Different Mechanism and Efficacy of Dextran-Sodium Sulfate and 2,4,6-Trinitrobenzene Sulphonic Acid in Modeling Ulcerative Colitis in C57BL/6 Mice

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Research Article

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Different Mechanism and Efficacy of Dextran-sodium Sulfate and 2,4,6-Trinitrobenzene Sulphonic Acid in Modeling Ulcerative Colitis in C57BL/6 Mice

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Abbreviations: UC, ulcerative colitis; IBD, inflammatory bowel disease; DSS, Dextran-sodium sulfate; TNBS, 2,4,6-Trinitrobenzene sulphonic acid; DAI, disease activity index; HE, hematoxylin-eosin; IL-1, interleukin-1; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor α; IL-8, interleukin-8; IL-2, interleukin-2; IL-10, interleukin-10; phosphate buffer saline, PBS; propidium iodide PI; enzyme-linked immunosorbent assay, ELISA; Eppendorf, EP; hematoxylin-eosin(HE).
Abstract

**Background and Aims:** Dextran-sodium sulfate and 2,4,6-trinitrobenzene sulphonic acid are common modeling methods in studying ulcerative colitis. Little attention has been paid to the mechanism differences between the two approaches. Here, we aim to compare the mechanisms and efficacy of these two models and wish to provide fundamental proves for choosing ideal ulcerative colitis models.

**Methods:** Dextran-sodium sulfate and 2,4,6-trinitrobenzene sulphonic acid were applied to induce the colitis in C57BL/6 mice for seven days. Body weight and disease activity index were assessed. Hematology was detected by routine blood test. Histopathology was analyzed by hematoxylin-eosin staining section. Enzyme-linked immunosorbent assay, Western blot and quantitative real-time PCR were used to detect the cytokines protein levels and mRNA levels. Flow cytometry were used to detect the cycles and subsets of splenic cells.

**Results:** Dextran-sodium sulfate induced colitis in C57BL/6 mice showed higher acute immune activities, while 2,4,6-trinitrobenzene sulphonic acid induced colitis showed chronic immune activities with high platelet amounts and activation. Dextran-sodium sulfate is more suitable for modeling acute ulcerative colitis. On the contrary, 2,4,6-trinitrobenzene sulphonic acid is more appropriate for modeling chronic ulcerative colitis.

**Conclusions:** Dextran-sodium sulfate treatment within 7 days in C57BL/6 mice is a suitable experimental model for studying human acute ulcerative colitis with immune response, fecal blood and acute pathogenic damage. Conversely, 2,4,6-trinitrobenzene sulphonic acid treatment within 7 days is more appropriate for studying human chronic ulcerative colitis with hypercoagulable state, IL-2 over-expression state and chronic pathogenic damage.
Keywords: dextran sulfate sodium; 2,4,6-trinitrobenzene sulphonic acid; ulcerative colitis; mechanism; efficacy.

1. Introduction

Ulcerative colitis (UC) is a typical inflammatory bowel disease (IBD) with recurring and complicated specifics. \[1,2\] Compared with industrializing countries, industrialized countries have shown higher incidence and prevalence with industrialization and social development. \[3,4\] With the development of industrializing countries, UC is becoming an inescapable challenge for global public health. Recent studies showed that IBD is caused by a dysregulated three-way relationship between genetic susceptibility, microbiota, and immune dysregulation, which are influenced by genetic factors, environmental factors, sex, geography, age, dietary structure, lifestyle and breastfeeding, etc.. However, the etiology and mechanisms of UC have not been comprehensively revealed. \[4,5,6\]

UC is characterized by bloody stools, diarrhea, mucus stools, and urgency. The typical clinical treatments for UC include anti-inflammatory, biologic, immunomodulatory, and surgical therapies. \[5,7\] However, several unsolved problems including the adverse reaction of traditional drugs, the high threshold, incomplete stem cell therapy, lack of medical evidence of herbal medicine, and the prognosis of surgical treatment associated with colon cancer risk make it an urgent need to develop new therapies for UC. \[8,9,10\] Hence more academic research on UC will be carried out in the future.

The establishment of the UC model is essential in UC research. The widely known modeling methods are dextran sodium sulfate (DSS), 2,4,6-trinitrobenzene sulfonic acid (TNBS), oxazolone, acetic acid, and sulfhydryl inhibitors \[11\], yet the oxazolone, acetic acid, and sulfhydryl inhibitors are
not frequently applied due to their high lethality. DSS causes direct chemical damage to the intestinal
epithelium, exposing the submucosa and lamina propria to bacteria, thus triggering an inflammatory
response.\textsuperscript{[12]} The TNBS induced colitis tends to develop laterally, eventually developing into
transmural colitis, which is a feature common to human Crohn's disease. Repeated administrations
of TNBS enemas to mice lead to cobblestone-like ulcers in the distal colon. This intestinal ulcer is
usually considered characteristic of the grossly altered colon in patients with Crohn's disease.
Therefore, the pathogenesis of TNBS model is considered to be more inclined to Crohn's disease
than UC model.\textsuperscript{[13]} However, TNBS is also considered to be an effective modeling method of
ulcerative colitis.\textsuperscript{[14]} Overall, the controversy that whether DSS or TNBS is more suitable chemical
methods in UC’s research is exist.

The modeling mechanism of DSS and TNBS is not revealed completely. Only a few literatures have
described the efficacy of UC animal models from single perspectives. There is a lack of description
of the criteria for modeling success and model selection. To better understand the efficacy of these
two models, we investigated the pathological injury and the immune responses accordingly. The
results showed that DSS-induced colitis tends to trigger acute immune activities in C57BL/6 mice,
while TNBS induced colitis showed chronic immune activities with high platelet amounts and
activation in C57BL/6 mice. This study can provide multidimensional evidence for the selection of
experimental UC animal models.

