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Roxadustat protect mice from DSS-induced colitis in vivo by up-regulation of TLR4

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ABSTRACT

Background: The incidence of inflammatory bowel disease (IBD) is growing in the population. At present, the etiology of inflammatory bowel disease remains unclear, and there is no effective and low-toxic therapeutic drug. The role of the PHD-HIF pathway in relieving DSS-induced colitis is gradually being explored.
Methods: Wild-type C57BL/6 mice were used as a model of DSS-induced colitis to explore the important role of Roxadustat in alleviating DSS-induced colitis. High-throughput RNA-Seq and qRT-PCR methods were used to screen and verify the key differential genes in the colon of mice between normal saline (NS) and Roxadustat groups.
Results: Roxadustat could alleviate DSS-induced colitis. Compared with the mice in the NS group, TLR4 were significantly up-regulated in the Roxadustat group. TLR4 KO mice were used to verify the role of TLR4 in the alleviation of DSS-induced colitis by Roxadustat.
Conclusion: Roxadustat has a repairing effect on DSS-induced colitis, and may alleviate DSS-induced colitis by targeting the TLR4 pathway and promote intestinal stem cell proliferation.

1. Introduction

Inflammatory bowel disease (IBD) is divided into ulcerative colitis (UC) and crohns disease (CD), which can seriously interfere with the normal function of the digestive tract and lead to the destruction of the intestinal epithelial barrier, followed by the activation the immune system of intestinal mucosa [1]. Exposure to antigenic substances leads to inflammation and further disruption of the intestinal barrier [2]. With the development and widespread application of science and technology, the incidence of inflammatory bowel disease in the population is gradually increasing. In the latest issue of the Lancet, the prevalence of inflammatory bowel disease (IBD) was estimated at 7 million worldwide [3]. Currently, due to the limited treatment options available, the ultimate treatment is the surgical removal of large amounts of chronically inflamed intestinal tissue [4]. This therapy is damaging, so it is extremely necessary to find suitable, high-efficiency and low-toxic drugs for the relief of inflammatory bowel disease.

In the gastrointestinal tract, the impact of hypoxia on transepithelial permeability has been implicated in inflammatory bowel disease (IBD) [5–7].Physiological oxygen gradients exist in the intestinal mucosa due to the anatomical juxtaposition of the gastrointestinal mucosa with the oxygen-depleted lumen of the gut and the counter-current oxygen exchange system in the intestinal villi [8].Oxygen can physiologically regulate the barrier and absorption functions of the intestinal epithelium. Although the oxygen level in the intestinal tissue has been changing dynamically, in the presence of the hypoxia-inducible factors

Abbreviations: IBD, inflammatiory bowel disease; DSS, dextran sulfate sodium; NS, Normal saline; PBS, Phosphate buffer saline; UC, ulcerative colitis; CD, crohns disease; PHD, Proline hydroxylase; HIF, hypoxia-inducible factor; DMOG, dimethyloxalylglycine; LPS, Lipopolysaccharide; NAC, N-Acetyl-L-cysteine; HE, Hematoxylin and Eosin; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick-end labelling; DEGs, differentially expressed genes; ANOVA, Analysis of Variance; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT –PCR, Quantitative real-time PCR; EP, Eppendorf; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; MCHC, mean corpuscular hemoglobin contentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; WBC, white blood cell.

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HIF-1α and HIF-2α, the intestinal tissue can maintain the intestinal homeostasis of the environment, and hypoxia-inducible factors can regulate various genes required to maintain intestinal barrier function [9,10]. In the presence of oxygen, HIF is degraded by pVHL-containing E3 ubiquitin ligase, which binds to human pVHL when the conserved proline hydroxyl group in the core region of the HIF-derived peptide is hydroxylated [11].Proline hydroxylase (PHD) can post-translationally modify HIF-1α, enabling it to interact with the VHL complex. Proline hydroxylase is an iron-containing structure, so iron chelators can inhibit its activity [12]. Inhibition of HIF by hypoxia or proline hydroxylase inhibitors can modulate cell metabolism and survival, induce tissue remodeling, increase epithelial integrity, and promote stem cell survival [13].

The PHD-HIF pathway has been a research hotspot in recent years [11,14,15]. Multiple studies have demonstrated that the effect of pharmacological HIF activation through hydroxylase inhibition is profoundly anti-inflammatory [16]. Inflammation can destroy the metabolic microenvironment, activate HIF and other inflammatory transcription factors. In terms of inflammatory disease, a number of preclinical studies have demonstrated that hydroxylase inhibitors can anti-inflammatory [17]. This effectiveness has been shown in models of IBD [18,19]. Karhausen et al. constructed intestinal HIF-1α conditional knockout mice and HIF-1α conditional overexpression mice. It was found that mice with colon epithelial deficiency of HIF-1α had more severe colitis, more weight loss, shorter colon, and more severe intestinal mucosal immune barrier damage than wild-type mice. Conversely, mice with conditional overexpression of HIF-1α in the gut were protected [20]. These reports indicate that the PHD-HIF pathway plays an important role in alleviating intestinal inflammation.

In this study we used a dextran sulfate sodium (DSS)-induced mouse model of colitis, which has many features of human IBD. In this model, DSS damages colonic epithelial cells, thereby disrupting the intestinal barrier and allowing gut bacteria to penetrate the damaged mucosa, perpetuating colonic inflammation. Roxadustat as a proline hydroxylase inhibitor. It finds in our study to play an important role in relieving DSS-induced colitis. And its low toxicity can be used as clinical medicine.

2. Materials and methods

2.1. Animals and treatment

Male wild type C57BL/6 mice were obtained from China Academy of Science (Shanghai, China). TLR4 KO mice aged 6–8 weeks old were purchased from Model Animal Research Center, Nanjing University. All mice were housed in a laboratory animal room under standard conditions. The experiments were approved by the Laboratory Animal Center of the Naval Medical University, China in conformance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Roxadustat treatment

Roxadustat was purchased from Cayman Chemical Company (http://www.caymanchem.com). Roxadustat was dissolved in DMSO to prepare the original solution, the mouse administration was diluted with normal saline to prepare different concentration gradient solutions. The mice were treated with Roxadustat (25.0 mg/Kg, dissolved in NS) via peritoneal injection.

2.3. Other materials

Normal saline (NS) was obtained from ChangHai Hospital (Shanghai, China). Phosphate buffer saline (PBS) was obtained from Boguang Bio (Shanghai, China). Dextran Sulfate Sodium was purchased from Sigma (USA).

2.4. Model of DSS- induced colitis

Colitis was induced by treatment of mice with 2.5% DSS (Sigma) dissolved in the drinking water for 7 days.

