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Effects of *Ginkgo biloba* leaf polysaccharides on immune function in immunosuppressed mice

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Received August 13, 2022, accepted September 20, 2022

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Pharmazie 77: 326-330 (2022) *doi: 10.1691/ph.2022.2488*

Ginkgo biloba leaf polysaccharides (GBLP) are bioactive compounds with immunomodulatory activity. In this work, the effects of GBLP on the immune system in immunosuppressed mice were examined. Mice were divided into normal control, model, positive control, and high, medium, and low GBLP (800, 400, and 200 mg/kg·bw) groups, with 12 mice in each group. An immunosuppressed mouse model was prepared using cyclophosphamide. After 28 days of gavage, peripheral blood lymphocytes were collected, and the thymus and spleen were removed and weighed. Splenic lymphocyte proliferation, cytokine secretion, mRNA expression, and TLR/MAPK signaling pathway-related protein expression were detected by MTT assay, ELISA, qRT-PCR, and western blotting, respectively. In comparison to the normal control group, the indices were significantly lower in the model group ($p < 0.05$). GBLP restored spleen, thymus, and lymphocyte proliferation indices, secretion and mRNA expression of IL-4, IFN-γ, IL-2, and IL-10, protein expression of TLR2, TLR4, MyD88, and IRAK4, and phosphorylation levels of JNK, ERK, and p38 in cyclophosphamide-treated mice ($p < 0.05$). The findings indicate that GBLP improved cyclophosphamide-induced immunosuppression in mice. Its mechanism of action involved the TLR/MAPK signaling pathways, implying that GBLP can be further developed as an immunomodulatory functional food component.

1. Introduction

The immune system can resist the invasion of harmful foreign substances and is an important barrier for maintaining health (Rooks and Garrett 2016). Cyclophosphamide is an alkylating agent of purines, with characteristics of immunosuppression and immunoregulation, and is selective for T cells (Wang et al. 2022a). Immunosuppression is a state of immune dysfunction. When the immune system is destroyed and in the immunosuppression state, the body will be more sensitive to pathogens and prone to a variety of diseases (Horvat et al. 2015). Many individuals have weakened immunity and are in a state of immunosuppression due to congenital insufficiency, acquired malnutrition or other reasons, enabling the development of various chronic diseases (Bourke et al. 2016). Therefore, improving immune suppression and immunity is essential in preventing the occurrence of various chronic diseases.

Recently, non-toxic plant polysaccharides have attracted growing interest because of their various biological activities, both *in vivo* and *in vitro* (Wang and Zhang 2019). A key biological activity of plant polysaccharides is immunoregulation, characterized by the ability to reverse cyclophosphamide-induced immunodeficiency (Iwasaki and Medzhitov 2015). *Ginkgo biloba* leaf polysaccharides (GBLP) perform many biological functions, including immune regulation, and have the advantages of low toxicity, high safety, and a wide range of sources. Polysaccharides extracted from *G. biloba* leaves induce immunomodulatory effects by promoting the secretion of nitrous oxide and cytokines in mouse macrophage-derived RAW264.7 cells (Ren et al. 2019). Another study showed that GBLP exerts immunogenic and protective effects against an inactivated, highly virulent infectious bursal disease virus vaccine at a concentration of 20 g/L (Meng et al. 2019a). However, there is limited published data on the immune-related activity of GBLP, and its immunomodulatory activity is virtually unknown. Notably, most of the existing studies, to the best of our knowledge, have focused on the phagocytic ability of macrophages activated by GBLP, function of complement in serum, splenic T lymphocyte activity, and induction of bone marrow-derived dendritic cell maturation. There are no studies on the effects of GBLP on cyclophosphamide-induced immunosuppressed mice, particularly regarding the effects of GBLP on the TLR/MAPK signaling pathways (Fang et al. 2020).

In this study, to clarify whether GBLP can restore deviated immune parameters in immunosuppressed animals, we examined the effects of GBLP on immune organ indices, spleen lymphocyte proliferation, serum lymphocytokine levels, mRNA expression of related cytokines, and expression of TLR/MAPK signaling pathway-related proteins *in vivo*. These discoveries will improve our comprehension of the potential value of GBLP and its use in developing immunomodulatory functional foods to prevent chronic diseases caused by low immunity.