3. RESULTS
3.1 Differences in ordinary circumstances and DAI scores between the DSS and TNBS models

The general condition and disease activity of mice with colitis are the classical evaluation indexes of animal models. The ordinary circumstance showed that the DSS group mice had obvious symptoms such as decreased spirit, loss of appetite, hair disorder, loose stools and bloody stools.

In order to assess the effects of DSS and TNBS modeling, the body weights of mice were evaluated: As shown in Figure 1, the disease activity in the DSS group mice increased within 7 days, which was consistent with the studies by Jialing L et.al. Different from DSS-treated mice, the TNBS-treated mice had more obvious and severe symptoms in diarrhea and loose stools at the first three days of modeling with less blood in the feces, which gradually subsided afterward.

Their DAI gradually decreased in the late stage of modeling and increased in the early stage of modeling. Significant blood stools were seen in those mice from the DSS group at the late modeling stage, while the other two groups weren’t.
Figure 1 Body weight changes, fecal shape, fecal occult blood and disease activity score (DAI) of different groups of C57 mice during the feeding period

The body weight of DSS group gradually decreased from day 4. In contrast, the body weight of the TNBS group gradually decreased from day 1 to day 3, and gradually increased from day 4. The DAI of the TNBS group was higher than the other two groups. Yet DAI scores of the DSS group became highest afterward. *: Compared with control group; #: Comparison between DSS group and TNBS group. Date are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.2 Differences in peripheral blood immune response between DSS and TNBS models
Colonic injury in UC is often inseparable from inflammation. And hematology analysis is a classic test for inflammatory diseases in clinical practice. To explore the inflammation in mice, we examined hematological indices. First, we tested the blood routine of mice. As Figure 2 showed that red blood cells count, hematocrit, hemoglobin, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were decreased significantly in the DSS group, while those in the TNBS group increased significantly.

Figure 2 Results of routine blood tests: We performed routine blood tests in three groups of
mice to study the effects of DSS and TNBS-induced colitis on peripheral blood

A. Red blood cell count; B. Hemoglobin assay; C. Mean cell hemoglobin; D. hematocrit; E. Mean cell hemoglobin concentration; F. Standard deviation (SD) of red cell distribution width; G. Coefficient variation (CV) of red cell distribution width; H. Platelet count; I. Mean platelet volume; J. Lymphocyte count; K. Lymphocyte percentage; L. Granulocyte count; M. Granulocyte percentage; N. Intermediate cells count; O. Intermediate cells percentage. Date are means ± SD.

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

On the other hand, as Figure 3 showed that white blood cells count, containing lymphocyte, granulocyte, and intermediate cell, was significantly increased in the DSS group. Meanwhile, DSS and TNBS showed an upregulating trend of lymphocyte ratio. TNBS significantly increased the ratio of intermediate cells (including monocytes, eosinophils, and basophils 3 types). Moreover, the total platelet counts and mean platelet volume were significantly increased in the TNBS group. Instead, the mean platelet volume in the DSS group was significantly decreased.

As a result, DSS-treated mice showed an acute immune response in peripheral blood and anemia with a bleeding state while TNBS treated mice showed a hypercoagulable state. To further investigate the immune response in peripheral blood, the expression of serum cytokines was examined. As is shown in Figure 3, serum IFN-γ, IL-2, TNF-α were significantly downregulated in the DSS group, while serum TNF-α was the only cytokine that significantly decreased in the TNBS group. Additionally, serum IL-1β and IL-2 were significantly increased in TNBS group. Serum IL-8 and IL-10 were not significantly changed in both groups. Consequently, IFN-γ and TNF-α were
decreased in DSS-treated mice, which might be relative to the movement of macrophages. IL-2 and IL-1β were increased after TNBS treatment, which might link to the immune response of related immune cells.
To further investigate the peripheral blood immune cell subgroups of the two UC model mice, flow cytometry analysis was performed. The results showed that the percentage of total leukocytes was

Figure 3 The expression of immune cytokines and percentages of immune cell subsets in
peripheral blood: The expression of IFN-γ, IL-1β, IL-2, TNF-α, IL-8 and IL-10 are showed in A–F; Total leukocytes, CD45+granulocyte percentage, CD3+T cell percentage, NK1.1+ T cell percentage, F4/80+macrophage percentage, and B cell percentage are showed in G–L. Date are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

significantly increased in the DSS group. On the contrary, the percentage of total leukocytes was significantly decreased in the TNBS group. Subsequently, the percentage of CD45+granulocytes was significantly increased in the DSS group, which dispel the over-response of CD45+granulocytes. However, percentages of CD3+T cells, NK1.1+ T cells, and F4/80+macrophages were significantly decreased in the DSS group. In addition, CD19+ B cells were significantly reduced in the TNBS group, while that in the DSS group showed an upregulating trend. In a comprehensive analysis, the increased total leukocytes in the DSS group, compatible with blood routine tests, were mainly covered increased granulocytes and B cells, confirming the short-term modeling of DSS as a feature of acute inflammation. Whereas an increasing trending of CD3+T cells response was appeared in TNBS group, as well as F4/80+ macrophages.

3.3 Differences in morphological damage in the colon of mice between the DSS and TNBS models

First, an immunological assessment of the colon locally was performed. As shown in Figure 4: no significant pathological damage was seen in Control group; the proximal cecum segment of the colon in DSS group was heavily congested and edematous, with varying degrees of mucosal necrosis and ulcer formation visible in each sample. The proximal cecum segment in TNBS group was mildly to moderately congested, with necrotic areas visible in some samples. Next, the CMDI
score was calculated: colonic weight and CMDI scores were significantly higher in both of DSS group and TNBS group; CMDI scores in DSS group were significantly higher than those in TNBS group, while colonic weight in DSS group was significantly lower than that in TNBS group. The length of the colon was also measured. The colonic length was significantly shorter in DSS group. Nevertheless, no significant change of colonic length appeared in TBNS group. Next, the colon of mice in pathological sections was evaluated, and the results showed that the colon tissues of mice in DSS group all showed damage to the mucosal layer, submucosal layer and muscular layer; local loss of epithelium in the mucosal layer; some of the mucosal layer epithelium was necrotic and hemorrhagic; the structure of the lamina propria was damaged; a large number of inflammatory cells
or new granulation tissue was seen in all layers; the crypts were lost.

