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Impact of T-2 toxin on intestinal inflammation and transcriptional regulation of inflammatory response in mouse macrophages

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ABSTRACT

T-2 toxin, a fungal secondary metabolite produced by toxigenic *Fusarium* species, poses a significant threat to grain food and feed due to its potential to cause intestinal inflammation in livestock and poultry. Macrophages play a crucial role as integral components of the body's immune system during intestinal inflammation. This study aimed to elucidate the mechanism behind the inflammatory response triggered by T-2 toxin in macrophages. Compared to the control group, gavage administration of T-2 toxin (0.33, 1, and 4 mg kg^{-1}) led to a decrease in body weight and feed intake, along with histopathological alterations in the colon of mice. In addition, T-2 toxin induced the upregulation of macrophage-derived cytokines like IL-1β, IL-6, and TNF-α, as well as a rise in the population of $F4/80^{+}$ macrophages in the colon. T-2 toxin also led to the upregulation of IL-1β, IL-6, and TNF-α in mouse bone marrow-derived macrophages (BMDMs). Furthermore, the transcriptomic analysis of BMDMs exposed to T-2 toxin (10 nM) identified the "TNF signaling pathway," "Lipid and atherosclerosis," "Epstein-Barr virus infection," "MAPK signaling pathway," and the "NF-kappa B signaling pathway" as the top five significantly enriched pathways. Subsequently, twelve inflammation-related genes were randomly chosen for validation through quantitative reverse transcription PCR (RT-qPCR), with the results corroborating those from the transcriptomic analysis. The comprehensive analysis of transcriptome data highlights the activation of several signaling pathways associated with the inflammatory response following T-2 toxin-induced BMDMs, offering potential therapeutic targets for the prevention and treatment of T-2 toxin-induced intestinal inflammation.

1. Introduction

T-2 toxin, a fungal secondary metabolite produced by various toxigenic *Fusarium* species, has been extensively investigated due to its early discovery, high toxicity, and wide presence in food and animal feed [1]. Its potent toxicity leads to intestinal inflammation, posing significant threats to human health and animal husbandry [2]. Considering the immune system as a major target of T-2 toxin toxicity, considerable attention has been given to studying its impact on immune responses in recent years.

T-2 toxin can distribute throughout the body and reach various organs without the need for transporters or adjuvants [3]. The toxin undergoes rapid absorption in the gastrointestinal (GI) tract [4], leading to damage in nearly all GI tract cells and influencing nutrient absorption even at minimal toxin concentrations [5]. Obremski et al. found the impact of T-2 toxin on the percentages of $CD4^{+}$ and $CD8^{+}$ T lymphocytes, $CD21^{+}$ B cells, as well as the mRNA expression levels of related cytokines in porcine ileum [6].

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Macrophages are an important part of the body's defense against infection and disease, playing a pivotal role in the immune system [7,8]. They are distributed throughout various tissues, including in the lamina propria of the gut. Most adult intestinal macrophages originate from the monocytic cell lineage that emigrates from the bone marrow (BM) and play a crucial role in maintaining intestinal homeostasis. Noel et al. established a co-culture model of the gut and macrophages, demonstrating that the depletion of macrophages adversely affects the subsequent differentiation of intestinal epithelial cells [9]. Sehgal A et al. demonstrated that colony-stimulating factor 1 (CSF1)-dependent macrophages within the gut wall are capable of repairing enteritis induced by inflammation or chemotherapy and are essential for sustaining the small intestinal stem cell niche [10]. In inflammatory conditions, circulating monocytes originating from the BM infiltrate the inflamed intestinal tissue and differentiate into macrophages [11]. Research by Nanthakumar indicated that neonatal colitis is characterized by a high rate of macrophage infiltration in the inflamed regions, underlying the role of macrophages as the first line of defense [12]. Additionally, Swirski et al. revealed that the spleen serves as a critical reservoir for monocytes in mice, exhibiting a phenotype similar to that of blood-derived monocytes [13]. These findings suggest that the spleen serves as an additional primary source of monocytes, which differentiate into macrophages and facilitate their migration to the inflamed intestinal tissue.

