 24 h later, were given 1.5% (A) or 3% (B) DSS in the drinking water and for WT and *TLR4*-KO mice given 1.5% (C) or 3% (D) DSS in their drinking water.

USA), *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8/A9*, *MMP3*, Jo-2 (PeproTech Corporation, USA) for 24 hours, and were then exposed to 0.5% DSS. After 24 h, the CT-26 cells were stained with Annexin V-FITC/propidium iodide (PI) (eBioscience Corporation, USA), and apoptosis rates were detected by flow cytometry (Beckman Coulter, USA).

Transwell migration assay

CT-26 cells were treated with 100 ng/mL LPS (Sigma Corporation, USA), *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8*, *S100A9*, *Mmp3* and Jo-2 (PeproTech Corporation, USA) for 24 h. The cells were then harvested and suspended in cell culture medium without FBS. The cells were seeded onto membranes in the absence of Matrigel. Complete medium containing FBS was added to the lower chamber, and non-migrated cells were gently removed with a cotton swab after 24 or 48 h. Migratory cells located on the lower surface of the insert were fixed in 4% paraformaldehyde (Solarbio, Beijing, China), stained with 0.1% crystal violet (Sigma Corporation, USA), and counted for 53 s under flow cytometry (Beckman Coulter, USA) (17).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). All experiments were performed at least in triplicate. Comparisons between WT and *TLR4*-KO mice groups were performed using the Student's *t*-test. Differences in survival of the various mice groups were assessed using the Kaplan-Meier analysis and Cox regression analysis. The GraphPad software generated a P value and statistic for each analysis; $P < 0.05$ was considered statistically significant.

Results

Exogenous LPS aggravated DSS-induced acute intestinal injury. Moreover, mice deficient in *TLR4* showed even higher susceptibility to DSS-induced intestinal injury

The WT mice were injected with vehicle (PBS) or different doses of LPS 24 h before oral administration of 1.5% or 3% DSS. Animals that received LPS showed significantly higher mortality than did WT mice that were given PBS (Figure 1A,B). These data suggest that LPS administration

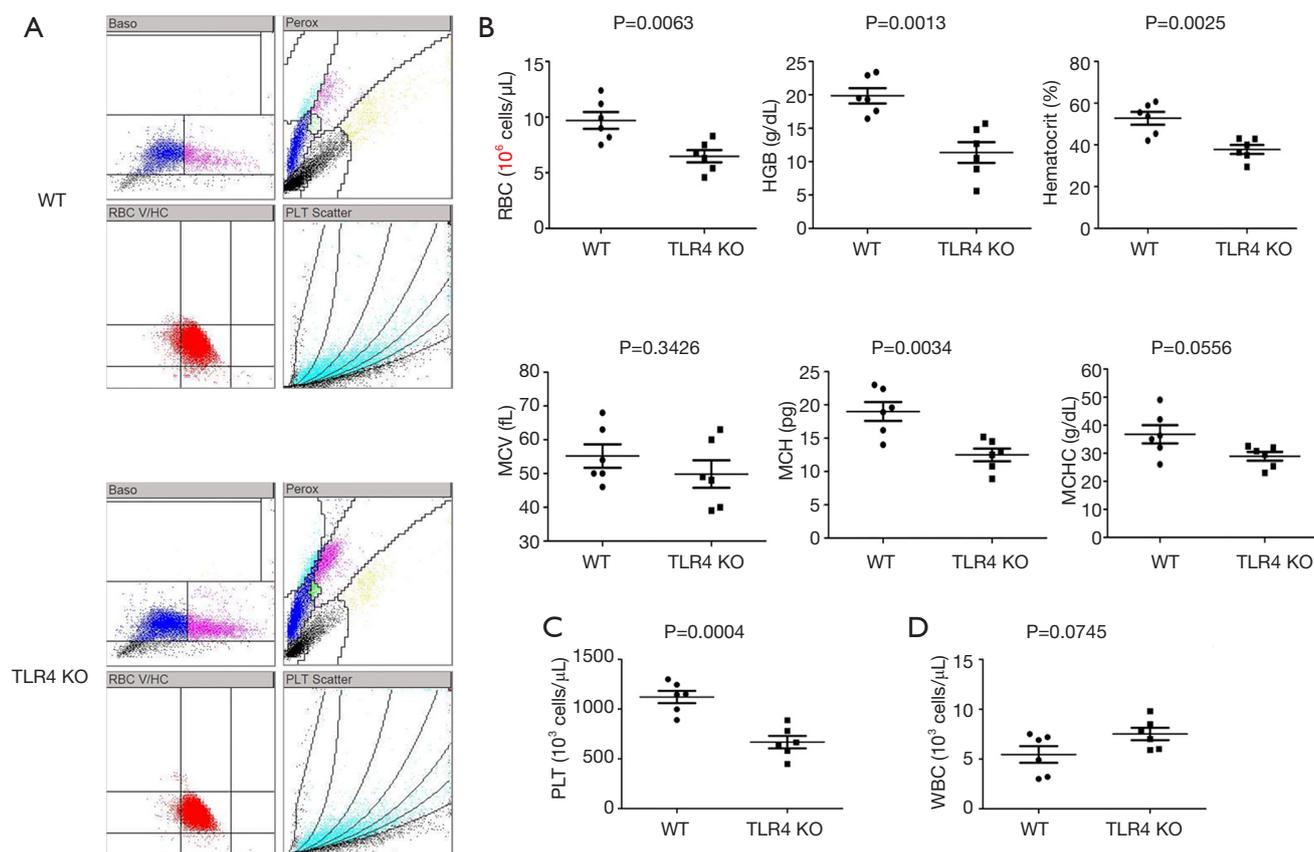


Figure 2 Mice deficient in *TLR4* were more susceptible to anemia. (A) Representative peripheral blood cell analysis patterns of WT and *TLR4-KO* mice. In Baso pattern (top left), blue dots represent mononuclear cells, pink dots represent nucleated red blood cells or cell with lobed nuclei, the dots in the upper section of the quadrant represent basophilic granulocytes, and black dots represent primitive cells. In Perox pattern (top right), dark blue dots represent lymphocytes, green dots represent monocytes, pink dots represent neutrophil granulocytes, yellow dots represent eosinophilic granulocytes, and light blue dots represent unstained large cells. In the RBC V/HC pattern (bottom left), the Y axis denotes RBC volume and the X axis denotes hemoglobin concentration. In the PLT Scatter pattern (bottom right), the Y axis denotes platelet volume and the X axis denotes platelet content. Comparison of peripheral red blood cells (B), WBC (C), and platelets (D) in WT and *TLR4-KO* mice that received 2.5% dextran sulfate sodium (DSS) for 7 days.

aggravates DSS-induced acute intestinal injury. Yet, *TLR4-KO* mice displayed more severe mortality than WT mice after receiving 1.5% or 3% DSS (Figure 1C,D). It is known that LPS can activate *TLR4* signaling (18), so this result surprised us. We therefore analyzed the cause of death and morbidity of *TLR4-KO* mice, using several parameters. Measuring the peripheral blood index on day seven after receiving 2.5% DSS, *TLR4-KO* mice were more prone to anemia than WT mice (Figure 2A). They exhibited a significant decrease in red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit, and platelet (PLT) count compared to WT mice (Figure 2B,C). However, white blood cells (WBC) displayed higher level

trend in the peripheral blood of *TLR4-KO* mice, which may be related to the aggravated intestinal inflammation (Figure 2D). Consistent with symptoms of anemia, *TLR4-KO* mice intestinal bleeding was more serious than in WT mice after ingesting 2.5% DSS for 7 days (Figure 3A,B). Histological study, performed at the end of the 7-day treatment with 2.5% DSS, indicated that extensive superficial ulceration with severe acute inflammation in *TLR4-KO* mice colon was more severe than that in the colon of WT mice. The colon of *TLR4-KO* mice also displayed extensive surface epithelium denudation (Figure 3C). Immunohistological Ki67 assay showed that, compared to WT mice, the proliferation of IEC in *TLR4-KO* mice