Figure 4 Pathogenic damage of mice colon from each groups: Mice were executed on the 8th day of modeling, and the colon tissue was collected, photographed, weighed, length measured and CMDI scored. Colon tissue were fixed in 4% paraformaldehyde through Swiss rolls shapes, embedded and sectioned for pathological diagnosis. A. Weight of colon from each groups; B. Length of colon from each groups; C. CMDI assessment of colon from each group; D. microscope F

Length of colon from each groups; C. CMDI assessment of colon from each group; D. microscope
observation after hematoxylin-eosin staining. E. Histopathological score of colon; F. Colon from each groups observed by nick eyes. Date are means ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Partial colonic inflammation penetrated the entire intestinal wall. Meanwhile, we evaluated the pathological tissue sections of mice with HS scores, which showed that (1) inflammatory cell infiltration score: DSS=3, 3 mice with 1 score in TNBS, and the rest with 0 score; (2) crypt damage score: DSS=2, TNBS=0. Thus, there are differences in damage to the colon of mice by both DSS and TNBS modeling methods, in which the inflammatory cell infiltration and colonic injury in the late stage of DSS modeling were the most serious, while the inflammatory cell infiltration and colonic injury in the late stage of TNBS modeling were irregular.

3.4 Differences in synthesis and expression of colonic inflammatory cytokines in DSS and TNBS models

The involvement of a series pro-inflammatory factors promotes the expansion of colitis. In human IBD, various immune cells and stromal cells such as macrophages, dendritic cells (dc), effector T cells produce increased amounts of soluble and membrane-bound TNF, which in turn promotes macrophage secretion of IL-1 and TNF-α. \cite{18} INF-γ, IL-2, IL-8, IL-10 are typical cytokines produced by different immune cells. In order to investigate the cytokines activities in colitis induced by different methods, RT-qPCR was used to explore the mRNA expression of colon cytokines. As shown in Figure 5, the IL-1β mRNA and IL-10 mRNA were significantly upregulated in DSS group. INF-γ mRNA, IL-2 mRNA, TNF-α mRNA, IL-8 mRNA expressed as a slight upregulating trend in DSS group. What’s more, only IL-2 mRNA showed a trend of upregulation in TNBS group. Thus,
colonic mRNA of IL-1β, IL-10 in DSS group showed a significantly increase, which showed a potential mechanism of macrophages and regular cells related with IL-10. Meanwhile, IL-2 showed an upregulating trend in TNBS treated mice, which might explain the vital role of activated Th2 or NK1.1+ cells in TNBS treated mice. Due to the potential mechanism of IL-1β, IL-2 and IL-10,
Western blot was used to detect these colon cytokines.

Figure 5 The mRNA expression of colonic cytokines in different groups of C57 mice: After 7 days of continuous feeding with DSS or a single enema with TNBS, we detected the mRNA expression of colonic cytokines by RT-qPCR. A. The mRNA expression of colonic IFN-γ; B. The mRNA expression of colonic IL-1β; C. The mRNA expression of colonic IL-2; D. The mRNA expression of colonic TNF-α; E. The mRNA expression of colonic IL-8; F. The mRNA expression of colonic IL-10; G. The mRNA expression of splenic IFN-γ; H. The mRNA expression of splenic IL-1β; I. The mRNA expression of splenic IL-2; J. The mRNA expression of splenic TNF-α; K. The mRNA expression of splenic IL-8; L. The mRNA expression of splenic IL-10.
mRNA expression of colonic IL-1β; C. The mRNA expression of colonic IL-2; D. The mRNA expression of colonic TNF-α; E. The mRNA expression of colonic IL-8; F. The mRNA expression of colonic IL-10. G. The mRNA expression of splenic IFN-γ; H. The mRNA expression of splenic IL-1β; I. The mRNA expression of splenic IL-2; J. The mRNA expression of splenic TNF-α; K. The mRNA expression of splenic IL-8; L. The mRNA expression of splenic IL-10; Date are means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

As is shown in Figure 6, IL-1β: Compared with control group and TNBS group, DSS group shows a significant increase, while no significant change happened in Control group and TNBS group; IL-10 also shows the same condition as IL-1β. However, IL-2 shows an upregulating trend in TNBS groups when compared with control group and DSS group.

Figure 6 The expression of cytokines in different groups of C57 mice (colonic and splenic cytokines): After 7 days of continuous feeding with DSS or a single enema with TNBS, we detected the expression of splenic cytokines by Western Blot. The samples derive from the same experiment and those blots were processed in parallel. A. The expression of colonic IL-1β; B. The expression of colonic IL-2; C. The expression of colonic TNF-α; D. The expression of splenic IL-1β; E. The expression of splenic IL-2; F. The expression of splenic TNF-α; G. The expression of splenic IL-8; H. The expression of splenic IL-10;
3.5 Differences in the effects of DSS and TNBS staging on the spleen