Mycotoxins have the potential to influence the activity and function of macrophages. For example, elevated levels of gibberellins can induce apoptosis in macrophages, consequently diminishing their ability to release inflammatory cytokines [14]. Trichothecene mycotoxins, among the naturally occurring food contaminants identified as the most dangerous [15], profoundly impact the immunomodulatory function of macrophages. Even low concentrations of T-2 toxin disrupt the recognition of pathogen-associated molecular patterns by porcine primary macrophages, resulting in reduced Toll-like receptor expression and impairing the immune system [16]. Further research is warranted to elucidate the molecular mechanisms responsible for the immunotoxicity of T-2 toxin in inducing intestinal inflammation in macrophages. Transcriptome sequencing also called as RNA sequencing or RNA-Seq is a high-throughput sequencing technology that enables the characterization of all RNA transcripts present in a given sample at a specific moment [17].

In this study, we assessed intestinal inflammation in mice treated with T-2 toxin. Given the crucial role of macrophages in maintaining intestinal homeostasis and medicating the immune response, we evaluated macrophage infiltration in the intestines of mice and the inflammatory response in mouse bone marrow-derived macrophages (BMDMs). Additionally, we employed RNA-seq technology to sequence the transcriptome of BMDMs in response to T-2 toxin-induced inflammation, aiming to gain further insights into the regulatory mechanisms and molecular pathways underlying macrophage function affected by T-2 toxin.

2. Materials and method

2.1. T-2 toxin solution preparation

T-2 toxin (CAS number: 21259-20-1, 99.0 % purity) was purchased from Qingdao Pribolab Biotechnology Company (Qingdao, China). The T-2 toxin was dissolved in dimethyl sulfoxide (DMSO, CAS number: 67-68-5, 99.0 % purity, Sangong Bioengineering, Shanghai, China) to create a solution with a concentration of 25 mg mL^{-1}, and stored at −20 °C, protected from light.

2.2. Mice, treatments, and samples collection

SPF 8-10-weeks-old C57BL/6 male mice were purchased from Hangzhou Qizhen Laboratory Animal Science and Technology Co., LTD. All mice were maintained in pathogen-free conditions with standard laboratory chow and water *ad libitum*. The protocol was approved by the Committee on the Ethics of Animal Experiments of Zhejiang Academy of Agricultural Science. Project Proposal Number 2023ZAASLA020. After one week of acclimatization, the mice were randomly divided into 4 groups, with 5 mice in each group. The mice received gavage administration of normal saline (Control), or T-2 toxin at a dose of 0.33, 1, or 4 mg kg^{-1}, respectively. Body weight and food intake were monitored daily starting from the initial day of gavage. On day 7, spleen and colon tissues were collected from mice for subsequent Hematoxylin-Eosin (H&E) staining or RT-qPCR analysis.

2.3. H&E staining

Colon tissues were fixed in a 4 % paraformaldehyde solution for 24 h, dehydrated using a HistoCore PEARL tissue processor (Leica Biosystems, Nussloch, Germany), and subsequently embedded in a paraffin block using a HistoCore Arcadia paraffin embedding machine (Leica Biosystems, Nussloch, Germany). Tissue sections with a thickness of 5 μm were stained with H&E to observe colonic inflammation and histopathological damage. Staining was carried out as per the manufacturer's protocol using an H&E staining kit (Golden Clone Biotechnology, Beijing, China).

2.4. Immunofluorescence staining

Colon tissue sections were deparaffinized using xylene and a graded series of ethanol. Antigen retrieval was performed by immersing the sections in a 10 mmol/L EDTA solution (pH 8.0) and heating them in a water bath at 95 °C for 20 min. Subsequently, the sections were rinsed with phosphate-buffered saline (PBS, pH 7.2) and incubated with a blocking buffer (1 % BSA/0.3 % Triton X-100/1 % normal goat serum in PBS) at room temperature for 30 min. The sections were then incubated with anti-mouse F4/80 primary antibody (Invitrogen, Carlsbad, USA) at 4 °C overnight. After washing with PBS, the sections were incubated with Alexa Fluor 488-conjugated (IgG) secondary antibody (Invitrogen, Carlsbad, USA) for 1 h at room temperature. Finally, the sections were incubated with hoechst 33342 at room temperature for 5 min. Fluorescence images were captured using a confocal laser scanning microscope (Zeiss, LSM 900, Oberkochen, Germany). The F4/80-positive cells were quantified using ImageJ software (National Institutes of Health, USA).