2. Investigations and results

2.1. Determination of organ indices

As shown in Fig. 1A, the organ indices in the SC group were significantly lower than those in NC, GL, GM, and GH groups ($p < 0.05$), indicating that cyclophosphamide treatment significantly

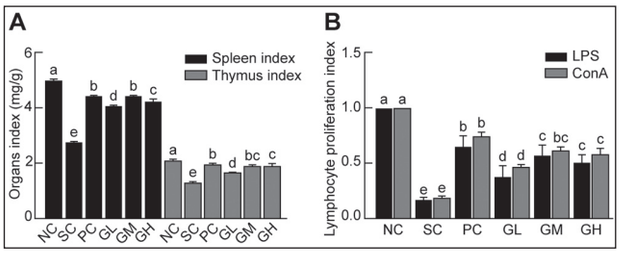

Fig. 1: The effects of GBLP on organ indices and spleen lymphocyte proliferation in cyclophosphamide-treated mice. Values are displayed as mean (x) ± SD (n = 3).

decreased spleen and thymus weight. No critical distinction existed between the organ indices in the GM and PC groups ($p > 0.05$). These outcomes demonstrate that the immunosuppressive model has been adequately established. Furthermore, GBLP was able to reverse the cyclophosphamide-induced decay of these organs.

2.2. Evaluation of the effect of GBLP on lymphocyte proliferation

T and B lymphocyte proliferation in SC was lower than that in NC, as demonstrated in Fig. 1B. Compared to SC, mice in GL, GM, GH, and PC exhibited significant proliferation of lymphocytes induced by concanavalin A (ConA) or lipopolysaccharide (LPS) treatment ($p < 0.05$). GM demonstrated a stronger proliferation of spleen cells following ConA treatment than the other GBLP groups, with no noteworthy distinction compared to PC ($p > 0.05$). The results revealed that GBLP combined with ConA or LPS had synergistic effects on spleen cell proliferation.

2.3. Evaluation of the effect of GBLP on cytokine secretion

As shown in Figs. 2 and 3, IFN-, IL-4, IL-2, and IL-10 production were lower in SC than in NC ($p < 0.05$), demonstrating that cyclophosphamide treatment restricted immunomodulatory capacity of the mice. Cytokine secretion was greater in GL, GM, GH, and PC than in SC ($p < 0.05$). Between GM and PC, there was no discernible difference in the release of IL-4 or IL-10 by lymphocytes ($p > 0.05$). IFN- and IL-2 secretion levels in GM and GH, however, were higher than those in PC ($p < 0.05$). These findings demonstrated that GBLP might boost the control of cytokine release, counteract cyclophosphamide-induced immunosuppression, and enhance immunological activity.

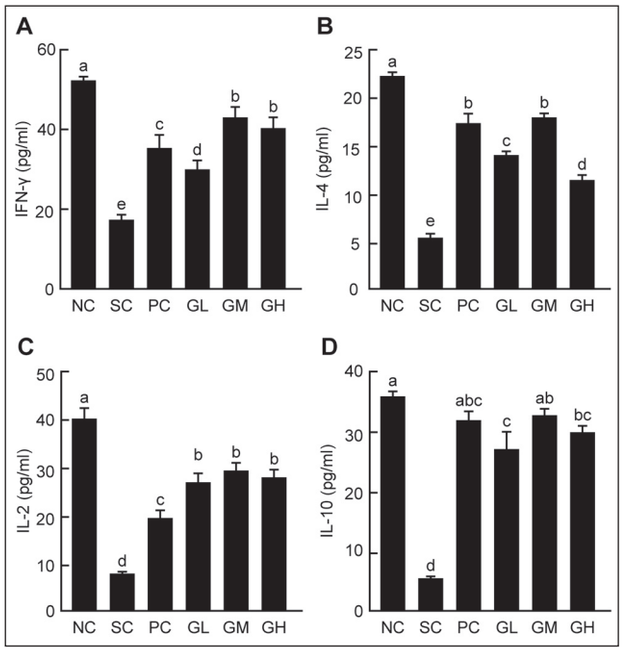

Fig. 2: The effect of GBLP on cytokine secretion. Values are displayed as mean (x) ± SD (n = 3).

2.4. Evaluation of the effect of GBLP on mRNA expression

The mRNA expression levels of IFN-, IL-4, IL-2 and IL-10 in SC group were significantly lower than those in NC, GL, GM, GH and PC groups ($p < 0.05$). There was no significant variation between GM group and PC group ($p > 0.05$). These findings demonstrated that GBLP was able to boost the expression of immune-related genes in order to counteract cyclophosphamide-induced immunosuppression and increase immunological function.