Spleen, as a center of systematic immune, is reported to be influenced after colitis induced in animal models. To better understand the difference of systematic immune activity between these two modeling methods, we also investigated the splenic immune cells subsets by flow cytometry, and the expression levels of several cytokines by Western blot and RT-qPCR. As shown in Figure 5, the mRNA expression of IL-2 and IL-10 were significantly upregulated in TNBS group. The mRNA expression of IL-10 was significantly upregulated in DSS group. Also, we detected the expression of IL-1β, IL-2 and IL-10 in splenic issues through western blot. As shown in Figure 6, IL-10 significant upregulation in DSS group can be seen, and significant increase of IL-2 also showed in TNBS group, which provide the best agreement with the situation of mRNA expression. Interestingly, a significant downregulation of IL-1β was observed in DSS group. Besides, no significant change of IL-10 expression was detected in TNBS group, which means IL-10 transcription was upregulated but failed to finish its generation. Sequencely, we investigated the splenic immune cells subsets. As shown in Figure 7, the splenic CD45+ granulocytes were significantly decreased in DSS group and TNBS group. CD3+ T cells were significantly decreased in DSS group; NK1.1+ cells in TNBS group were significantly increased. F4/80+ Macrophages in TNBS group were significantly increased. CD19+ B cells were significantly increased in DSS group,
while significantly decreased in TNBS group.

Figure 7 The percentage of splenic immune cells in different groups of C57 mice: Different immune cells subsets were detected through flow cytometry. A. The percentage of CD45+ granulocytes; B. The percentage of CD3+ T cells; C. The percentage of NK1.1+ T cells; D. The percentage of F4/80+ macrophages; E. The percentage of CD19+ B cells; F. The proliferation of splenic cells; G. Splenic resting cells; H. Splenic disintegrating cells; I. Splenic proliferating cells. Date are means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
Furthermore, cell cycle of splenic cells was detected by flow cytometry. As shown in Figure 7, the percentage of resting phase cells was significantly decreased in DSS group with the significant increasing percentage of mitosis phase cells. The percentage of proliferating cells was increased significantly in DSS group. Splenic Cells from TNBS treated mice showed a tendency of proliferating and dividing, but the results have no statistical significance with control group.

Therefore, DSS induced colitis can lead to over-expression of splenic function, which is related with the systematic immune response.

4. Discussion

Animal experiments on ulcerative colitis have been hot topics of researches. Most experimental animal modeling methods are based on DSS and TNBS, which are water-soluble, negatively charged sulfated polysaccharides that induce colitis by causing direct chemical damage to intestinal epithelial cells mucosa. In 1990, Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y and Nakaya R successfully induced colitis in mice with DSS for the first time. The TNBS chemical solution, when applied, requires mixing with ethanol solutions of different concentrations, the principle of which is to activate the immune response by connecting ethanol stimulation to burn the colonic mucosa so that TNBS enters the mucosa. In 1966, Little JR and Eisen HN first proposed the TNBS modeling method, which has since been used in various experimental animals. Some studies have initially explored the differences between the two models of DSS and TNBS through endoscopy, pathological diagnosis, or a class of bio-signaling molecules alone. Hitherto, some scholars believe that TNBS is more like human Crohn's disease in
some pathological mechanisms. However, there is no study to explore the effect of these two models on the efficacy of human UC disease model from the perspective of local colon and overall immune response. In our study, morphological damage, colonic immune response, and overall immune response of C57BL/6 mice were investigated by modeling with DSS and TNBS, respectively. Importantly, we found significant differences in morphology, local and overall immune response between the two models, which directly guide the different applicability of the two models. The ordinary circumstances of DSS group mice showed a progressive aggravation, which were similar to the period of human IBD activity. And the circumstances of TNBS group mice were more like the remission period of human IBD.

Colonic injury is a typical lesion common to IBD. The common features of the colon in patients with ulcerative colitis are erythema and fine-grained surface seen in mild inflammation, mucosal bleeding, edema, and ulceration in severe lesions.\[^5\] In this experiment, the colons of DSS group mice were significantly wrinkled, with obvious macroscopic mucosal hemorrhage, edema and ulceration. In contrast, there was no significant change in colon length in TNBS group. The colon weight increased in both models, which was caused by colonic edema and congestion. Both models resulted in increased CMDI scores, among which the degree of colonic mucosal damage was more severe in the mice of the DSS group. Meanwhile, differences in colorectal pathogenic development direction also exist between the two models. DSS induced colitis started from the rectum and gradually extended to the proximal end, which was consistent with the direction of human UC.\[^27\] However, TNBS showed a progression from the cecum end to the colon end and mostly showed damage to the entire intestinal end, which was similar to CD in humans. The pathological changes
of UC patients under the microscope showed that the lesions were limited to the mucosa and
submucosa layer, sometimes deeper. For patients with chronic diseases, the lesions show distortion
of the colonic crypt structure and may have reduced crypt branching and number, often accompanied
by the increased distance between the base of the crypt and the mucosal muscle layer. Some
individuals have basal plasma cell and lymphocyte aggregation, and may have mucosal vascular
congestion, accompanied by edema and focal hemorrhage, and infiltration of inflammatory cells
such as neutrophils, lymphocytes, and macrophages. The present results showed that DSS-induced
severe colonic mucosal injury was characterized by massive inflammatory cell infiltration through
mucosal, submucosal, and muscular layers and severe crypt damage. DSS-induced damage in the
intestinal barrier further allowed the entry of bacteria and antigens from the intestinal lumen into
the intestinal mucosal layer, causing local inflammation throughout the intestinal wall, which was
in line with the lesions in human patients with fulminant or severe UC.\textsuperscript{[5,6,17]} The TNBS induced
epithelial damage in the mucosal layer, accompanied by loss of necrotic cell and partial crypt
damage. A small amount of inflammatory cell infiltration was seen in the lamina propria,
manifesting as mucosal erosion and localized congestion in the lamina propria, which is inseparable
from the cascade immune response caused by TNBS. The TNBS entered the mucosa and bound to
the lysine ε-amino group to form histone protein, which together formed a complete antigen to cause
an immune response and further the cascade immune response in the mucosa.\textsuperscript{[12,13]} From the above
intestinal mucosal injury, TNBS injury is more like human UC patients in the chronic or remission
lesions.