2.5. Cell culture and treatment

Bone marrow-derived macrophages (BMDMs) were obtained from the tibia and femoral bone marrow of C57BL/6 male mice, and maintained in Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific, Grand Island, USA) supplemented with 10 % (vol/vol) fetal bovine serum (FBS, Thermo Fisher Scientific), L-glutamine, sodium pyruvate, 100 U mL^{-1} penicillin-streptomycin, and 20 ng mL^{-1} macrophage colony-stimulating factor (M-CSF, R&D Systems, Minneapolis, USA) for 7 days. For experiments, BMDMs were seeded into plates containing IMDM supplemented with 1 % FBS and incubated overnight. Subsequently, the BMDMs were exposed to T-2 toxin for 6 h for further RT-qPCR analysis and RNA-seq analysis.

2.6. cDNA synthesis and RT-qPCR analysis

Total RNA was extracted from mouse tissues or BMDMs using TriPure Isolation Reagent (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. cDNA synthesis was performed using the PrimeScript RT reagent Kit (Takara, Japan) for reverse transcription. Real-time PCR was conducted using the iTaq™ Universal SYBR® Green kit (Bio-Rad, USA) on a Real-Time PCR detection system (Bio-Rad, CFX96, USA). The primer sequences of IL-1β, IL-6,

TNF-α, and the internal reference gene β-actin are provided in Supplementary Table 1. The relative expression level of target genes to β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the control group.

2.7. RNA-seq bioinformatics analysis and RT-qPCR verification

Total RNA was extracted from mouse BMDMs using TriPure Isolation Reagent, and mRNA with poly tails was enriched with magnetic beads with an Oligo (dT). The enriched mRNA was further purified, reverse transcribed, and synthesized into double-stranded cDNA. Subsequently, a library was constructed and subjected to quality control undergoing paired-end sequencing (PE150). The initial step involved performing data quality control on the raw data obtained from the sequencing machine. Afterward, the raw data underwent de-junctioning and low-quality filtering. The filtered data was then compared and aligned to the reference genome sequence using HISAT2 software [18], resulting in the generation of a comparison reference genome. Transcript