2.5. Histological examination

When the mice were sacrificed, approximately 1 cm length of the distal colon was removed and fixed in 4% paraformaldehyde. After embedded in paraffin, the tissues were stained with hematoxylin and eosin (HE). TUNEL (Terminal deoxynucle otidyl transferase dUTP nick-end labelling) staining and Ki67 assay were did according to the manufacturer's instructions. The $TUNEL^{+}$ cells were counted in 10 crypts per section.

2.6. Analysis of peripheral blood cells

Wild-type mice treated with 2.5% DSS were eyebled 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.

2.7. Flow cytometry

The femur of mice was washed repeatedly with 1 ml PBS for 3 times. Then the cell suspension was centrifuged at 1200 rpm/min for 5 min. After the supernatant discarded, the pellet was lysed with 1 ml Red Blood Cell Lysis Buffer for 10 min at 4 °C to remove the RBCs. The leaving BMCs were washed and resuspended with 1 ml PBS. After fixation with Binding Buffer, CD71-PE and TER119-FITC were stained, and detected by flow cytometer after 20 min.

2.8. RNA sequencing and functional enrichment analysis

Total RNA was isolated from intestine of mice using Trizol (Invitrogen, USA) after Roxadustat intraperitoneal injection. NanoVue (GE, USA) was used to assess RNA purity. Each RNA sample had an A260: A280 ratio >1.8 and an A260:A230 ratio >2.0. Sequencing was performed at Oebiotech (Shanghai, China) with the Illumina HiSeq X Ten. Prior to sequencing, the raw data were filtered to produce high-quality clean data. All the subsequent analyses were performed using the clean data.

Differential expression analysis was performed using the DESeq2. Q value <0.05 and foldchange >2 or foldchange <0.5 was set as the threshold for significantly differential expression gene (DEGs). Hierarchical cluster analysis of DEGs was performed using R (v 3.2.0) to demonstrate the expression pattern of genes in different groups and samples. The radar map of top 30 genes was drew to show the expression of up-regulated or down-regulated DEGs using R packet ggradar.

Based on the hypergeometric distribution, GO and KEGG pathway enrichment analysis of DEGs were performed to screen the significant enriched term using R (v 3.2.0), respectively. R (v 3.2.0) was used to draw the column diagram of the significant enrichment term.

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software. The analysis was used a predefined gene set, and the genes were ranked according to the degree of differential expression in the two types of samples. Then it is tested whether the predefined gene set was enriched at the top or bottom of the ranking list.

2.9. Intestine immunofuorescence

Immunofuorescence analysis was used to detect TLR4. The intestinal tissues was fixed in 4% paraformaldehyde for 20 min and permeabilized

Table 1
qRT-PCR primers of six genes.

Gene symbol	Forward	Reverse
TLR4	AAATGCACTGAGCTTTAGTGGT	TGGCACTCATAATGATGGCAC
Tifa	GACGCTGATACAGAGGAGACG	GAACGGAGCTGTTGAACTGTT
Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Muc1	AGTGCCAAGTCAATACCCTGT	CTGGGGTGAACTGTTACTGGA
Fzd9	TTGCTCTATTATTTCGGGATGGC	CAGGACCACGATAGTTTTGAGTG
CD44	AGAAAAATGGCCGCTACAGTATC	TGCATGTTTCAAAACCCTTGC

in 0.5% Triton X-100 for 10 min. After blocking in BSA, the intestinal tissueswas stained with antibodies, followed by the secondary antibody (11000). The images were obtained with a fluorescent microscope.

2.10. Quantitative real-time PCR(qRT -PCR)

Total RNA from tissue samples was isolated using RNA isolation and purification reagent kit provided by Solarbio (Beijing, China). RNA concentration was measured using NanoVue (GE, USA), and was subsequently stored at −80 °C pending analysis. The primers were synthesized by Shenggong Biotech Corporation (Shanghai, China). cDNA was synthesized using TaKaRa kit(RR036A). Fluorescence qRT-PCR reactions were performed using TaKaRa Kit (RR420A). Primers used for qRT-PCR are listed in Table 1.

2.11. Statistical analysis

Data were expressed as means ± the standard errors of means. Two-tailed Student's *t*-test was used to analyze the difference between two groups. One-way ANOVA was employed to analyze the difference among three groups. Kaplan–Meier analysis was applied to estimate the difference of overall survival between two groups. The data were analyzed using SPSS ver. 19 software (IBM Corp, Armonk, NY, USA). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for control versus Roxadustat treatment. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Roxadustat can prolong the survival of mice with DSS-induced colitis with low toxicity

First, we selected several compounds clearly reported in the literature to alleviate DSS-induced colitis and the proline hydroxylase inhibitor Roxadustat, which were administered intraperitoneally to mice 24 h before and 24 h after DSS treatment, including Roxadustat, Dimethyloxalylglycine (DMOG), Lipopolysaccharide (LPS), and N-Acetyl-L-cysteine (NAC)(Fig. 1A) [18,21,22]. We continuously monitored body weight of mice fed DSS for one week in five groups (Fig. 1B). In our experimental system, we found that both Roxadustat and LPS can alleviate the weight loss of mice caused by DSS-induced colitis, while NAC and DMOG have no obvious effect. Further, we observed the survival of DSS-induced colitis mice within 20 days (Fig. 1C). We found that Roxadustat had the best alleviative effect among several compounds. Roxadustat significantly prolonged the survival of DSS-induced colitis mice, which was better than the other three compounds. So we further gave mice an intraperitoneal injection at 20 times the protective dose to compare the toxicity of several compounds. We found that the toxicity of