The involvement of a series of immune cells and pro-inflammatory factors promotes the expansion
of colitis. In human IBD, various immune cells and stromal cells, such as macrophages, dendritic
cells (dc) and effector T cells, produce increased amounts of soluble and membrane-bound TNF,
thereby promoting the secretion of IL-1 and TNF-α by macrophage. [28] Therefore, in order to better
understand the difference between these two modeling methods, we also compared the immune cells
subsets and pro-inflammatory factors. The results showed that the counts of peripheral blood
granulocyte, lymphocyte, and intermediate cells in DSS mice increased significantly along with a
decrease in the intermediate cell ratio.

Increased leukocytes total amount have been detected in DSS group but not in TNBS group, which
is also a common manifestation of human UC, suggesting a more hyperactive immune response
associated with colitis induced by DSS. However, percentage changes of leukocytes were
discovered in TNBS group. The RT-qPCR results of the DSS group showed that the significantly
upregulation of IL-1β and upregulating tendency of TNF-α were locally in the colon. This
phenomenon is driven by the recognition of antigens from dendritic cells and macrophages,
stimulating themselves to secrete IL-1β and TNF-α and triggering an inflammatory cascade
response. [29,30] DSS can induce caspase-1 activation to promote IL-1β synthesis through NLRP3
and macrophages, thus participating in the induction and amplification of colitis. [31,32,33] TNF-α and
IL-1β were mainly secreted by macrophages, and TNF-α blockers were effective for DSS-induced
colitis, while DSS mice showed an upregulating in colonic IL-1β and TNF-α along with a
downregulating in serum TNF-α. [34,35] Therefore, we speculate that DSS may activate a mechanism
that leads to the migration and accumulation of macrophages from the peripheral circulation to the
colonic lesion. The decreased tendency of proportion of CD3+ T cells and NK1.1+ T cells in
peripheral blood of DSS group, and the increased proportion of CD19+ B cells, suggesting that DSS
induced colitis may stimulate the proliferation and activation of CD45+ granulocytes and CD19+ B
cells in peripheral blood. Obviously, the decrease of F4/80 macrophages, which further supported
the mechanism by which DSS-induced colitis causes peripheral macrophages to migrate and
accumulate in the colon. It’s reported that UC is considered to have a Th2 profile.\textsuperscript{[36]} IL-2 is also
mentioned to be one of the specific product of Th2. In this study, colonic IL-2 upregulating tendency
was observed in TNBS group mice, while IL-2 expression in peripheral blood was increased.
Conversely, colonic and serum IL-2 of DSS group shows no significant change, which is
interestingly different with human UC. It’s also reported that IL-2 can stimulate the proliferation
and activation of NK1.1+ cells.\textsuperscript{[35,37]} Significant increases in both peripheral blood T cell subsets
and splenic NK1.1+ T cells happened in TNBS group, while peripheral blood NK cells did not show
a significant increase, which to some extent indicates that peripheral blood IL-2 has an effect on
splenic NK1.1+ T cells, but had little effect on peripheral blood NK1.1+ T cells. In addition, IL-1β
and TNF-α did not show significant increases, which might led by the chronic inflammatory activity
induced by TNBS. It has been reported that in human IBD, TNF-α promotes IL-8 release by
stimulating monocytes \textsuperscript{[38,39]}, thus recruiting granulocytes to infiltrate colon tissue, but IL-8 levels
in DSS and TNBS groups were not significantly increased. Interestingly, the expression of IL-10
was altered in DSS-induced colitis mice, which may be a compensatory increase after the onset of
DSS-induced colitis. IL-10 is a cytokine that inhibits the development of inflammation by targeting
a variety of white blood cells, attenuates excessive immune responses, and protects the epithelium
from inflammation-induced damage.\textsuperscript{[40,41,42]} It can prevent mitochondrial dysfunction by inhibiting
mTOR, thus avoiding the inflammatory expansion triggered by mitochondrial abnormalities.\textsuperscript{[42,43]}
Accumulated damaged mitochondria in macrophages had been reported in IL10-knockout mouse model of patients with colitis and inflammatory bowel disease, and mitochondrial dysfunction further leads to dysregulated activation of NLRP3 inflammatory vesicles and accumulation of IL-1β. Thus, IL-10 plays a key negative feedback role in DSS-induced colitis. However, the IL-10 expression level in the spleen of DSS group showed an upregulation. Therefore, it can be speculated that the induction of pro-inflammatory factors such as IL-1β and TNF-α in DSS-induced colitis model was accompanied by the local compensatory upregulation of IL-10 in the colon, which may be one of the mechanisms of the transition from acute inflammation to chronic inflammation after continuous treatment of DSS for 7 days. DSS had no significant effect on the secretion of other splenic immune cytokine. However, CD45+ granulocytes and CD3+ T cells in spleen of DSS group were significantly decreased, while CD19+ B cells were significantly increased. B cells are mainly distributed in the splenic marrow and lymph nodes. Therefore, it’s reasonable to assume that DSS induced the increase of B cell rather than their cytokines. This mechanism may be induced by DSS-induced colitis, which prompted the splenic B cells activation and proliferation.