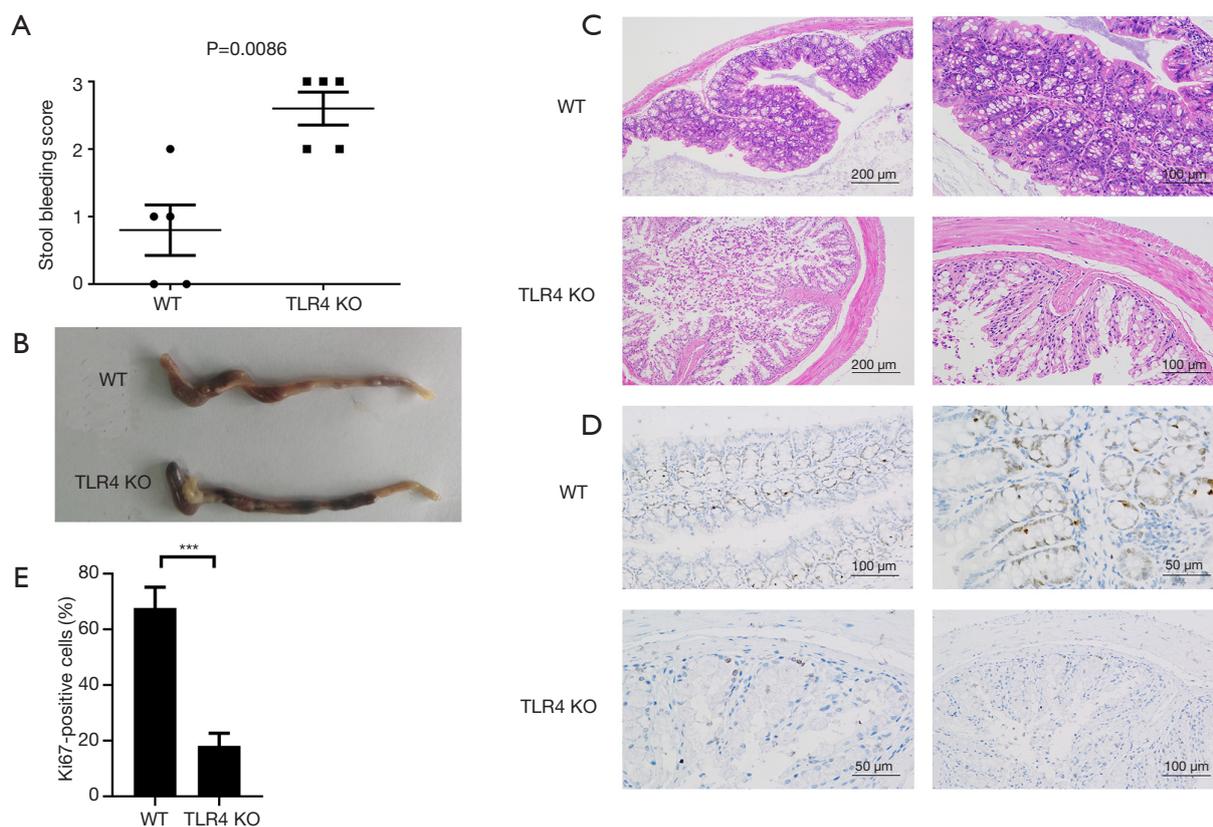


Figure 3 Compared with WT mice, the intestinal damage of *TLR4*-KO mice was more severe. (A) Stool bleeding score: 0, no gross blood visible throughout the entire colon; 1, gross blood present in <1/3 of the colon; 2, gross blood visible in >1/3 but <2/3 of the colon; 3, gross blood visible in $\geq 2/3$ of the colon. (B) Image of representative colons from WT and *TLR4*-KO mice after 7 days of treatment with 2.5% dextran sulfate sodium (DSS). (C) H&E-stained images (magnification, $\times 100$, $\times 200$) of colons from WT and *TLR4*-KO mice after 7 days of treatment with 2.5% DSS treatment. (D) Immunohistological Ki67 assay images (magnification, $\times 200$, $\times 400$) of colons from WT and *TLR4*-KO mice after receiving 2.5% DSS treatment for 7 days. (E) Comparison between WT and *TLR4*-KO mice based on Ki67-positive cells in intestines. The comparisons were performed after treating the mice with 2.5% DSS for 7 days. ***, $P < 0.001$.

was significantly weakened after oral administration of 2.5% DSS (Figure 3D,E).

Differential genes expression patterns analysis in guts from WT and *TLR4*-KO mice after DSS treatment

To determine how *TLR4* exerts a protective effect in DSS-induced colitis, we compared the global gene expression profiles in the gut between WT and *TLR4*-KO mice. A cut-off value of a \log_2 (fold change) greater than 0.58 and $P < 0.05$ were used to detect the differentially expressed genes. A total of 7,273 differential genes were detected by RNA-Seq in 4 pairs of colon samples from WT and *TLR4*-KO mice. Hierarchical clustering analysis indicated that the gene expression patterns in the colon were distinguishable

between WT and *TLR4*-KO mice after DSS treatment (Figure 4A). Compared with WT mice, there were 3,503 significantly up-regulated and 3,769 significantly down-regulated genes in guts of *TLR4*-KO mice. Volcano plot helped visualize the significantly different up-regulated and down-regulated genes in the guts of *TLR4*-KO mice (Figure 4B). We selected 54 genes that we pinpointed based on our RNA-Seq data sets, such as *IL6*, *CSF3*, *CCL2*, *Fas* and *Fas-L*, and created a heat map of their expression (Figure 4C). These 54 genes are associated with inflammatory responses and *TLR4* signaling. We are familiar with these genes as we frequently use them in our experimental system. As expected, the selected genes expression patterns in the colons were significantly distinguishable between the WT and *TLR4*-KO mice after DSS treatment (Figure 4C).