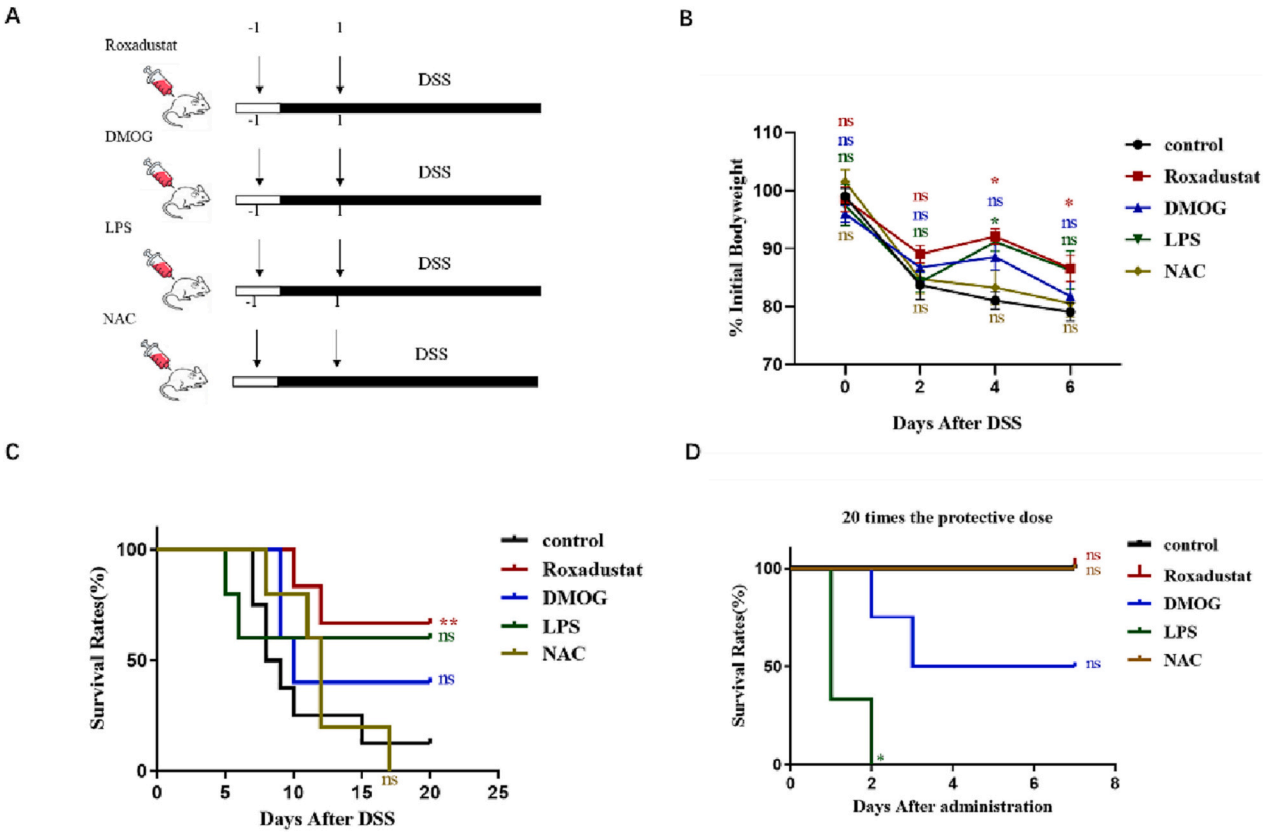

Fig. 1. Roxadustat has advantages over several other DSS-induced colitis treatment compounds. A. Intraperitoneal injection of four compounds in DSS-induced colitis mice. B. Body weight monitoring in mice with DSS-induced colitis within one week after administration of four compounds. (control, $n = 6$; Roxadustat, n = 6; DMOG, n = 6; LPS, n = 6; NAC, $n = 6$.) C. 20 days survival observation of DSS-induced colitis mice after administration of four compounds. (control, $n = 8$; Roxadustat, n = 6; DMOG, $n = 5$; LPS, n = 5; NAC, $n = 5$.) D. 20 times protective doses administration dose for one week survival observation. (control, $n = 3$; Roxadustat, n = 3; DMOG, $n = 4$; LPS, n = 3; NAC, $n = 3$.) Different colors represent the results of the comparison of the corresponding groups with the control group.

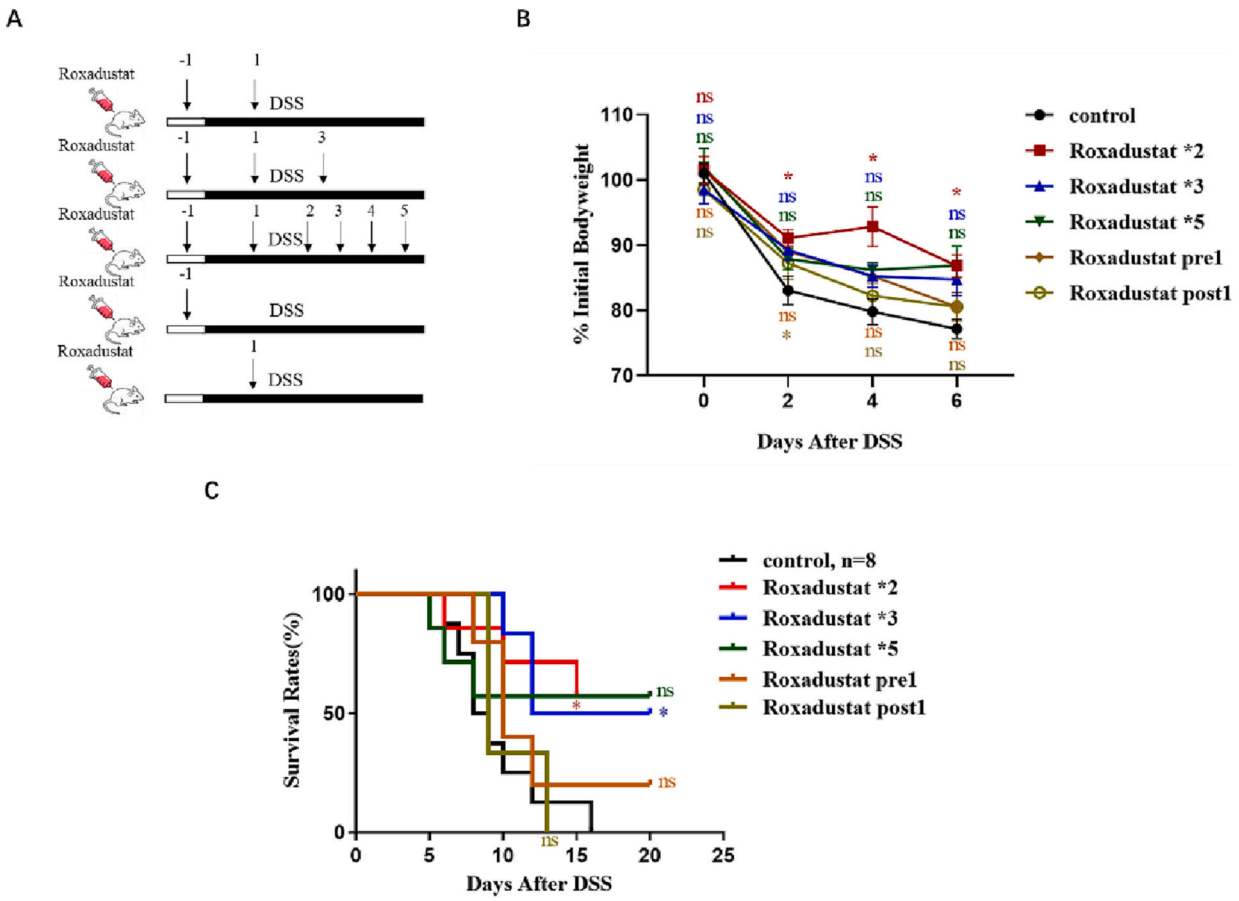

Fig. 2. Optimal dosing regimens of Roxadustat to relieve DSS-induced colitis in mice. A. Five Roxadustat dosing regimens. B. One-week body weight monitoring in mice with DSS-induced colitis after five dosing regimens. (control, $n = 8$; Roxadustat*2, $n = 7$; Roxadustat*3, $n = 6$; Roxadustat*5, n = 7; Roxadustat pre1, $n = 5$; Roxadustat post1, $n = 3$.) C. 20 days survival observation of DSS-induced colitis mice after five Roxadustat dosing regimens. (control, n = 6; Roxadustat*2, n = 6; Roxadustat*3, n = 6; Roxadustat*5, n = 6; Roxadustat pre1, n = 6; Roxadustat post1, $n = 6$.) Different colors represent the results of the comparison of the corresponding groups with the control group.