In order to test this hypothesis, we examined the cell cycle of splenic tissue cells. The percentage of cells in the resting phase of DSS decreased significantly. In contrast, the percentage of cells in the dividing and proliferating phases increased significantly. Meanwhile, the peripheral blood B cells of C57 mice induced by DSS showed an increasing trend, which may be led by DSS-induced colon inflammation. The local inflammation activated the proliferation of splenic B cells, and B cells migrate to the peripheral blood. The activation of B cells in peripheral blood has been reported to be specific to CD patients rather than UC patients, and IL-8 secreted by B cells is one of the causes
of aggravation of CD.\textsuperscript{[45]} In contrast, there is no significant upregulation of IL-8 in peripheral blood and colonic localization but upregulation of IL-10 in colonic localization, which indicates that the B cells that responded in the DSS group of mice may be regulatory B cells. Regulatory B cells can secrete IL-10 to regulate inflammatory activities.\textsuperscript{[46]} Although IL-10 upregulation was shown in mucosal T cells of patients with active ulcerative colitis, human UC shows a decline of regulatory B cells, which is totally different from that of DSS group.\textsuperscript{[47,48,49,50]}

According to the pathogenesis of human UC, the blood routine often shows an anemic, hypercoagulable state with reduced hemoglobin and increased platelet count, especially in active stage. Our blood routine results showed a decrease in hemoglobin and platelet macrophage ratio in the DSS group, and no significant changes in platelets. This suggested that DSS led to continuous bleeding and anemia in mice, similar to the spontaneous bleeding seen clinically in human UC. In TNBS group, hemoglobin slightly increased and platelets significantly increased. This was similar to the hypercoagulable state observed in patients with active UC, suggesting that TNBS-induced colitis did not resolve its hypercoagulable condition despite a trend of remission on the 7\textsuperscript{th} day of modeling. The hypercoagulable state of blood in UC is often one reason for ulcer formation and extension. Upregulation and activation of platelets can cause blood hypercoagulation in UC patients. Also, platelets activation has been proved to be involved in inflammatory response. Therefore, further studies are still needed to explore the role of the platelet activation factors in UC.

In conclusion, DSS-induced colitis is a suitable experimental method for the study of human acute ulcerative colitis. This recruitment of macrophages and granulocytes in the model is more significant,
showing an approached phenomenon related to human ulcerative colitis. The TNBS-induced colitis more appropriately restore chronic UC in humans. Moreover, the TNBS-induced colitis in C57 mice replicates the mechanism of IL-2 elevation in human UC more effectively. TNBS is more suitable for the analysis of platelet activation and hypercoagulable state associated with colitis than DSS. In human acute and chronic colitis, there are often undifferentiated colitis and indistinguishable colitis. In this study, both DSS and TNBS have their applicable areas. Therefore, the selection of animal models of ulcerative colitis needs to be based on the potential mechanism of model and the research direction accordingly.

4. Methods

4.1 Animals

C57BL/6 male mice weighed 20~22g and aged about 8 weeks, were purchased from the laboratory animal center of Sichuan University. Mice were bred at the experimental animal center in Chengdu University of Traditional Chinese Medicine, with the 20~23°C, 50%~60% relative humidity condition under a natural light-dark cycle. Mice were allowed free access to laboratory animal chow (Dashuo Co., Chengdu, Sichuan, China) and drinking water for acclimation. During the experimental period, each mouse was controlled the daily feeding of 8g ± 2.75g. After feeding adaptively for 7 days, mice were divided randomly into three groups, i.e. control group, DSS group, and TNBS group. The weight differences of the mice from each group are not significant ($P > 0.05$).

4.2 Colitis Induction

Mice from the DSS group were given distilled water containing 4% DSS (wt/vol) (MP Biomedicals
Co., Illkirch, France) for seven days to induced colitis. Mice from the TNBS group were fasted for 24h and anesthetized with Zoletil (Virbac SA Co., Carros, France) before colitis induction. TNBS solution (0.4ml/100g body weight), which was mixed with 5% Trinitrobenzene sulfonic acid (Sigma Chemical Co., St. Louis, MO, USA) and 50% ethanol in a 1:1 ratio, was injected slowly into mice's colon via an 8cm thin catheter. And the mice were held in a head-side-down posture for thirty seconds to prevent the leakage of TNBS solution. After waking up from anesthesia, the mice were fed with drinking water and laboratory animal chow ad libitum.

4.3 Assessment of ordinary circumstances and DAI

Mice's ordinary circumstances were daily observed during the study. Mental state, hair gloss, and feces were observed by naked eyes. The weight on the first day of the experiment was used as the base value to calculate weight loss, and the percentage of weight loss was calculated by the formula:

\[
\text{The percentage of weight loss} = \frac{\text{Initial weight} - \text{weight on the day}}{\text{Initial weight}}.
\]

Fecal properties were assessed by the means of Table (1) \cite{15}. Weight loss scores were calculated with the assessment of the percentage of weight loss. And fecal property scores were calculated in accordance with Table (2). Occult blood/ Fecal blood scores were evaluated according to the manual of fecal OB test card (Baso Diagnostics inc. Zhu Hai). DAI of IBD was calculated as follows:

\[
\text{DAI} = \frac{\text{weight loss score} + \text{fetal attribute score} + \text{occult blood}}{3}. [16]
\]

4.4 Blood samples collection and hematology analyzes

Mice were fasted overnight on the seventh day to prepare for sampling. Blood samples were collected from the eyeballs of mice for blood routine examination and flow cytometry, while others...
were obtained with the common tubes for serum retention. The blood samples were analyzed by hematology analyzer (Shenzhen Prokan Electronics inc. Shenzhen, China) with Diluent for blood cell analysis (PE-D01) and cleaning solution for blood cell analysis (PE-C02).

4.5 Histological analysis

4.5.1 Assessment of colon mucosa damage index

The general damage of colon tissues of mice were assessed by colon mucosa damage index (CMDI).

The contents of CMDI are as follows: 0=No pathological damage to colon tissue; 1= Colonic mucosal epithelial structure is intact with mild hyperemia and edema, but, no erosion or ulcer formation; 2=Moderate hyperemia, edema, erosion, or intestinal adhesion can be seen; 3= Severe hyperemia and edema of colonic tissue, mucosal necrosis, and ulcer formation of which the biggest ulcer is up to 1 cm longitudinal diameter; or total colonic necrosis can be seen.