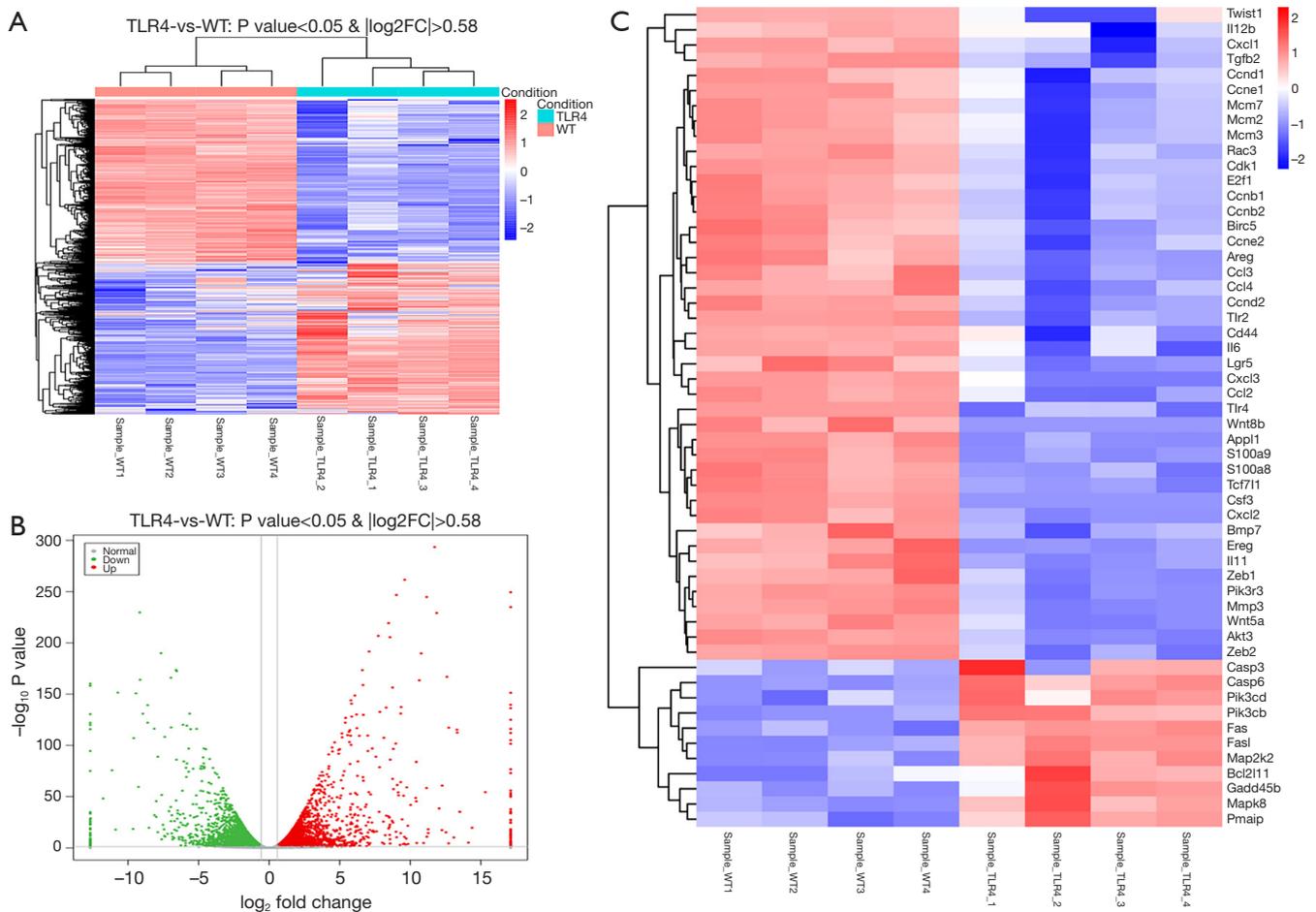


Figure 4 Differential genes expression between WT and *TLR4*-KO mice colons after dextran sulfate sodium (DSS) treatment were analyzed by high-throughput RNA-Seq. (A) Heat map of differential genes expression between WT and *TLR4*-KO mice colons after oral administration of 2.5% DSS for seven days. (B) Scatter plot of differentially expressed genes in colon tissue. Each dot stands for a gene. Red and green color dots indicate an increase or decrease, respectively, of $>\log_2$ (fold change) of 0.58 and $P < 0.05$ in *TLR4*-KO group compared to the WT group. (C) Heat map of selected 54 genes with differential expression between WT and *TLR4*-KO mice after oral administration of 2.5% DSS for 7 days.

TLR4-KO aggravates DSS-induced intestinal damage by attenuating cell cycle, cytokine-cytokine receptor interaction and TLR signaling pathways, and enhancing the apoptosis pathway

We performed genes KEGG pathway enrichment analysis. Analysis of the KEGG database revealed differences in 319 pathways between the two groups following DSS treatment. Out of the total differential KEGG pathways between WT and *TLR4*-KO mice, we selected 15 signaling pathways that are closely related to DSS-induced colitis and performed KEGG enrichment. The four pathways with the highest enrichment scores were cell cycle ($P < 0.0001$), cytokine-

cytokine receptor interaction ($P = 0.0036$), apoptosis ($P = 0.0429$) and toll-like receptor signaling pathways ($P = 0.0615$) (Figure 5). The expressions of important regulatory cyclins of the cell cycle pathway (*CycB*, *CycD*, *CycE*, and *CDK1*) were significantly lower, and *P15* and *P21* were significantly higher in the *TLR4*-KO gut tissue than in the WT control. Other important regulatory molecules in the cell cycle pathway, such as *CDK4*, *CDK6* and *P16*, were not significantly different between WT control and *TLR4*-KO (Figure S1A). According to previous reports, down-regulated cyclins (*CycB*, *CycD*, *CycE* and *CDK1*) in the gut of *TLR4*-KO mice could promote the cell cycle pathway (19,20), while up-regulated *P15* (21) and *P21* (22)

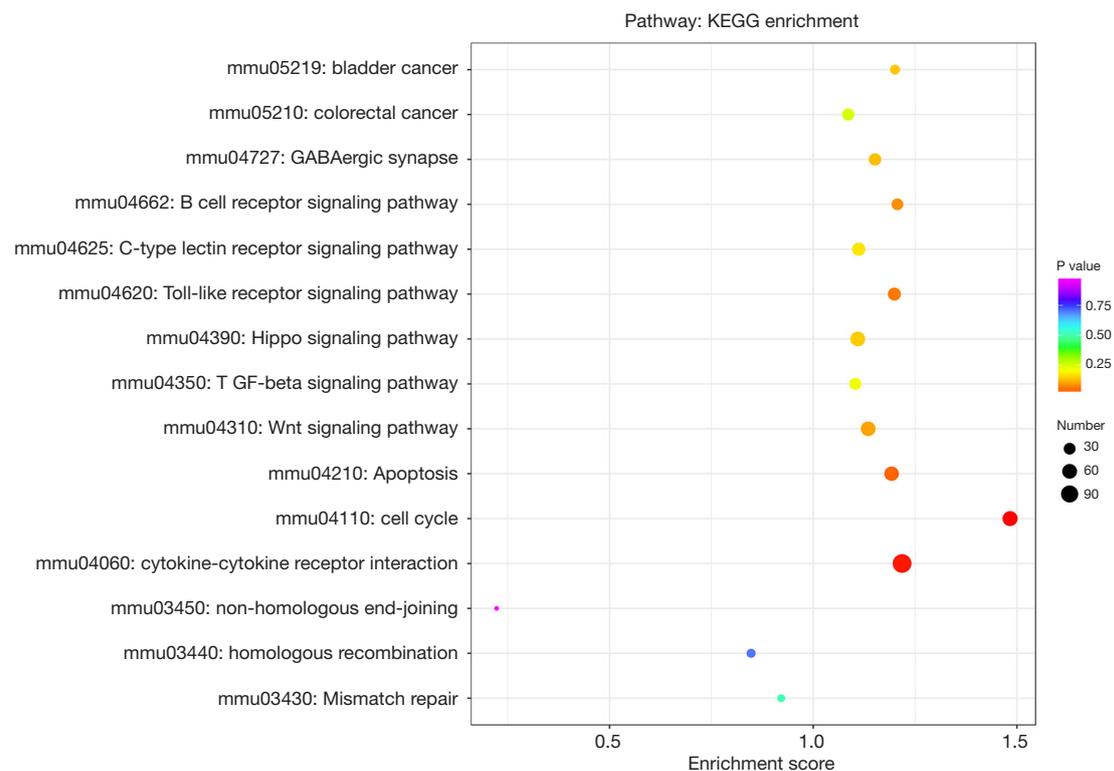


Figure 5 Fifteen signaling pathways related to dextran sulfate sodium (DSS)-induced colitis KEGG enrichment. Deletion of *TLR4* activated cell cycle, cytokine-cytokine receptor interaction, and apoptosis signaling pathways, and enhanced DSS-induced intestinal damage.