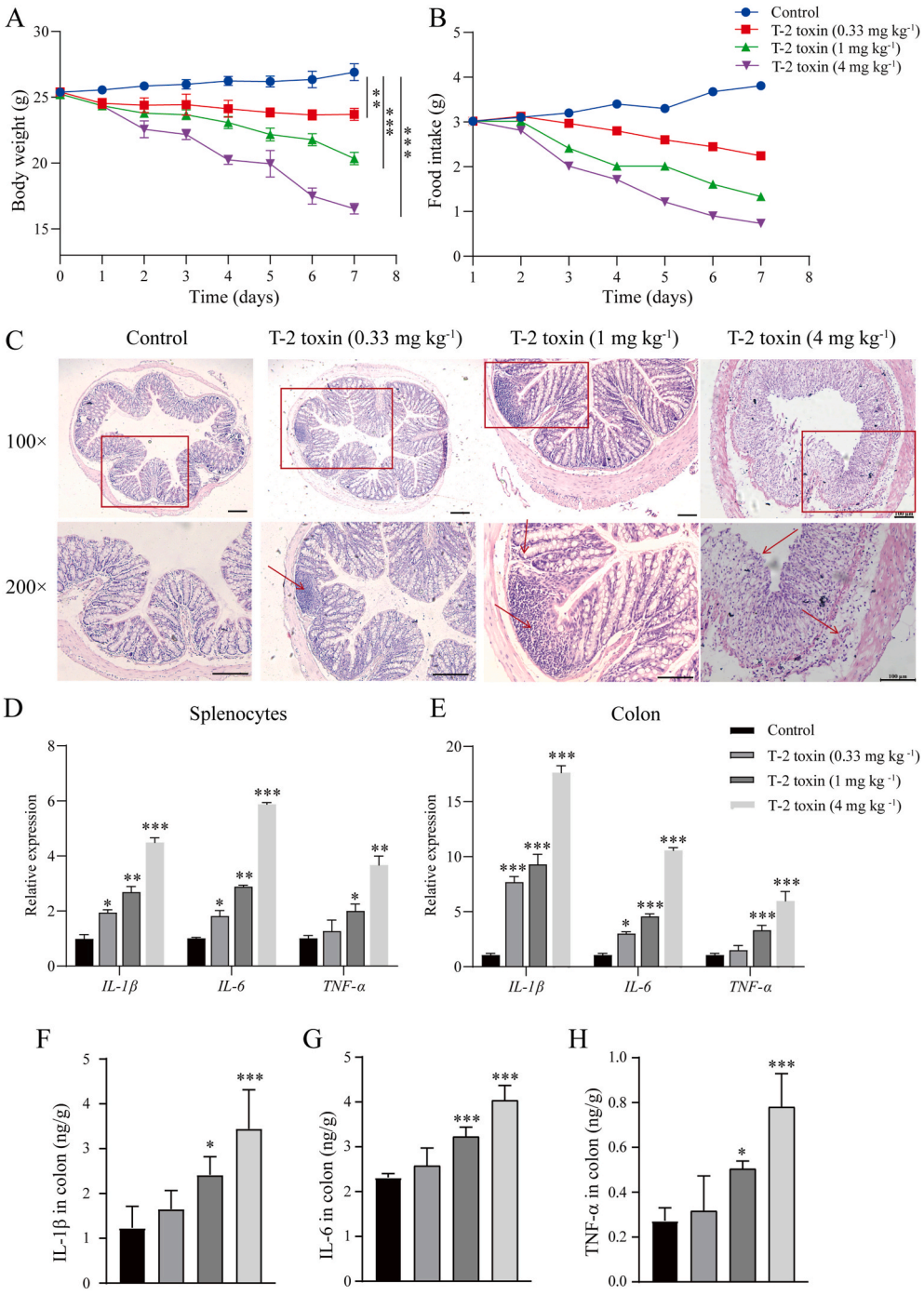

Fig. 1. The impact of T-2 toxin on intestinal inflammation in mice. The mice received oral administration of normal saline (Control), or T-2 toxin (0.33, 1, or 4 mg kg^{-1}), and body weight (A) and food intake (B) were monitored daily for 7 days. Data are mean ± SEM ($n = 5$). $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA test). (C) H&E staining of paraffin sections of colons isolated from Control or T-2 toxin-treated mice on day 7. Scale bar, 100 μm. RT-qPCR analysis of *IL-1β*, *IL-6*, and *TNF-α* expression in splenocytes (D) and colon (E) isolated from Control or T-2 toxin-treated mice on day 7. Data are mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus Control (one-way ANOVA test).

reconstruction was carried out using Stringtie software [19] to accurately assemble transcripts and quantify the expression levels of each gene or transcript. Finally, differential expression analysis was conducted using DESeq2 [20] and relevant pathways were discovered by enrichment analysis.

By analyzing the transcriptome sequencing data with KEGG pathway analysis, 12 immunity-related differentially expressed genes (DEGs) were randomly selected and the results were verified using RT-qPCR. The primer sequences of the 12 genes are provided in Supplementary Table 2.

2.8. Statistical analysis

Data analysis was performed using GraphPad Prism 9 software. Data are represented as mean ± SEM. To examine the statistical differences in the quantitative analysis of the intensity of F4/80 in the sections of colons between control and T-2 toxin treatment group, an unpaired two-tailed Student's *t*-test was conducted. The other statistical differences between groups were assessed using a Tukey post-hoc test, one-way analysis of variance (ANOVA). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. T-2 toxin induces intestinal inflammation in mice

To investigate the impact of T-2 toxin on intestinal inflammation in mice, we administered varying doses of T-2 toxin (0.33, 1, and 4 mg kg^{-1}) orally to the mice for 7 consecutive days. The low dose of 0.33 mg kg^{-1} T-2 toxin significantly reduced the body weight and food intake of mice. Similarly, increasing doses of T-2 toxin at 1 and 4 mg kg^{-1} led to a corresponding decrease in body weight and food intake in mice (Fig. 1A and B). On day 7, the experiment concluded with histological examination of the mice's colon tissues. Mice treated with 0.33 mg kg^{-1} T-2 toxin exhibited observable inflammatory cell infiltration in the colon, whereas those treated with 1 mg kg^{-1} T-2 toxin displayed a more pronounced inflammatory cell infiltration compared to the control group. Remarkably, the group treated with 4 mg kg^{-1} T-2 toxin experienced a loss of the colon's histological structure, with inflammatory cells infiltrating the mucosa and submucosa (Fig. 1C). The expression levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α were significantly upregulated in both splenocytes and colon tissues in a dose-dependent manner due to T-2 toxin exposure (Fig. 1D and E). These findings indicate that T-2 toxin induces significant intestinal inflammation in mice and stimulates the expression levels of inflammatory cytokines.