Roxadustat was similar to that of NAC, and much lower than that of DMOG and LPS (Fig. 1D). All the results indicated that Roxadustat has good research prospects for relieving DSS-induced colitis with low toxicity.

3.2. Two administrations of Roxadustat had a good alleviating effect on DSS-induced colitis

Therefore, we focused on the proline hydroxylase inhibitor Roxadustat to find the optimal dose for relieving DSS-induced colitis. We designed five dosing frequency regimens. Scheme 1 is administered 24 h before and 24 h after DSS feeding. Scheme 2 is administered 24 h before, 24 h and 72 h after DSS feeding. Scheme 3 is 24 h before DSS administration and four consecutive days after DSS feeding. Scheme 4 is administered 24 h before DSS is given. Scheme 5 is administered 24 h after DSS is given (Fig. 2A). We continuously monitored body weight of mice fed DSS for one week in six groups (Fig. 2B). We found that the three regimens of twice administration, three administrations and daily administration were more effective and had a good alleviating effect on weight loss in mice caused by DSS-induced colitis. Further, we monitored the effect of five dosing regimens on the survival of mice after 20 days of DSS feeding. More consistently, the three regimens of twice administration, three administrations and daily administration significantly improved survival in mice with DSS-induced colitis (Fig. 2C). Therefore, Roxadustat can alleviate DSS-induced colitis in mice, and the specific relief depends on the number of administration. The three regimens of twice administration, three administrations and daily administration were effective.

3.3. Two administrations of Roxadustat have a good relieving effect on DSS-induced colitis

Further, we observed the therapeutic effect by using three regimens respectively. Scheme 1 is administered 24 h before and 24 h after DSS administration. Scheme 2 is administered 24 h before, 24 h and 72 h after DSS administration. Scheme 3 is 24 h before DSS administration and four consecutive days after DSS administration. On the fifth day after administration of DSS, the mice were dissected, and the colons of the mice were taken out to compare the differences in the length of the colons of the mice in each group (Fig. 3A/B). The effects of the three dosing regimens were comparable. In the same way, the intestinal pathology of mice was taken for HE staining (Fig. 3C/D). The pathological score found that the colon pathological HE staining score of mice administered twice was better than that of mice administered three times and every day (Fig. 3E). The above results suggest that two administrations have a good relieving effect on DSS-induced colitis in mice, and the volume of administrations is low, causing little damage to the mice.

3.4. Roxadustat inhibits intestinal stem cell apoptosis and promotes intestinal stem cell proliferation

Further, we selected the NS group and Roxadustat twice administration group for TUNEL staining and Ki67 staining of intestinal tissue.