in the gut of *TLR4*-KO mice could inhibit the cell cycle pathway. So, the cell cycle pathway was down-regulated in *TLR4*-KO mice following DSS treatment. In the cytokine-cytokine receptor interaction pathway, expressions of the important regulatory factors *IL11*, *IL6*, *CCL2*, *CSF3*, and *TNF* was significantly lower in *TLR4*-KO mice gut tissue compared to WT control (Figure S1B). All these regulatory factors play important roles in promoting the cytokine-cytokine receptor interaction pathway (23,24). It is thus clear that the cytokine-cytokine receptor interaction pathway was down-regulated in the guts of *TLR4*-KO mice following DSS treatment. *Fas* and *Fas-L* are very important cytokines in the apoptosis signaling pathway, upregulation of which could significantly enhance the apoptosis signaling pathway (25). Expression of *Fas* and *Fas-L* significantly increased in the gut of *TLR4*-KO mice compared with WT control (Figure S2A). However, expression of *TNF- α* in the apoptosis signaling pathway significantly decreased in gut tissue of *TLR4*-KO mice compared to WT control. *TNF* receptor signaling has an important role in repairing intestinal epithelium in acute intestinal injury (26)

(Figure S2A). These findings suggest that the apoptosis signaling pathway was generally up-regulated in *TLR4*-KO mice that were treated with DSS. The toll-like receptor signaling pathway showed a downward trend in the gut of *TLR4*-KO mice ($P=0.0615$), however expression of *TLR1*, *TLR2*, *TLR4*, *TLR5* and their downstream cytokines *IL6*, *IL12* and *TNF- α* significantly decreased in this pathway (Figure S2B). It can be concluded from all the above KEGG data that *TLR4*-KO might aggravate DSS-induced intestinal damage by attenuating the cell cycle, cytokine-cytokine receptor interaction, and toll-like receptor signaling pathways, and enhancing the apoptosis pathway.

Twenty-five key genes screened by RNA-Seq and verified by qRT-PCR and ELISA showed that IL-6, CCL2, and CSF3 are mediated by TLR4 in CT-26 cells

qRT-PCR was performed for the 25 concerned genes, selected from all identified dysregulated differential genes. These 25 genes were significantly dysregulated in *TLR4*-KO mice colon as determined by RNA-Seq analysis, and were

Table 2 Information on 25 differentially dysregulated genes is listed

| Gene symbol | Fold change | P value | Representative pathway |
|---------------|-------------|-------------|--|
| <i>TLR4</i> | 0.000585335 | 3.3723E-152 | Toll-like receptor signaling pathway |
| <i>TLR2</i> | 0.115586127 | 6.00E-41 | Toll-like receptor signaling pathway |
| <i>S100A8</i> | 0.049062896 | 2.72E-08 | IL-17 signaling pathway |
| <i>S100A9</i> | 0.042650897 | 1.79E-13 | IL-17 signaling pathway |
| <i>IL6</i> | 0.007980337 | 8.11E-27 | Cytokine-cytokine receptor interaction |
| <i>IL12b</i> | 0.156359004 | 0.000543509 | Cytokine-cytokine receptor interaction |
| <i>Ccne1</i> | 0.339806818 | 1.73E-08 | Cell cycle |
| <i>Ccne2</i> | 0.419347761 | 7.03E-05 | Cell cycle |
| <i>Ccnb1</i> | 0.312930703 | 1.50E-09 | Cell cycle |
| <i>Ccnb2</i> | 0.408863048 | 2.34E-07 | Cell cycle |
| <i>Ccl2</i> | 0.026705626 | 1.00E-13 | Cytokine-cytokine receptor interaction |
| <i>Ccl3</i> | 0.108806643 | 4.21E-06 | Toll-like receptor signaling pathway |
| <i>Ccl4</i> | 0.132664573 | 1.28E-08 | NF-kappa B signaling pathway |
| <i>Cdk1</i> | 0.437774729 | 3.37E-09 | Cell cycle |
| <i>IL11</i> | 0.046172897 | 6.19E-05 | Cytokine-cytokine receptor interaction |
| <i>Ccnd1</i> | 0.321812344 | 1.39E-05 | Wnt signaling pathway |
| <i>Lgr5</i> | 0.463694421 | 5.54E-05 | Wnt signaling pathway |
| <i>Mmp3</i> | 0.036079083 | 8.24E-22 | TNF signaling pathway |
| <i>CSF3</i> | 0 | 3.62E-10 | Cytokine-cytokine receptor interaction |
| <i>Wnt5a</i> | 0.31949403 | 4.08E-13 | Wnt signaling pathway |
| <i>CXCL3</i> | 0.00270957 | 2.09E-20 | Cytokine-cytokine receptor interaction |
| <i>Casp3</i> | 2.136599012 | 9.85E-05 | Apoptosis |
| <i>Casp6</i> | 6.73370506 | 1.10E-16 | Apoptosis |
| <i>Fas</i> | 2.831701919 | 1.14E-13 | Cytokine-cytokine receptor interaction/apoptosis |
| <i>FasL</i> | 13.96978841 | 1.47E-09 | Cytokine-cytokine receptor interaction/apoptosis |

The related information presented include: gene symbol, fold change, P value, and representative pathway. Gene names written in green indicate that the genes were down-regulated in colons of *TLR4-KO* mice, when compared to the WT control. Gene names written in red indicate that the genes were up-regulated in colons of *TLR4-KO* mice, when compared to the WT control.

commonly used in our experimental system. The related information on these 25 differentially dysregulated genes is presented in *Table 2*. qRT-PCR results were consistent with the results of the RNA-Seq study, and, in comparison to the WT mice, expressions of *TLR4*, *IL6*, *CCL2*, and *CSF3* were especially lower and *Casp3*, *Casp6*, *Fas* and *FasL* were higher in the gut of *TLR4-KO* mice (*Figure 6*). ELISA was performed to measure expression levels of *IL-6*, *CCL2*, and *CSF3* in the colon of WT and *TLR4-KO* mice. The results suggest that, compared with WT mice, *IL6*, *CCL2*,

and *CSF3* were significantly down-regulated in *TLR4-KO* mice (*Figure 7A*). To explore how *TLR4* signaling regulated the expression levels of *IL6*, *CCL2*, *CSF3*, *Fas*, and *FasL*, we performed qRT-PCR and ELISA in CT-26 cells treated with LPS. We found that the expression levels of *IL6*, *CCL2*, and *CSF3* significantly increased while the expression levels of *Fas* and *FasL* only slightly increased in CT-26 cells stimulated by LPS (*Figure 7B,C*). As far as *Fas* and *FasL*, the results were in disagreement with the results of the RNA-seq and qRT-PCR analyses that showed a significant increase