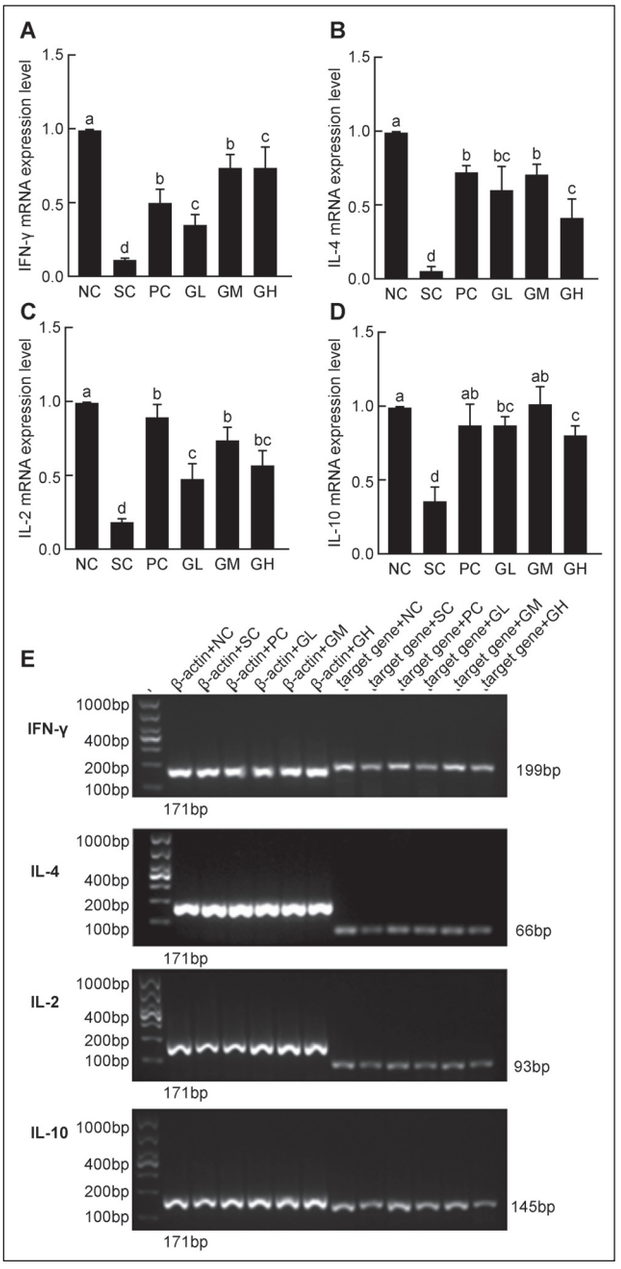

Fig. 3: The effect of GBLP on mRNA expression of cytokines. Values are displayed as mean (x) ± SD (n = 3).

2.5. Effect of GBLP on TLR and MAPK signaling pathways

As illustrated in Figs. 4 and 5, TLR2, TLR4, MyD88, and IRAK4 protein expression levels, as well as the ratios of p-JNK/JNK, p-ERK/ERK, and p-p38/p38, were all considerably lower in SC than in NC ($p < 0.05$). The expression and phosphorylation levels of the correlated proteins in GBLP-treated groups were essentially increased compared with those in SC ($p < 0.05$). Regarding the protein expression levels of TLR4, MyD88, and IRAK4 and ratio of p-JNK/JNK, there were no noteworthy differences between GM and PC ($p > 0.05$). These findings revealed that GBLP elevated protein expression and phosphorylation levels, indicating that GBLP was able to counter cyclophosphamide-induced immunosuppression in mice by activating the TLR/MAPK signaling pathways.

3. Discussion

Cyclophosphamide is the most common immunosuppressant used to treat autoimmune diseases. A high dose of cyclophosphamide can suppress immunocyte proliferation and damage the immune