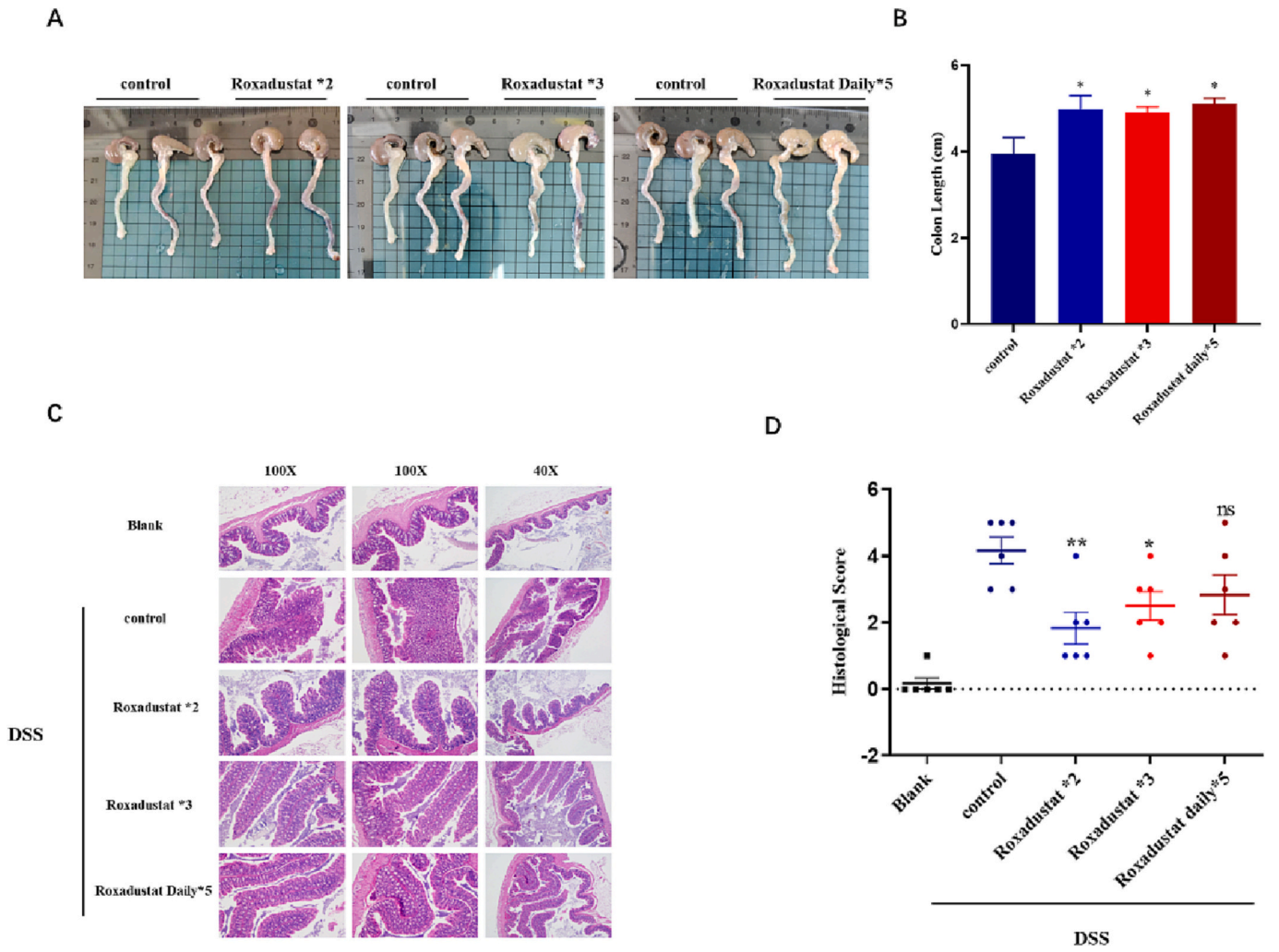

Fig. 3. Effects of three administration regimens on relieving DSS-induced colitis in mice. A. Comparison of colon lengths in mice on the fifth day after three dosing regimens. B. Colon length statistics of mice on the fifth day after three dosing regimens. *C.* Colon pathology in mice on the fifth day after the three dosing regimens. D. Colon pathology scores in mice on the fifth day after the three dosing regimens.

We observed the intestinal effects of Roxadustat on DSS-induced colitis mice. The results showed that Roxadustat could alleviate DSS-induced colitis in mice. TUNEL staining showed that Roxadustat could inhibit apoptosis of intestinal stem cell in DSS-induced colitis mice (Fig. 4A). Ki67 staining results showed that Roxadustat could promote the proliferation of intestinal stem cell in DSS-induced colitis mice (Fig. 4B). These results suggest that Roxadustat can alleviate DSS-induced colitis in mice.

3.5. Roxadustat promotes the recovery of hematopoietic system in DSS-induced colitis mice

On the other hand, we detected the changes of hematopoietic system. Roxadustat was intraperitoneally injected twice 24 h before and 24 h after DSS administration, and 2.5% DSS was given for five days. On the fifth day, the mice were taken blood from the orbit, and their blood routine was detected. It was found that the mice fed with DSS the reduction of red blood cell (RBC) count, hemoglobin (HGB) concentration and hematocrit and platelet (PLT) was alleviated after administration of Roxadustat (Fig. 5A). Compared with the NS group, red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT) and platelet (PLT) count were significantly increased in the Roxadustat group. In addition, we took the mouse bone marrow and washed it into a single-cell suspension. Flow staining cytometry was used to detect the differentiation of hematopoietic stem cells. It was found that with the increase of the number of administrations, the proportion of differentiated into mature erythrocytes also increased (Fig. 5B). These results showed that Roxadustat could promote the recovery of hematopoietic system in DSS-induced colitis mice.

3.6. Identification of DEGs after Roxadustat treatment

In order to find the target of action of Roxadustat in alleviating DSS-induced colitis in mice, we performed transcriptome sequencing of colon tissues after two doses of the Roxadustat (3:3). To determine how Roxadustat exerts a protective effect in DSS-induced colitis, we compared the overall gene expression profiles in the intestine of mice between NS and Roxadustat groups. Differentially expressed genes were detected using the cut-off value log2 (fold change) >0.58, $P < 0.05$. In three pairs of colon samples from NS and Roxadustat groups, a total of 626 differential genes were detected by RNA-Seq. Hierarchical clustering analysis indicated that the gene expression patterns in the colon of NS and Roxadustat groups were distinguishable (Fig. 6A). Compared with NS group, 466 genes were significantly up-regulated and 160 genes were significantly down-regulated in the gut of Roxadustat group. The volcano plot can visually see that the up-regulated and down-regulated genes in the colon of Roxadustat group were significantly different (Fig. 6B). Among them, there are 13 genes in the RNA-Seq dataset that

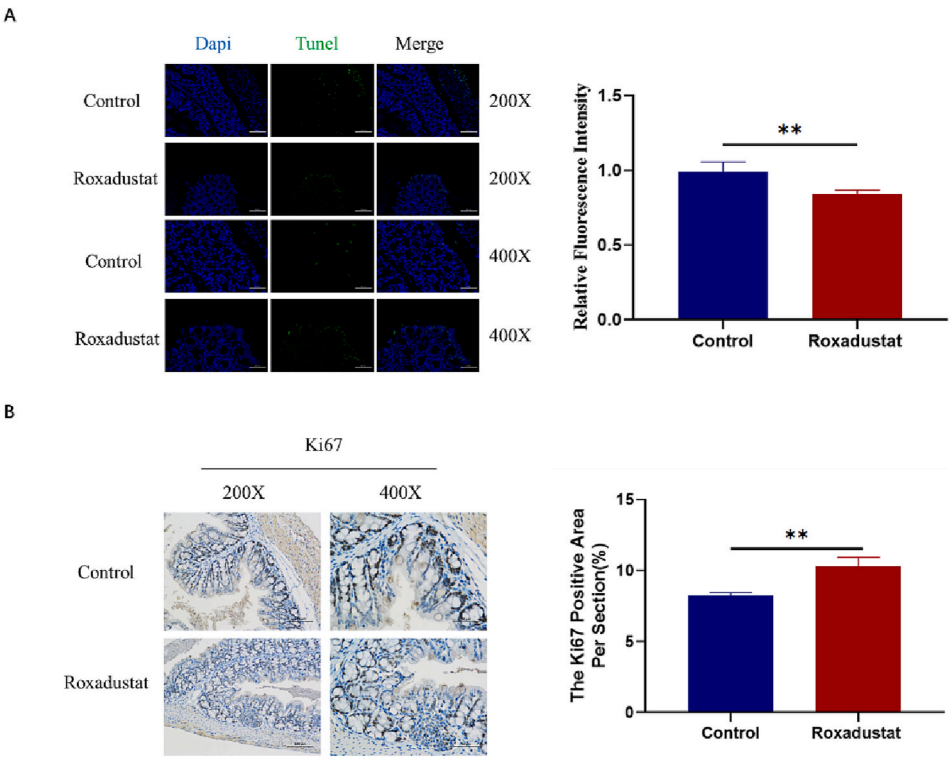

Fig. 4. Roxadustat protects intestinal tissue from injury. A. The representative images of TUNEL-stained intestinal sections with the indicated treatment at 5d after DSS feeding. B. The representative images of Ki67-stained intestinal sections with the indicated treatment at 5d after DSS feeding.

may play a role in Roxadustat to alleviate DSS-induced colitis, such as TLR4, Tifa, Sox2, Serpina10, S100a14, Muc1, Mal, Hgf, Gsn, Fzd9, Fgf10, Defa28 and Cd44 (Fig. 6C). These 13 genes were associated with inflammatory responses and TLR4 signaling [23–27]. We are familiar with these genes because we frequently use them in experimental systems. As expected, the expression patterns of selected genes in the colon were markedly different between NS and Roxadustat groups.