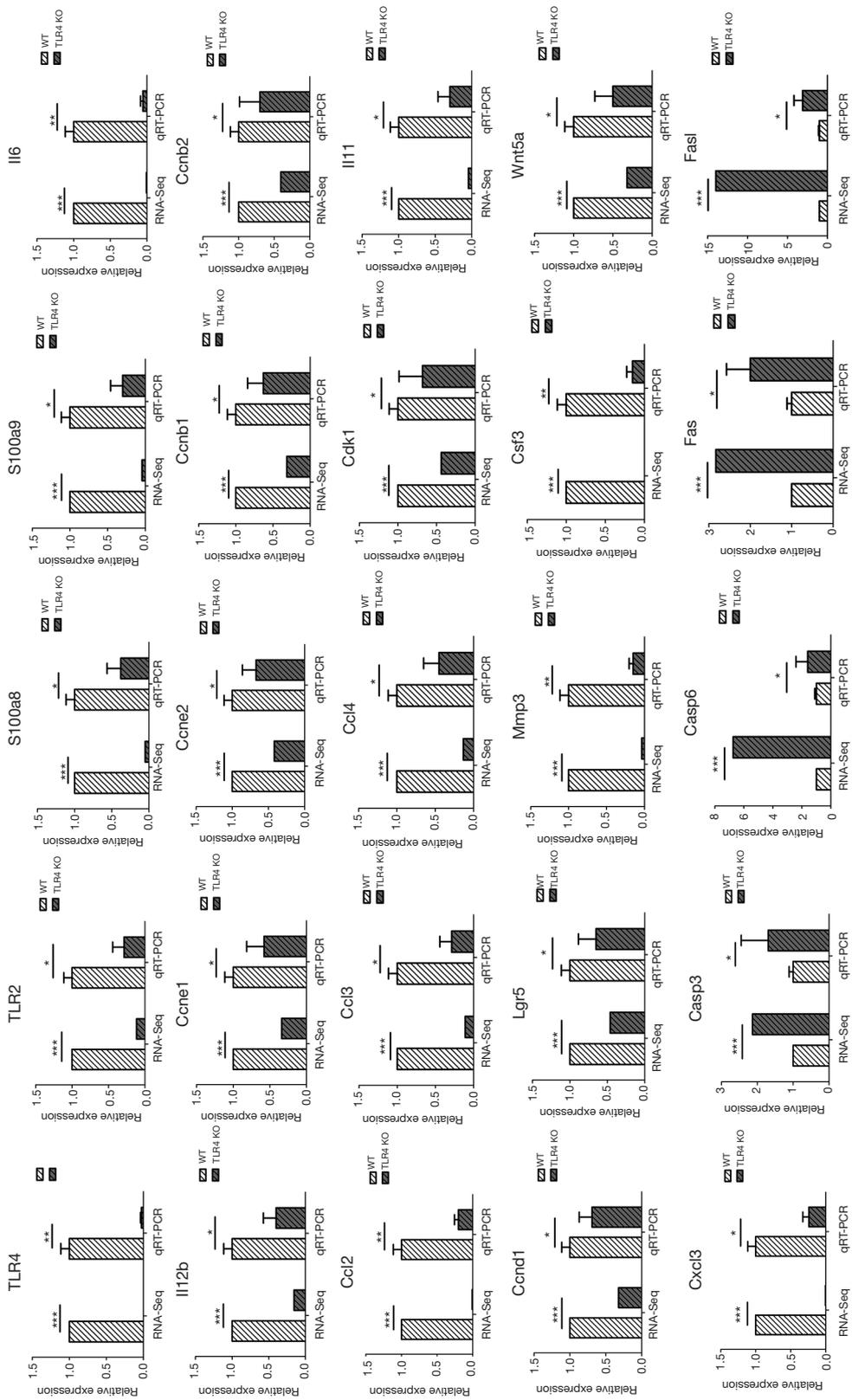


Figure 6 Validation of key genes screened by RNA-Seq. Validation of the relative expression of 25 differentially expressed genes in colons of WT and TLR4-KO mice was performed by qRT-PCR after oral treatment with 2.5% dextran sulfate sodium (DSS) for 7 days. *, P<0.05; **, P<0.01; ***, P<0.001. NS, no significant difference detected.

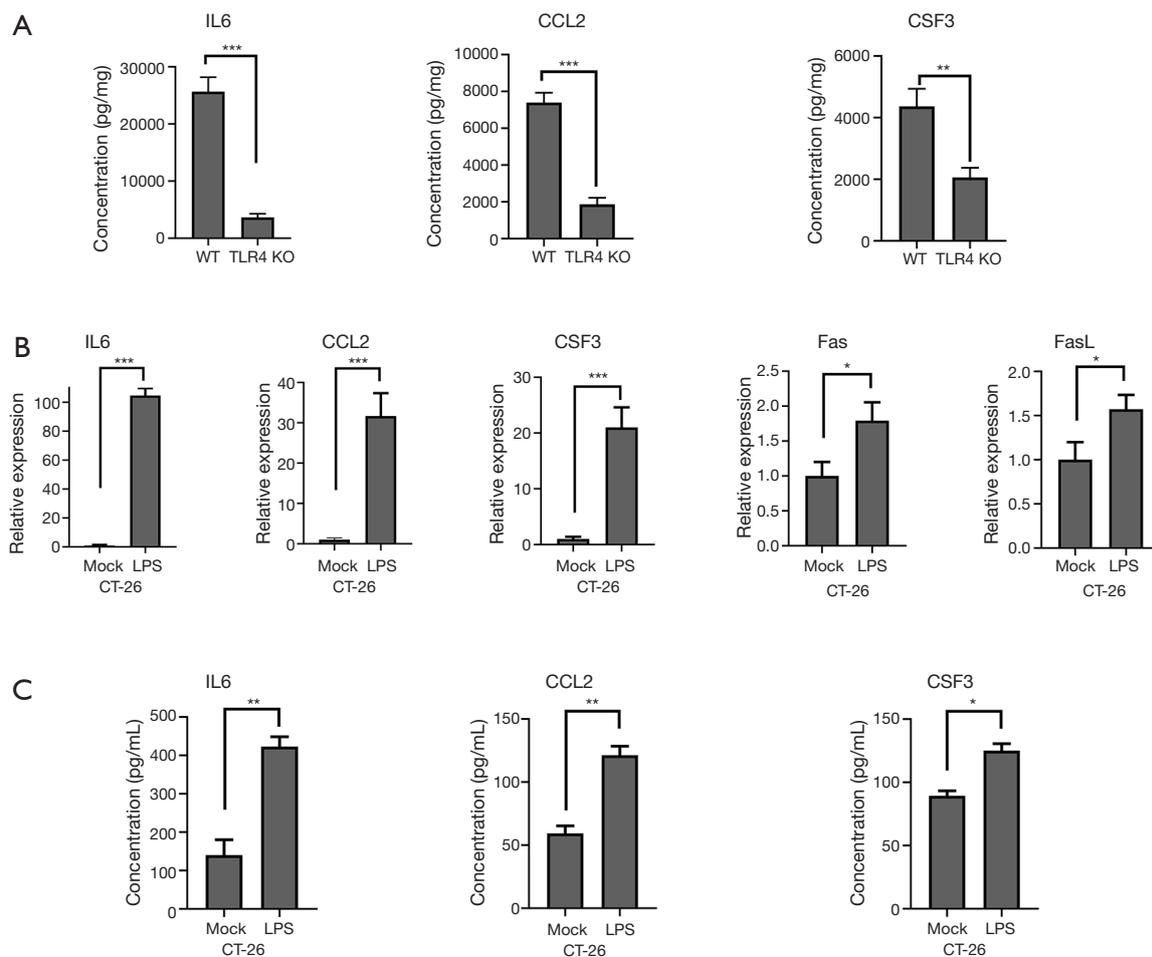


Figure 7 *IL6* was related to *TLR4* deficiency the most. The cytokines might be important in *TLR4* signaling regulation of DSS injury, and that *Fas* might also be involved in the process, but if so, the effect is minimal. (A) ELISA validation for the concentration of *IL6*, *CCL2*, and *CSF3* in colons of WT and *TLR4*-KO mice after treatment with 2.5% dextran sulfate sodium (DSS) for seven days. (B) qRT-PCR evaluation of the expression levels of *IL6*, *CCL2*, *CSF3*, *Fas*, and *FasL* in the CT-26 cells, with and without LPS treatment (100 ng/mL). (C) ELISA validation for the concentration of *IL6*, *CCL2*, and *CSF3* in CT-26 cells, with and without LPS treatment (100 ng/mL). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of *Fas* and *FasL* in the colon of *TLR4*-KO mice treated with DSS, because LPS has the effect of activating *TLR4*. These data suggested that *TLR4* might play a repairing role in DSS-induced intestinal damage by up-regulating the cytokines *IL6*, *CCL2*, and *CSF3*. *Fas* and *FasL* promoted DSS-induced colon injury in the mice, but they might have little to do with *TLR4* signaling.