3.2. T-2 toxin induces macrophages infiltration in the colon

Since T-2 toxin induced inflammatory cell infiltration and upregulation of macrophage-derived cytokines in the colon, we employed immunofluorescence staining to detect the infiltration of $F4/80^{+}$ mature macrophages in mouse colon tissues. As depicted in Fig. 2A, there was almost no presence $F4/80^{+}$ macrophages in the colon sections of the control group. Conversely, mice treated with 1 mg kg^{-1} T-2 toxin displayed a markedly observable infiltration of macrophages in the colon. Quantitative analysis of the results revealed a significant increase in F4/80-positive cells within the T-2 toxin treatment group (Fig. 2B).

3.3. T-2 toxin induces inflammatory response in BMDMs

Since T-2 toxin induced upregulation of macrophage-derived cytokines in splenocytes and colon, bone marrow-derived macrophages (BMDMs) were exposed to different concentrations of T-2 toxin (3.3, 10, and 30 nM), and the expression levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α were examined. The 3.3 nM concentration of T-2 toxin caused a significant upregulation of IL-6, but showed no effect on the expression levels of IL-1β and TNF-α compared to the control. However, the concentrations of 10 nM and 30 nM of T-2 toxin resulted in a dose-dependent significant upregulation of IL-1β, IL-6, and TNF-α (Fig. 3). These findings demonstrate that T-2 toxin at a concentration of 10 nM dramatically stimulates the inflammatory response in macrophages.

3.4. RNA-seq analysis of BMDMs exposed to T-2 toxin and RT-qPCR verification

To elucidate the regulatory mechanisms and molecular pathways underlying the T-2 toxin-induced inflammatory response in macrophages, we employed RNA-Seq analysis. The control and T-2 toxin-treated groups generated 40,283,345 and 38,539,477 clean raw reads, respectively. Through transcriptome sequencing, a total of 17,359 genes were identified, including 273 DEGs as illustrated in Fig. 3A. Among

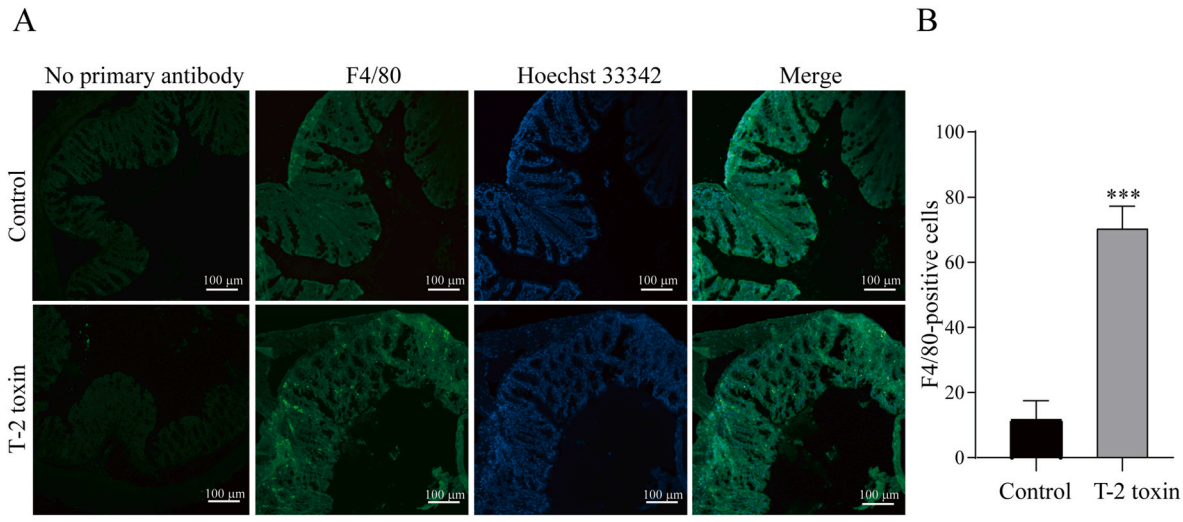

Fig. 2. The impact of T-2 toxin on macrophage infiltration in the colon. The mice received oral administration of normal saline (Control) or T-2 toxin (1 mg kg^{-1}) for 7 days. (A) Detection of F4/80 protein expression with immunofluorescence staining in the sections of colons of mice. The nuclei were stained with hoechst 33342 (blue) and F4/80 protein was labeled with Alexa Fluor 488 (green). Scale bar, 100 μm. (B) Quantitative analysis of the F4/80-positive cells in the sections of colons. Data are mean ± SEM ($n = 5$). $^{**}p < 0.01$ versus Control (unpaired two-tailed Student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