Further we performed gene KEGG pathway enrichment analysis. KEGG database analysis showed that there were differences in 255 pathways between NS and Roxadustat groups. Among the KEGG total differential pathways in NS and Roxadustat groups, we selected representative 30 signaling pathways and performed KEGG enrichment. The six most significant pathways among these 30 pathways are IL-17 signaling pathway ($P < 0.001$), TNF signaling pathway ($P < 0.001$), Inflammatiory bowel disease (IBD) ($P < 0.001$), Toll-like receptor signaling pathway($P < 0.05$), NF-kappa B signaling pathway($P < 0.05$) and HIF-1 signaling pathway ($P < 0.05$) (Fig. 6D). GO results showed negative regulation of inflammatory response (GO:0006954), cell wall disruption in other organism (GO:0044278), defense response to Gram-positive bacterium (GO:0050830), antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844) and defense response to Gram-negative bacterium (GO:0050829) were significantly enriched (Fig. 6E). These pathways may play a role in Roxadustat to alleviate DSS-induced colitis.

3.7. Roxadustat alleviates DSS-induced colitis mainly through several molecules represented by TLR4

Among the up-regulated molecules, we selected TLR4 as the key downstream molecule. In our previous work, TLR4 played a key role in the alleviation of DSS-induced colitis [28,29]. By qRT-PCR, we found that Roxadustat could significantly upregulate the mRNA level of TLR4. In addition, qRT-PCR showed that Roxadustat could also up-regulate the mRNA levels of Tifa, Sox2, Muc1, Fzd9 and CD44 (Fig. 7A). Next, the role of TLR4 in Roxadustat in alleviating DSS-induced colitis was validated by using TLR4 KO mice. TLR4 KO mice and wild-type mice were divided into NS group and Roxadustat group. By recording the 20 days survival of DSS-induced colitis mice, we found that the mortality of TLR4 KO mice was not statistically significant between the NS group and the Roxadustat group (Fig. 7B). However, the mortality of wild-type mice was statistically significant between the NS group and the Roxadustat group. In addition, mice were intraperitoneally injected with Roxadustat 24 h before and 24 h after DSS feeding, and the intestinal tissues of mice were taken for TLR4 immunofluorescence at 5 d after DSS feeding. The results showed that the expression of TLR4 in intestinal crypts in the Roxadustat administration group was significantly higher than that in the non-administration group (Fig. 7C). This further indicated that TLR4 may play an important role in the alleviation of DSS-induced colitis in mice by Roxadustat(Fig. 7D).

4. Discussion

In this study we showed that the proline hydroxylase inhibitor Roxadustat significantly ameliorates the severity of disease and accelerates recovery in mice model of DSS-induced colitis. The current therapeutic measures for IBD is significantly limited. At present, the problem that needs to be solved in the field of IBD treatment is to find high-efficiency and low-toxicity therapeutic drugs. The occurrence of IBD will lead to the impairment of intestinal epithelial function, which will lead to the non-specific entry of the intestinal mucosal luminal antigens into the lamina propria, activation of the mucosal immune system, and further aggravation of inflammation. It is extremely harmful to the intestine, so it is extremely necessary to find suitable therapeutic target pathways.

At present, there have been many reports on the prevention and treatment of IBD by compounds related to the PHD-HIF pathway. The main mechanisms by which proline hydroxylase inhibitors play a protective role in IBD are as follows. First, inhibits intestinal epithelial cell apoptosis and increases intestinal epithelial integrity to exert its barrier function [30]. Second ，enhanced expression of barrier protective genes