Functional study of the core dysregulated cytokines in intestinal cancer cell lines

We next conducted a study on CT-26 cells (mouse

intestinal cancer cell line), evaluating the functionality of the significantly dysregulated cytokines. CT-26 cells were treated with LPS (*TLR4* agonist), *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8*, *S100A9*, *MMP3*, and Jo-2 (Fas agonistic monoclonal antibody) for 24 h. The cells were then treated with 0.5% DSS. Cells viability was assessed after 24 h, using the MTT assay. Results suggest that, compared with control, viability of DSS-treated CT-26 cells have decreased following treatment with Jo-2 and increased when treated with LPS, *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8*, *S100A9*, and *MMP3*. Notably, LPS, *IL6*, and *CCL2* had a highly significant viability enhancement effect on DSS-

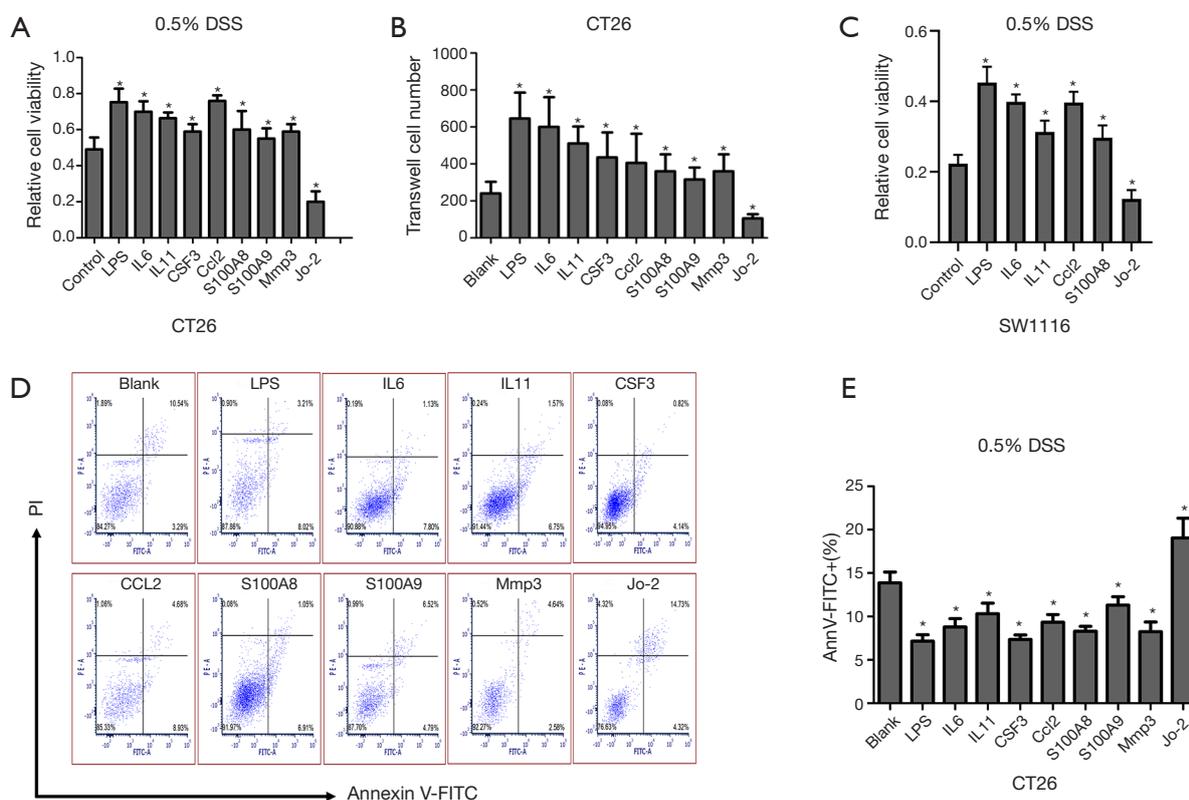


Figure 8 Functional study of the core dysregulated cytokines in CT-26 and SW1116 cells. (A) CT-26 cells were treated with 100 ng/mL LPS, IL6, IL11, CSF3, CCL2, S100A8/9, MMP3, and Jo-2 for 24 h, after which they were treated with 0.5% dextran sulfate sodium (DSS). Cells viability was evaluated after 24 h, using the MTT assay. (B) Migration assessment of CT-26 cells treated with LPS, IL6, IL11, CSF3, CCL2, S100A8/9, MMP3, and Jo-2. (C) SW1116 cells were treated with 100 ng/mL LPS, IL6, IL11, CCL2, S100A8, and Jo-2 for 24 h, after which they were treated with 0.5% DSS. Cells viability was evaluated after 24 h, using the MTT assay. (D) CT-26 cells were treated with 100 ng/mL LPS, IL6, IL11, CSF3, CCL2, S100A8/9, MMP3, and Jo-2 for 24 h, after which they were treated with 0.5% DSS. Apoptosis rate was assessed by flow cytometry after 24 h, using Annexin V/PI dual staining. (E) Statistical analysis of Annexin V-FITC-positive DSS-treated CT-26 cells. *, $P < 0.05$.

treated CT-26 cells (Figure 8A). We also performed the transwell migratory assay to study the effect of LPS, IL6, IL11, CSF3, CCL2, S100A8, S100A9, MMP3 and Jo-2 on intestinal cells migration. Consistent with previous experimental results, when compared with the blank control, LPS, IL6, IL11, CSF3, CCL2, S100A8, S100A9 and MMP3 promoted CT-26 cells' migration and Jo-2 inhibited it (Figure 8B). Additionally, SW1116 cells (human colorectal cancer cell line) were treated with LPS, IL6, IL11, CCL2, S100A8, and Jo-2 for 24 h, after which the cells were treated with 0.5% DSS. Cells viability was assessed after 24 h, using the MTT assay. Results suggest that, compared to the control, viability of DSS-treated SW1116 cells has decreased following treatment with Jo-2, and increased when treated with LPS, IL6, IL11, CCL2,

S100A8. The increase was especially notable when the cells were treated with LPS, IL6, and CCL2 (Figure 8C). Apoptosis rate of cytokines-treated CT-26 cells that were exposed to 0.5% DSS was then examined by Annexin V/PI dual staining. It was found that, compared to the blank control, apoptosis rates of LPS, IL6, IL11, CSF3, CCL2, S100A8, S100A9, and MMP3-treated CT-26 cells have decreased and the apoptosis rate of Jo-2-treated CT-26 cells has increased after 0.5% DSS administration (Figure 8D,E).