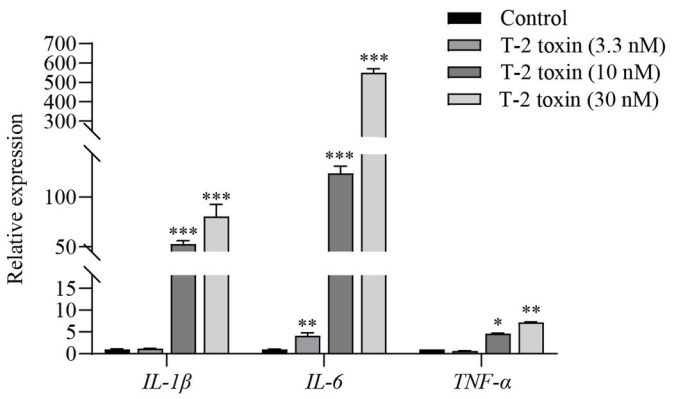

Fig. 3. The impact of T-2 toxin on inflammatory response in BMDMs. BMDMs were exposed to T-2 toxin (3.3, 10, and 30 nM) for 6 h, and RT-qPCR analysis of *IL-1β*, *IL-6*, and *TNF-α* were examined. Data are mean ± SEM ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus Control (one-way ANOVA test).

these, 194 genes exhibited up-regulation, while 79 genes demonstrated down-regulated expression.

The DEGs in BMDMs following T-2 toxin treatment were annotated using BLAST analysis of the GO database to investigate changes in overall gene function. Further analysis of the GO secondary pathways highlighted "I-κB kinase/NF-κB signaling," "Regulation of I-κB kinase/NF-κB signaling," and "Cellular response to molecule of bacterial origin" as the most significantly expressed pathways in term of "Biological Process". In term of "Cellular Component," "Endoplasmic reticulum protein-containing complex" and "Early endosome" were the most significantly expressed pathways. Notably, the pathways of "Ubiquitin protein ligase binding" and "Ubiquitin-like protein ligase binding" demonstrated the most significant expression in term of "Molecular Function" (Fig. 4B).

We conducted further analysis of DEGs in BMDMs following T-2 toxin stimulation and enriched them in KEGG pathway. Five metabolic pathways exhibited the most significant enrichment (Fig. 4C). The results revealed that a majority of the DEGs were associated with the "TNF signaling pathway," "Lipid and atherosclerosis," "Epstein-Barr virus infection," "MAPK signaling pathway," and "NF-kappa B signaling pathway".

To validate the reliability of the transcriptome data, we performed RT-qPCR on 12 randomly selected immunity-related DEGs (Supplementary Table 2). The results demonstrated a consistent up-regulation trend in the "TNF signaling pathway" for the DEGs *Rela*, *Cd40*, *Map3k14*, and *Sqstml* (Fig. 4D). Additionally, in the "JNK signaling pathway", the DEGs *Traf1*, *Traf2*, and *Trex1* showed up-regulation trends (Fig. 4E). Furthermore, genes associated with "Endoplasmic reticulum stress", such as *Ubxn7*, *Pdia6*, *Sdf211*, *Hspa5*, *Spcs3*, and *Vcp*, exhibited results consistent with those obtained from transcriptome sequencing (Fig. 4F). These results provide additional evidence for the credibility of the transcriptome sequencing data.

4. Discussion

T-2 toxin exerts deleterious effects on the gastrointestinal tract, and even trace amounts of the toxin are rapidly absorbed, leading to damage of the gastrointestinal mucosa and compromising nutrient absorption [21]. Marasas et al. reported that low doses of T-2 toxin administration led to significant weight loss in rats [22], which aligns with the findings of our study. Our histopathological examination revealed a notable loss of crypt and goblet cells in the colon of T-2 toxin-treated mice, accompanied by pronounced infiltration of inflammatory cells in the colon. These results are consistent with the research conducted by Zhang et al. [23], where they demonstrated the harmful impact of T-2 toxin on intestinal function in rats. Additionally, our investigation identified a significant upregulation of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the spleen and colon tissues of mice, implying that T-2 toxin has the potential to induce intestinal inflammation. However, the precise mechanism underlying T-2 toxin-induced intestinal inflammation has yet to be elucidated.