4.5.2 Histopathological analysis

The colonic tissues of mice were dissected out immediately and measured the length with a ruler. After that, the tissues were processed in the form of Swiss rolls, fixed with 4% paraformaldehyde, and followed by gradient ethanol dehydration, xylene transparency, paraffin embedding, tissue sectioning, and hematoxylin-eosin (HE) staining. Then, the pathological damages of the colon tissues were observed under light microscopy. According to the assessment system of pathological tissues raised by our team, we assessed the histopathological score with Table (3).

4.6 Western Blot

Whole-cell lysates were prepared from tissues. Briefly, tissues were weighed and cut on ice. The
Lysis buffer was pre-chilled on ice and added in the ratio of “tissues weight: lysis buffer volume = 1:10” and mixed as homogenate and centrifuged at 16,000 x g for 15 min. The supernatant was collected as samples. The protein concentration was detected using BCA protein kit (Beyotime). Samples were spotted into the wells (40 ug/lane). 1×SDS-PAGE running buffer was added into the inner and outer tanks. The electrophoresis was performed at 60V for 35min, and then electric pressure was turned to 100V for 90min. The PVDF membranes were activated with methanol for 5min, soaked until the PVDF membranes were completely soaked and translucent, and was transferred to the buffer. The membranes were then soaked in transfer buffer for 3-5min. These objects were clamped and loaded to the transfer tank at 100 V for 60 min. Then, the membranes were washed three times with TBST for 5 min each time, then blocked with 5% skimmed milk prepared with TBST at 37℃ and was shaken for 1.5h. And the membranes were washed six times with TBST for 5min each time and blot the water with filter paper. Antibodies were diluent with 5% BSA solution as follow: β-actin (1:1000, CST), IL-1β(1:1000, abcam), IL-2(1:1000), IL-10(1:1000, abcam). The antibodies were incubated overnight at 4°C and then washed eight times with TBST for 5 min each time. HRP-conjugated secondary antibodies were diluent with 5% BSA solution into 1:20000 final dilution. The membranes were sealed into the hybridization strip. Diluted antibodies were added and incubated in a shaker at 37°C for one hour. And the membranes were washed eight times with TBST for 5 min each time. PVDF membranes were placed into ECL chromogenic solution for 20-30 sec until blue-green fluorescence is visible in the dark. The PVDF film is exposed in the Chemi DOCTMMP imaging system tray and photographed. The grey value of each band was measured with Image J software, and the ratio of the target protein to the internal reference protein is calculated.
4.7 Real-Time qPCR

Quantitative real-time PCR (RT-qPCR) was performed to quantify the inflammatory cytokines gene expression. Total RNA of colon and spleen were extracted with RNAiso Plus reagent (Takara, Japan) according to manufacturer's instructions. Reverse transcription was performed to obtain cDNA according to the instruction of Transcriptor First Strand cDNA Synthesis Kit (Roche, German). RT-qPCR was performed using Stormstar SYBR Green qPCR Master Mix (DBI Bioscience, German) and detected by CFX96 Touch Real-Time PCR Detection System (BioRad, USA). The gene expressions of INF-γ, TNF-α, IL-10, IL-8, IL-2, IL-1β were normalized by β-actin, and the relative expression levels were calculated using $2^{-\Delta\Delta C_T}$ methods. The following primers were designed with Primer 5 software and used for real-time PCR analysis:

- Mouse INF-γ: forward primer: 5'-GATTGCCGGGTGTATCTGG-3', reverse primer: 5'-
  \[
  \text{ACTGCAGCTCTGAATGGTTTCTT-3'}; \\
  \]
- Mouse TNF-α: forward: 5'-TGATGGGAGGGTCTTCCT-3', reverse primer: 5'-
  \[
  \text{CACCTCAGGGAAGTCTGGAA-3'}; \\
  \]
- Mouse IL-10: forward: 5'-GGTCAGGTTCCTGTCTGC-3', reverse primer: 5'-
  \[
  \text{GGGGATGACAGTGGGGAA-3'}; \\
  \]
- Mouse IL-8: forward: 5'-CTCCCCCATCCCCATTTCTCTGA-3', reverse primer: 5'-
  \[
  \text{CAGCCCATAGTGAGTGGGAT-3'}; \\
  \]
- Mouse IL-2: forward: 5'-TGACCTCTGCAGCATGTT-3', reverse primer: 5'-
  \[
  \text{TGGCACTCAAATGTGTTGAC-3'}; \\
  \]
- Mouse IL-1β: forward: 5'-TGCCACCTTGGACAGTGG-3', reverse primer: 5'
4.8 Enzyme-linked immunosorbent assay (ELISA)

Immuno-cytokines levels of serum were performed by ELISA. ELISA plate wells were divided into standard wells and sample wells for contrast. Standard wells were each added with 50μl of different concentrations of standard reagent. Each sample well was added with a 10μl sample, which was mixed and shaken gently with 40μl sample dilution (The samples were finally diluted fivefold), and sample wells were added with 100μl conjugate reagent. All wells were sealed with a sealing membrane and incubated at 37°C for 60 minutes. The 20-fold concentrated washing solution was diluted 20-fold with distilled water for spare use. After incubation, the wells were carefully washed 5 times. Added 50 μl of chromogenic agent A and 50 μl of chromogenic agent B to each well in sequence, shake gently, and develop the color at 37°C for 15 minutes with protection from light. Added 50 μl of termination solution to each well to terminate the reaction (Blue immediately turns yellow). Zero the blank wells and measure the absorbance (OD) of each well sequentially at 450 nm. The measurement was performed within 15 minutes after the addition of the termination solution.