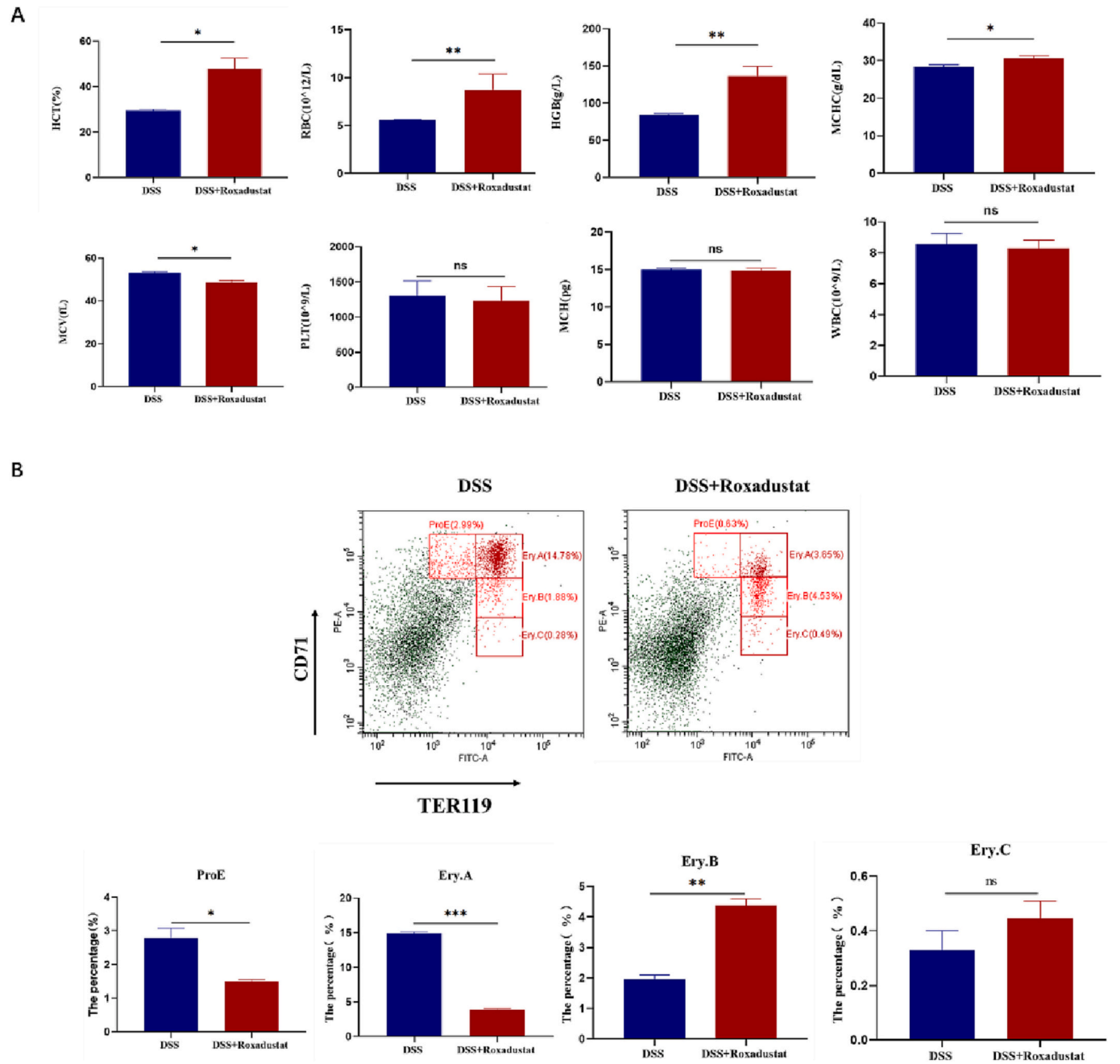

Fig. 5. Roxadustat promotes the recovery of hematopoietic system in DSS-induced colitis mice. A. Routine blood test was performed on the fifth day of DSS feeding in Roxadustat group and NS group. B. Differentiation of erythrocyte in mice on the fifth day in NS group and Roxadustat group. Ter119 (erythrocytes marker) and CD71 (erythroid precursors marker).

[31]. Finally, promotion of neutrophil apoptosis [32]. As a proline hydroxylase inhibitor, Roxadustat inhibits the apoptosis of intestinal epithelial cells, initiate inflammatory and immune responses, and promotes damage repair.

In this paper, we selected several compounds reported in the literature that can alleviate DSS-induced colitis and proline hydroxylase inhibitor Roxadustat. Then, we used the method of DSS-induced colitis to compare the alleviating effects of several compounds on DSS-induced colitis in terms of both body weight and survival. We concluded that Roxadustat has better alleviation effect and lower toxicity than the other three compounds. Therefore, we further explored the appropriate dosing schedule for Roxadustat. The five dosing schedules were also compared in terms of body weight and survival. We found that the two-dose regimen provided better relief of DSS-induced colitis than other dosing regimens. In addition, two administrations were economical and less harmful to the mice. The Roxadustat group had longer colonic length, more mature erythrocyte differentiation, more crypts and longer villi than the NS group. Roxadustat can reduce apoptosis and increase proliferation of intestinal tissue. All aspects suggest that Roxadustat has a good protection effect. Therefore, we deeply explored the mechanism of its intestinal protective effect. Transcriptome sequencing of mice intestine after two doses of Roxadustat. The sequencing results found that TLR4, Tifa, Sox2, Serpina10, S100a14, Muc1, Mal, Hgf, Gsn, Fzd9, Fgf10, Defa28 and Cd44 was significantly up-regulated. IL-17 signaling pathway, TNF signaling pathway, Inflammatiory bowel disease (IBD), Toll-like receptor signaling pathway, NF-kappa B signaling pathway and HIF-1 signaling pathway were significantly enriched. Therefore, we carried out further mechanism verification. We used qRT-PCR to verify

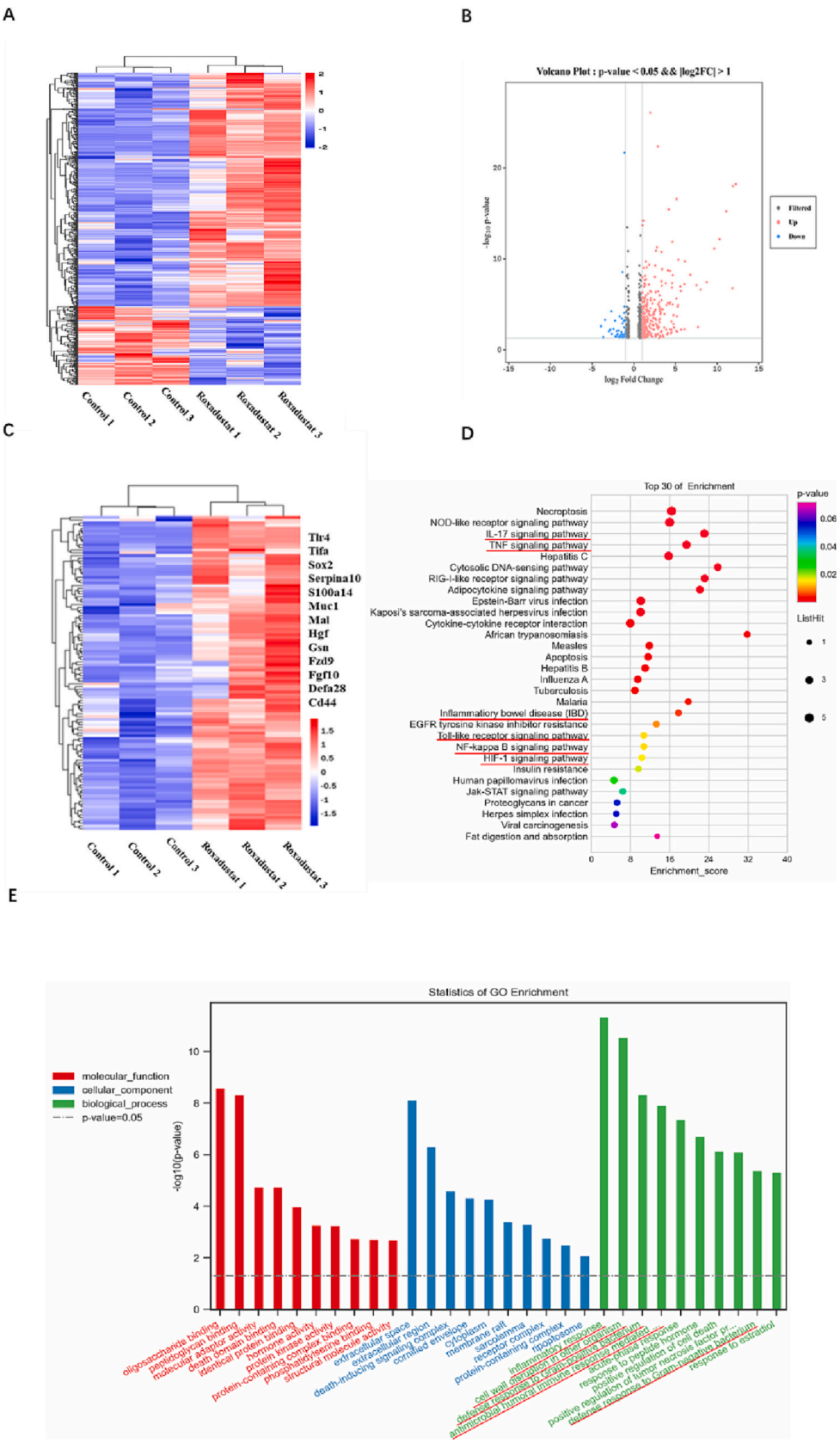

Fig. 6. Differential gene expression between the NS and Roxadustat groups of colon was analyzed by high-throughput RNA-Seq. A. Heat map of differential genes expression between NS and Roxadustat groups of colon. B. Scatter plot of differently expressed genes in colon tissue. Each dot stands for a gene. Red and blue color dots indicate an increase or decrease, respectively, of >log2(fold change) of 0.58 and $P < 0.05$ in Roxadustat group compared to the NS group. C. Heatmap of up-regulated genes after administration of Roxadustat. 13 representative genes were marked. D. Enrichment of the top 30 pathways after administration of Roxadustat. E. GO term analysis was performed on differentially expressed genes.