Macrophages are one of the most abundant types of immune cells in the colon, closely related to the pathogenesis of inflammatory bowel disease [24]. Activated macrophages contribute to the pathogenesis of DSS-induced colitis primarily through the secretion of proinflammatory cytokines and induction of tissue damage [25], thereby highlighting the significant impact of macrophages on intestinal inflammation. In this study, a significant increase in proinflammatory cytokines IL-1β, IL-6, and TNF-α was observed in mouse BMDMs treated with T-2 toxin. These findings were consistent with observations in the colon tissues of T-2 toxin-treated mice, including increased infiltration of $F4/80^+$ macrophages in the colon. These results suggest that T-2 toxin-induced macrophage activation may play a key role in the development of T-2 toxin-induced intestinal inflammation.

Transcriptome sequencing was utilized to reveal the potential molecular mechanisms underlying the immune response of macrophages to T-2 toxin. According to results of the GO enrichment analysis, several significantly expressed pathways were identified, including "I-κB kinase/NF-κB signaling," "Cellular response to biotic stimulus," and "Response to oxidative stress." These findings are consistent with the results of a previous study conducted by Ma et al., which investigated of aflatoxin B1-induced oxidative stress and inflammatory responses in mouse monocyte macrophages [26]. The observed DEGs may be linked to T-2 toxin influencing cell signaling and enzymatic activities in macrophages. Additionally, the KEGG pathway enrichment analysis revealed significant alterations in the "TNF signaling pathway," "MAPK signaling pathway," and "NF-kappa B signaling pathway" in BMDMs under T-2 toxin stimulation. It is well established that the "TNF signaling pathway" can activate several intracellular signaling pathways, including the MAPK and NF-κB mediated pathways, promoting inflammatory processes and regulating the expression levels of various inflammatory cytokines, chemokines, and mediators following T-2 toxin stimulation [27,28].

5. Conclusions

This study aimed to investigate the molecular mechanisms responsible for the immunotoxicity of T-2 toxin in inducing intestinal inflammation. We discovered that T-2 toxin triggers the upregulation of macrophages-derived cytokines, including IL-1β, IL-6, and TNF-α, as well as an increase in macrophages infiltration in the colon of mice. These observations strongly indicate the crucial involvement of macrophages in T-2 toxin-induced intestinal inflammation. Moreover, T-2 toxin induced an inflammatory response in mouse BMDMs. Transcriptomic analysis identified the "TNF signaling pathway," "Lipid and atherosclerosis," "Epstein-Barr virus infection," "MAPK signaling pathway," and "NF-kappa B signaling pathway" as the top five significantly enriched pathways. This study offers valuable insights into the interaction between T-2 toxin and macrophages, providing a reference for the development of strategies to mitigate the inflammatory response and intestinal inflammation induced by this toxin.

CRediT authorship contribution statement

Xinghui Yang: Writing – original draft, Software, Methodology. **Xiaoli Xu:** Visualization. **Qiuhong Zhong:** Methodology. **Haifeng Cui:** Supervision. **Junfeng Xu:** Funding acquisition, Conceptualization. **Wei Wei:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