Discussion

TLRs are sensors for pathogen-associated molecular patterns (PAMPs) and play important roles in immune

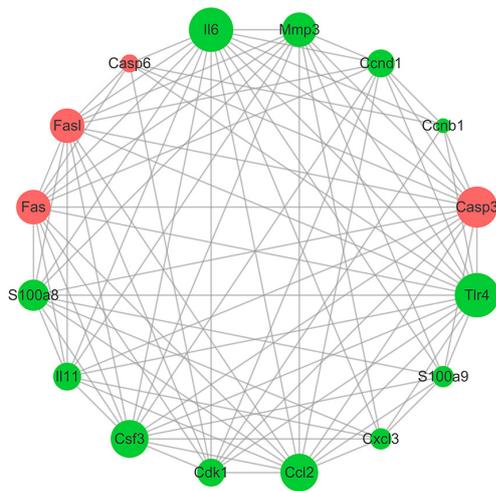


Figure 9 Network interaction map of genes in the guts of *TLR4*-KO mice.

responses (27). *TLR4* is a pattern recognition receptor (PRR) found in IEC that recognize microbial molecules. Research has shown that after *TLR4* combines with LPS, it triggers signaling cascades, mediated by MyD88, to activate the transcription factor *NF- κ B* and, eventually, leads to inflammatory response (28). However, in our work, we found that LPS and *TLR4*-KO aggravated intestinal damage in DSS-induced colitis in mouse model.

In order to study the key genes involved in *TLR4*-KO prompting DSS-induced intestinal injury, we used high-throughput RNA sequencing to screen for differentially expressed genes in the gut, comparing between WT and *TLR4*-KO mice. qRT-PCR was performed to verify our singled-out genes and results were consistent with the RNA-Seq data. Previous studies (26) have reported that the inflammatory cytokine *IL6* could protect IEC through the activation of *STAT3*. *TNF* receptor signaling was also shown to have an important role in repairing intestinal epithelium in acute intestinal injury (26). The repair genes, *Ccnb1*, *Cnd1*, *CSF3* and *CCL2*, are involve in mediating multiple pathways to repair intestinal epithelium in DSS-induced intestinal injury (29,30). Combined with the KEGG enrichment data, our study shows that *IL6*, *CCL2*, *CSF3*, *IL11*, *Ccnb1*, *Cnd1* and *TNF- α* have significantly decrease and *Fas* and *Fas-L* have increase in DSS-induced colitis related pathways. The expression levels of *IL6*, *CCL2*, *CSF3*, *Fas* and *FasL* have all increased in CT-26 cells stimulated by LPS. *Fas* and *FasL* have also increased in the

colon of *TLR4*-KO mice. These data suggest that *TLR4*-KO aggravates DSS-induced colon injury through down-regulation of the cytokines *IL6*, *CCL2*, and *CSF3*. *Fas* and *FasL* promoted DSS-induced colon injury in mice, but this might have little to do with *TLR4* signaling. Additionally, we confirmed that, when compared to the blank control, treatment with LPS (*TLR4* agonist), *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8*, *S100A9* and *Mmp3* enhanced CT-26 cells migration, and treatment with Jo-2 (*Fas* agonistic monoclonal antibody) inhibited it. LPS, *IL6*, *IL11*, *CSF3*, *CCL2*, *S100A8*, *S100A9* and *Mmp3* have also improved cell viability and reduced apoptosis rate in DSS-treated CT-26 cells. Jo-2, so we found, plays the opposite role. This indicates that promotion of cytokines activity, such as *IL6* and *CCL2*, or inhibition *Fas* activity could effectively improve the repair of intestinal epithelial damage. Based on these results, we built the genes network interaction map (Figures 9 and S3).

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by an institutional ethics committee and followed the tenets of the Declaration of Helsinki.

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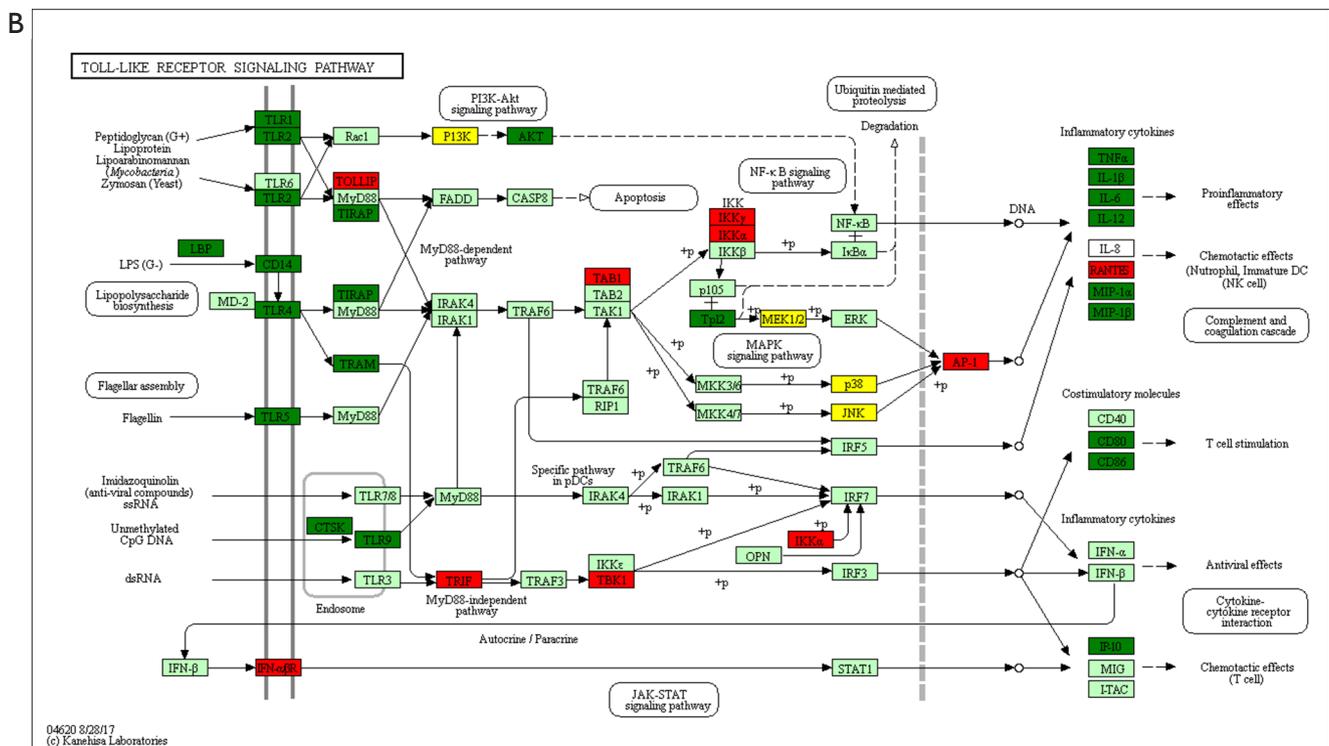
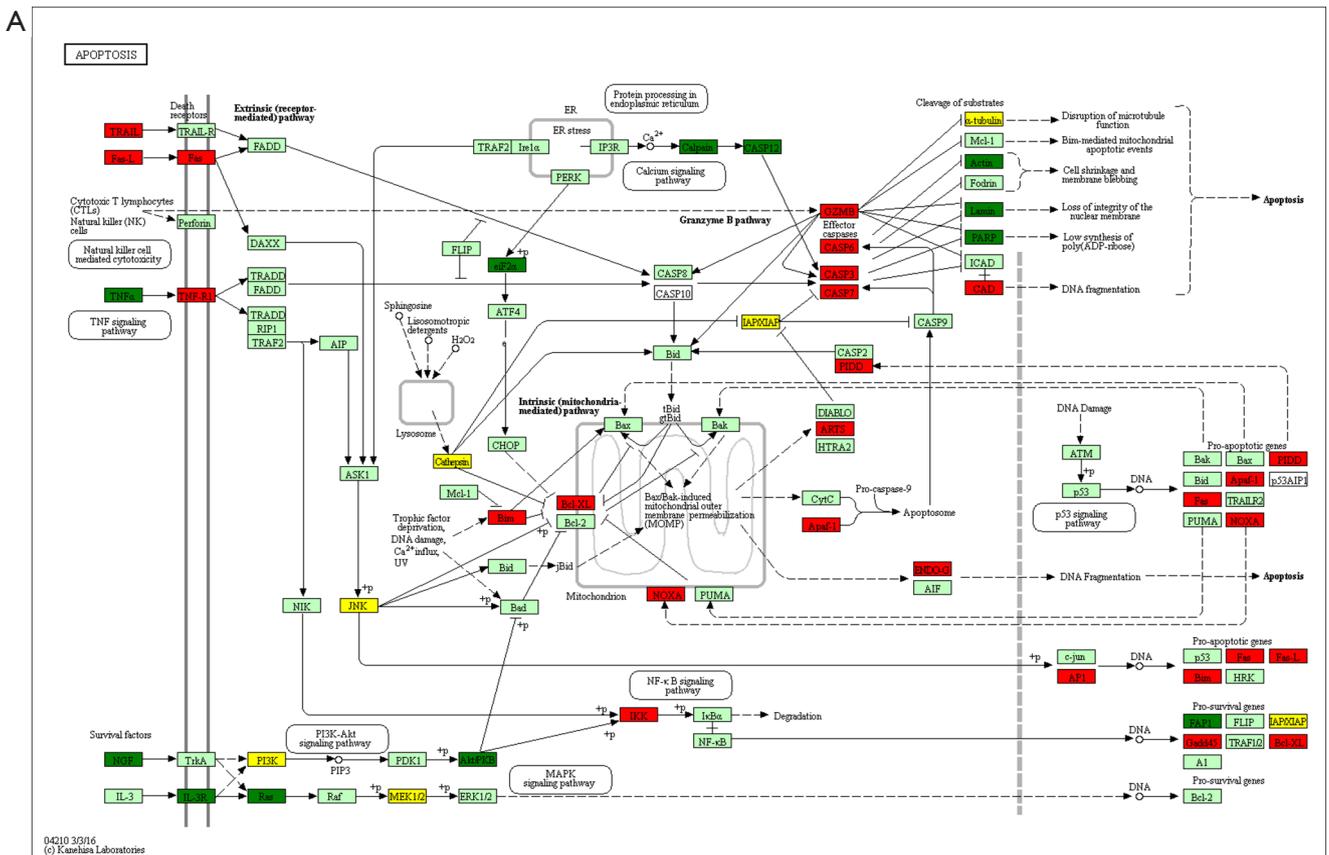