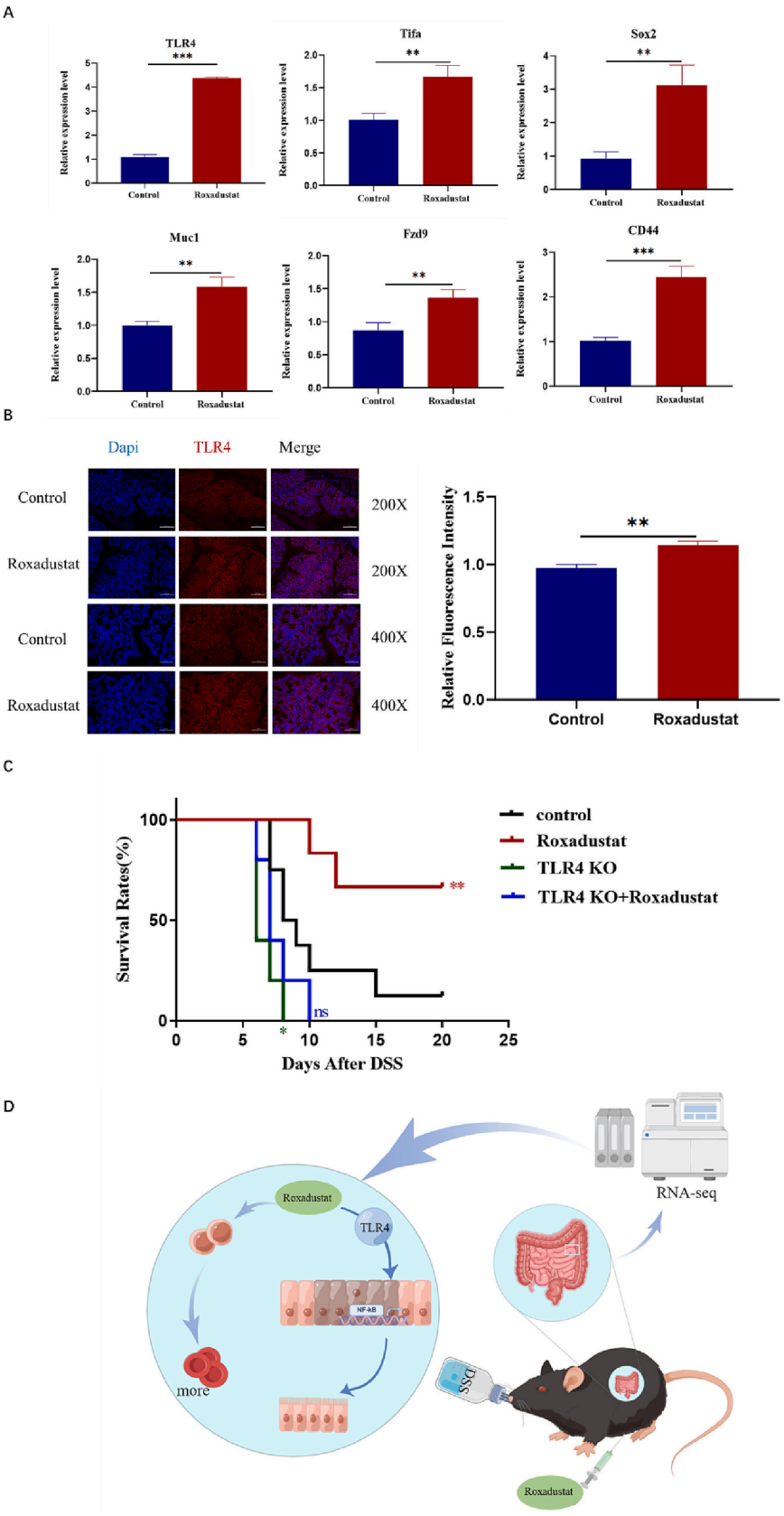

Fig. 7. TLR4 plays an important role in Roxadustat alleviating DSS-induced colitis in mice. A. Relative expression of TLR4 mRNA in NS group and Roxadustat group, including Tifa, SOX2, Muc1, Fzd9 and CD44. B. Immunofluorescence expression of TLR4 after DSS feeding between wild-type mouse gut and Roxadustat mice guts. C. Survival observation of wild-type mice and TLR4 KO mice after administration of Roxadustat within 20 days of DSS-induced colitis. (control, $n = 4$; Roxadustat, $n = 6$; TLR4 KO, $n = 5$; TLR4 KO+ Roxadustat, n = 5) D. The pattern of Roxadustat in treating colitis.

that TLR4, Tifa, Sox2, Muc1, Fzd9 and CD44 was significantly upregulated in the Roxadustat group. Then, We used TLR4 KO mice to validate the role played by TLR4 in Roxadustat in alleviating DSS-induced colitis. In addition, TLR4 immunofluorescence assay confirmed that Roxadustat significantly upregulated the expression of TLR4 in intestinal crypts after DSS feeding. These results demonstrate that Roxadustat exerts a role in alleviating DSS-induced colitis that is dependent on TLR4.

5. Conclusion

In conclusion, in this study we found that Roxadustat could alleviate DSS-induced colitis mainly by targeting TLR4 in vivo. Roxadustat has low toxicity and offers a new direction for the treatment of IBD.

Ethics approval and consent to participate

All animal experiments conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 85–23, National Academy Press, Washington, DC, revised 1996), with the approval of the Laboratory Animal Center of the Naval Medical University, Shanghai.

Consent for publication

Written informed consent for publication was obtained from all participants.

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Authors' contributions

Jianming Cai, Qi Wang, and Cong Liu designed the study. Zhenlan Feng, Ying Cheng and Yuedong Wang performed the experiments. Shugen Qu , Jicong Du and Fu Gao analyzed the data. Zhenlan Feng wrote the paper, Jianming Cai,Fu Gao and Cong Liu supported fund assistance. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors confirm that there are no conflicts of interest.

Data availability

All data generated or analyzed during this study are included in this published article.

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