4.9 Flow cytometry

4.9.1 Sample prepared

Tissue samples were washed with phosphate buffer saline (PBS) at 4°C, placed in a surface dish and
cut into homogenized form with curved ophthalmic scissors, added with 1 ml PBS at 4°C, and

filtered the tissue with 300 mesh filter cloth in a 5 ml flow tube. 1 ml PBS at was added into the

flow tube 4°C. Then, centrifuge the solution at 300g for 5 min. Blew off the cell wall after

centrifugation, and poured off the liquid to adjust the cell concentration to $10^6$ cells/ml by adding

PBS at 4°C. PBS at 4°C was added to adjust the cell concentration to $10^6$ cells/ml. Collect 100ul of

cell suspension from each group in a flow tube or EP tube, washed with 1 ml PBS (0.1 mmol/L, pH

7.4, 4°C) for 5 min per 300g, then discard the supernatant. Resuspend the cells with 190 ul of binding

buffer, added 5 ul of Annexin-V-FITC, and incubate for 10 min at room temperature in the dark.

The sample was then added 10 ul propidium iodide (PI), and incubate for 10 min at room

temperature in the dark. 300 ul binding buffer and assay were replenishing on flow cytometer within

1 h. Control setup: (1) Blank control: control or experimental cells without Annexin-V-FITC and PI;

(2) Single-labeled Annexin-V-FITC control: experimental cells plus Annexin-V-FITC; (3) Single-

label PI control: experimental cells plus PI Voltage was adjusted with a blank control and

fluorescence supplementation was adjusted with two single-label controls. The results were

Annexin-/PI- for normal living cells, Annexin+/PI- for apoptotic cells, and Annexin+/PI+ for

necrotic or advanced apoptotic cells.

4.9.2 Cell cycle analysis

Cells were harvested, and washed with PBS (pH 7.4 under 4°C) for one time (300g, 5 min), then

discard the supernatant. Added 2 ml of ice-cold 70% ethanol for 30 min, centrifuge at 300g for 5

min to remove the supernatant, and added 1 ml PBS (4°C) to centrifuge per 300g for 5 min and then

discard the supernatant, added 400 ul of PBS and resuspended the cells. 50 ul PI (PI 20 ug/ml,

RNase 20 ug/ml was added and incubated for 30 min at 37°C. Excitation light 488, detect forward
scattered light, laterally scattered light, and red light. Data were analyzed through ModFIT analysis software.

4.9.3 Lymphocye’s subsets analysis

2.5 μl fluorescent antibody was added to 100 μl sample in a flow-through tube, incubate for 30 min at 4°C in the dark; 2 ml lysis solution was added to each sample, then lysed for 10 min at room temperature in the dark, centrifuge for 5 min per 300g, discard supernatant; 2 ml PBS was added in a flow-through tube, centrifuge per 300 g for 5 min; 500 ul PBS was added to resuspend the cells, and the assay was performed on the machine. Detection content: ① CD45+ granulocytes; ② CD3+ T cells; ③ F4/80+ macrophages; ④ NK1.1+ T cells; ⑤ CD19+ B cells; FlowJo software was used to analyze the results.

4.10 Statistical Analysis

Data were presented as mean ± standard deviation. Data of mouse assays were obtained from eighteen mice with six mice per group (n=6). All statistical analysis was carried out using GraphPad Prism 9 for Windows (GraphPad Software, La Jolla California USA). The measurement data were analyzed by t-test and analysis of variance (ANOVA). All statistical tests were two-sided probability tests, and the data were judged as statistically significant results only when \( P < 0.05 \).
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Authors Contributions

Q.B. Y. and Z. Y. designed and performed the experiment and wrote manuscript; M.Q. W., K.H. Q., F.T. L. provided technical and performed data analysis; Q. D., J. W., Y.Q. S., Y. H. provided material support and helped with experiments; Z.P. H. and M.Y. Y. gave guide for animal experiments, X. P. supervised this study and critically revised the manuscript.

Ethics statement

This study was approved by the Chengdu University of Traditional Chinese Medicine Experimental Animal Ethics Committee and compliance with ethical requirements. All the procedures were carried out in compliance with the guidelines of the Council on Animal Care of the Academia Sinica and the ARRIVE guidelines.

Data availability statements

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
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Table (1)

| Fecal properties         | Evaluation Criteria                                |
|--------------------------|---------------------------------------------------|
| Normal feces             | Formed feces without blood                        |
| Poorly formed feces      | Semi-formed, paste-like feces that do not adhere to the anus |
| Loose feces              | Watery feces that adhere to the anus               |

Table (2)

| Points | Weight loss score | Fecal property score | Occult blood/ Fecal blood score |
|--------|-------------------|----------------------|---------------------------------|
| 0      | No decrease;      | Normal feces;        | Negative;                       |
| 1      | Decrease < 5%;    | Slightly poorly formed feces; | Negative;                       |
| 2      | Decrease from 5% to 10%; | Poorly formed feces; | Occult blood;                   |
| 3      | Decrease from 10% to 15%; | Loose feces;       | Slightly fecal blood;            |
| 4      | Decrease of ≥15%; | Anus sticked with loose feces; | Fecal blood;                    |

Table (3)

| 0 point | 1 point | 2 points | 3 points |
|---------|---------|----------|----------|
| (1)     | Inflammation | No inflammatory cell | Inflammatory cell |
|         | cell     | infiltration was seen in | inflammatory cell |
|         | mucosal  | the mucosal, submucosal, | infiltration in |
|         | layer    | muscular, and plasma | mucosal layer, |
|         | infiltrati | layers of the colon; | submucosa; |
|         | on       |                         | the colon; |
| (2)     | Sapheno  | Normal tissue structure, | Minor |
|         | us fossa | no crypt damage seen;   | localized |
|         | injury   | saphenous fossa injury; | saphenous fossa |
|         |          | severe injury;          | severe injury; |
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