Figure S2 Toll-like receptor 4 (TLR4)-KO aggravates dextran sulfate sodium (DSS)-induced intestinal damage by attenuating TLR signaling pathways and enhancing the apoptosis pathway. (A) Map of apoptosis signaling pathway; (B) map of Toll-like receptor signaling pathway.

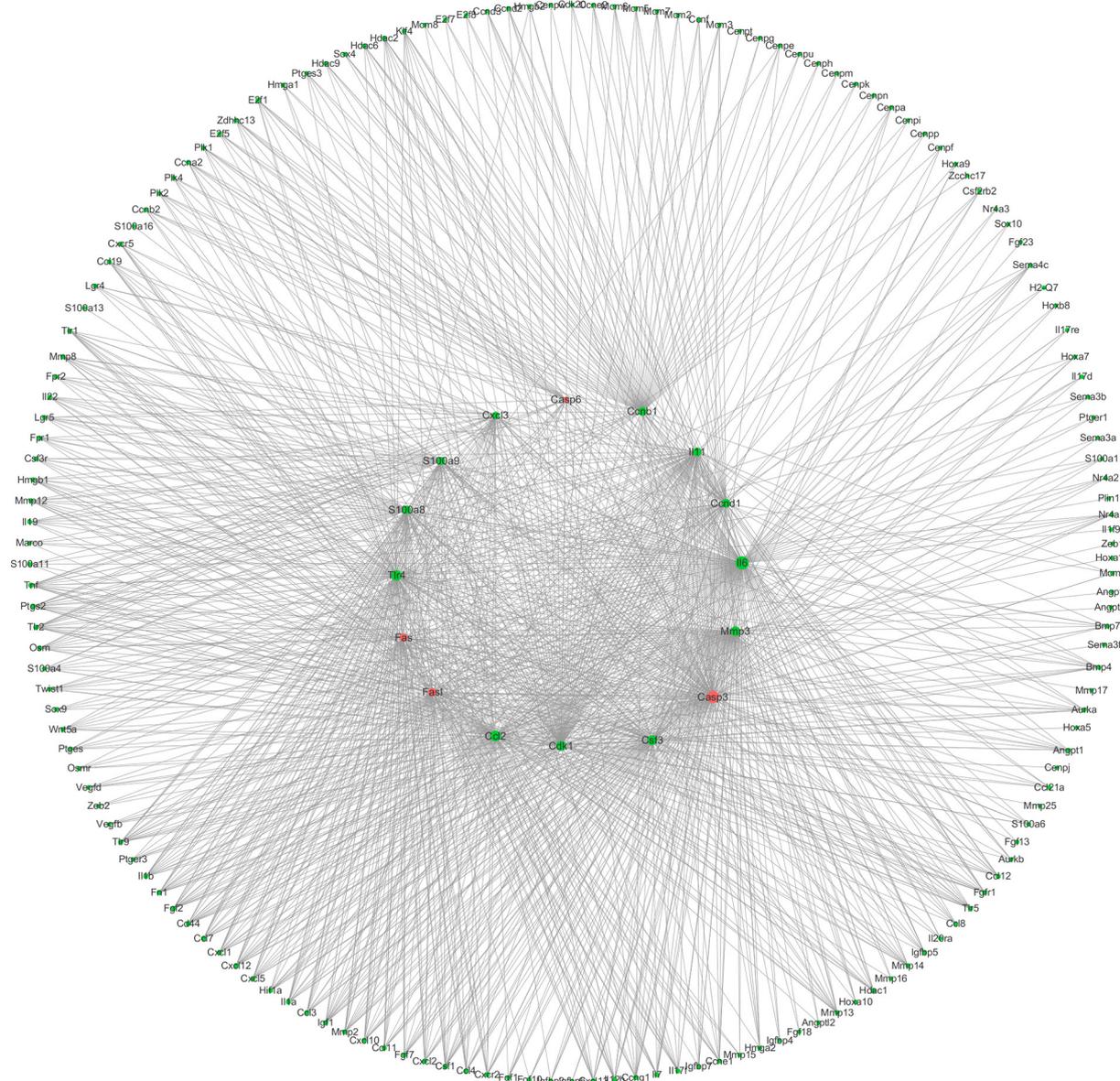


Figure S3 Network interaction map of genes in the guts of Toll-like receptor 4 (TLR4)-KO mice, and their core downstream factors, aggravating dextran sulfate sodium (DSS)-induced colitis.