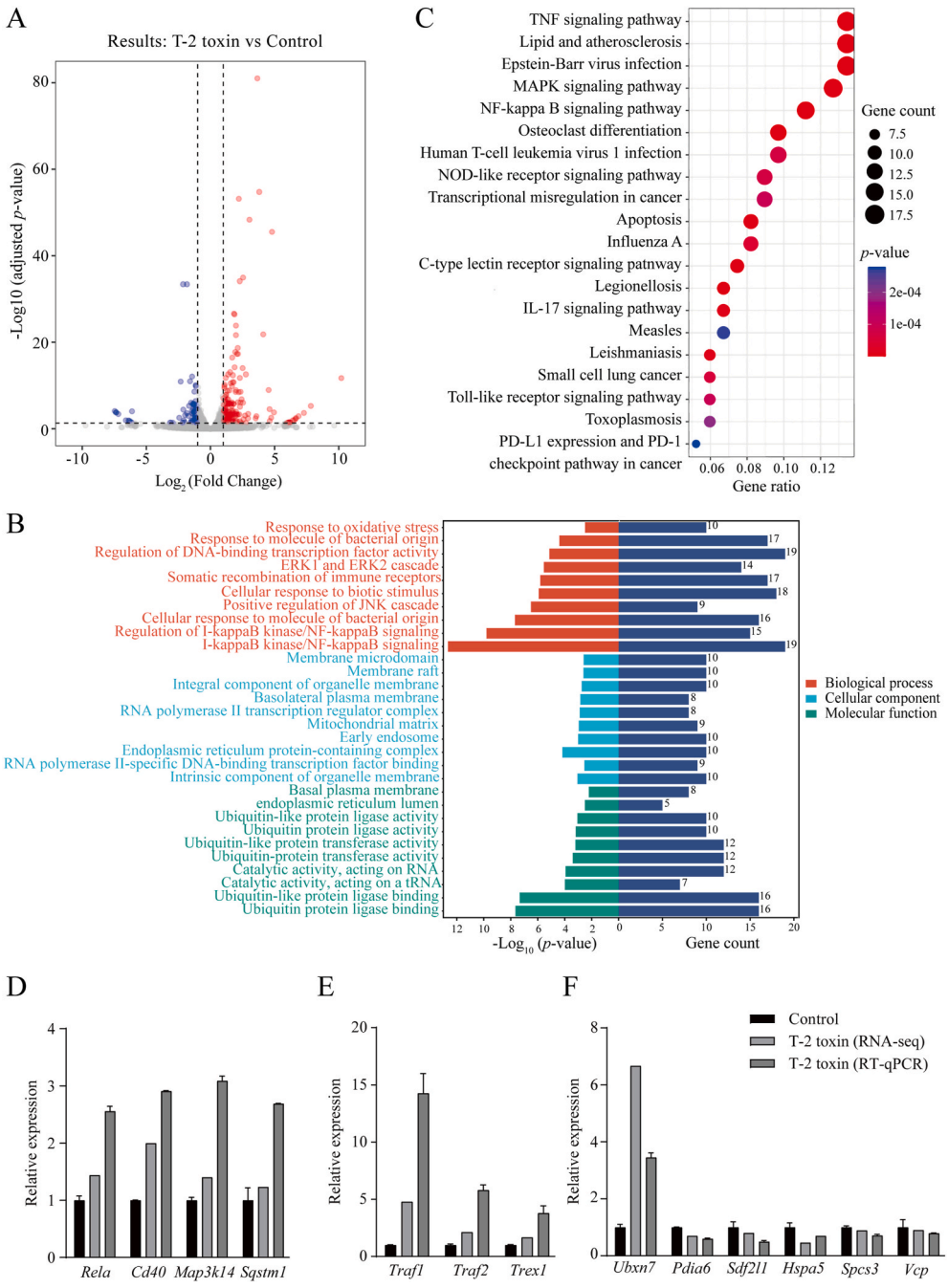

Fig. 4. RNA-seq analysis of BMDMs exposed to T-2 toxin. BMDMs were exposed to T-2 toxin (10 nM) for 6 h and RNA-seq analysis was examined. (A) Volcano plot of the DEGs between T-2 toxin and control groups. The y-axis corresponds to the mean expression value of -Log_{10} (adjusted *p*-value), and the x-axis displays the Log_2 (Fold Change) value. Up-regulated genes are denoted by red dots, down-regulated genes are indicated by blue dots, and genes with insignificant changes are represented by black dots between T-2 toxin and control groups. (B) GO enrichment analysis of the DEGs between T-2 toxin and control groups. GO terms are related to "Biological process," "Cellular component," and "Molecular function". The x-axis represents the respective -Log_{10} (*p*-value) of different pathways and the y-axis represents the pathway name. The bar graph on the right shows the number of genes in each cluster, highlighted in dark blue. (C) KEGG pathway analysis of DEGs between T-2 toxin and control groups. The size of the circle indicates the number of DEGs, with smaller *p* values represented by a redder color. A pathway with a larger and redder bubble indicates a higher number of DEGs. Expressions of 12 randomly selected immunity-related DEGs, including TNF signaling pathway-related genes (*Rela*, *Cd40*, *Map3k14*, and *Sqstm1*) (D), JNK signaling pathway-related genes (*Traf1*, *Traf2*, and *Trex1*) (E), and endoplasmic reticulum stress-related genes (*Ubxn7*, *Pdia6*, *Sdf2l1*, *Hspa5*, *Spcs3*, and *Vcp*) were validated by RT-qPCR. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101840.

Data availability

Data will be made available on request.

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