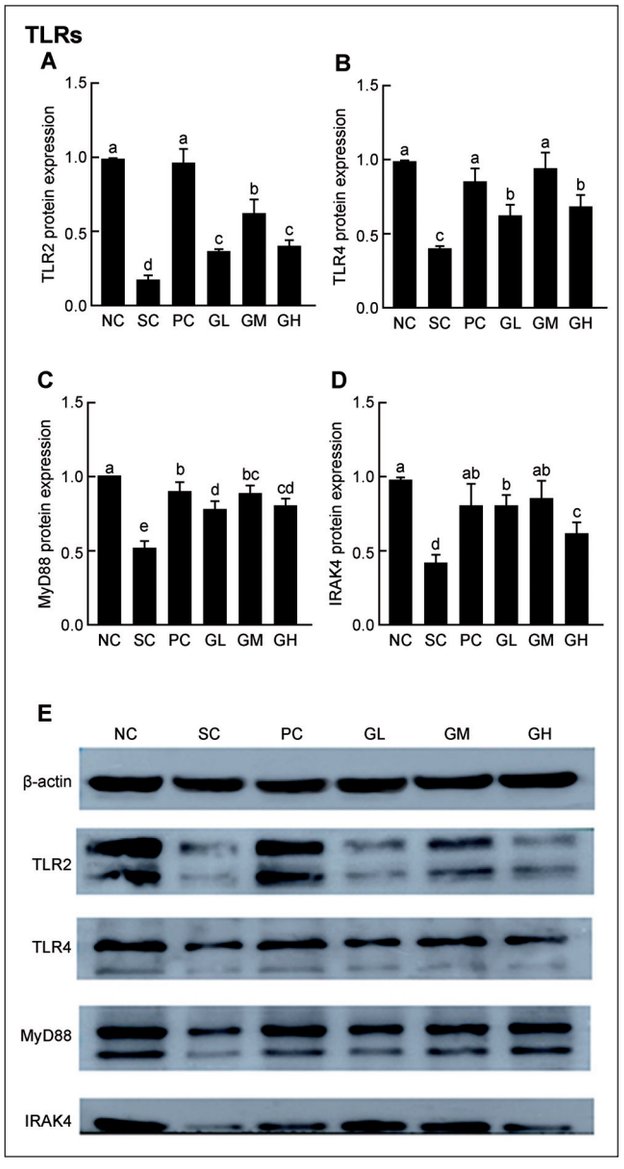

Fig. 4: The effect of GBLP on the TLR signaling pathway. Values are displayed as mean (x) ± SD (n = 3).

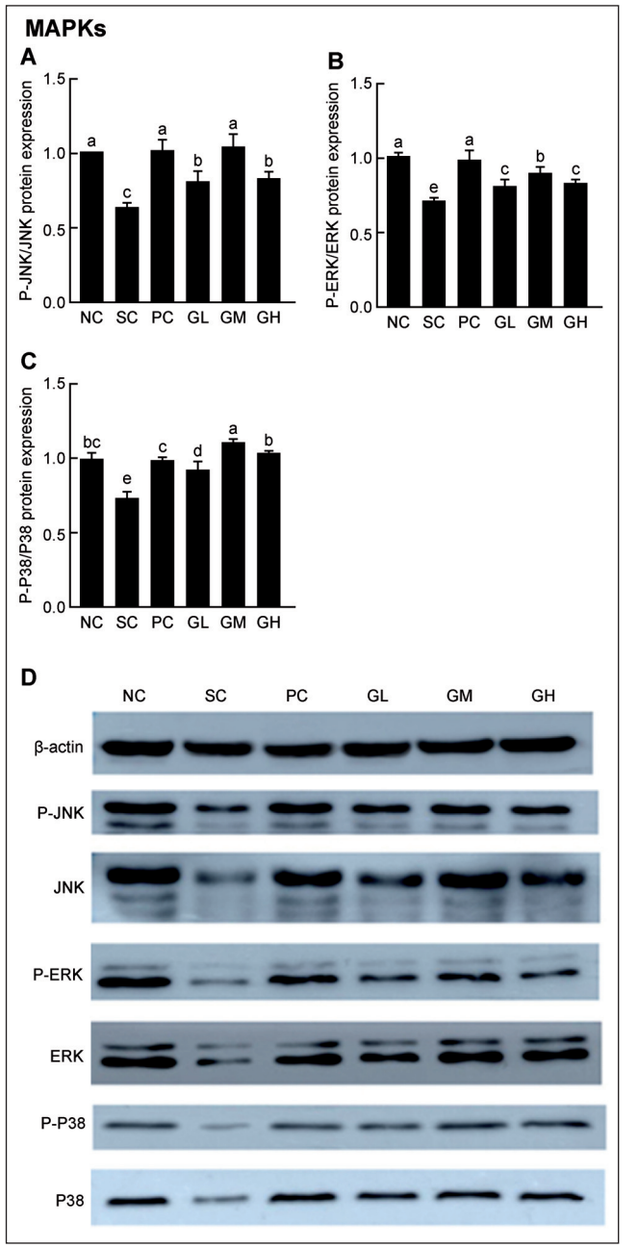

Fig. 5: The effect of GBLP on the MAPK signaling pathway. Values are displayed as mean (x) ± SD (n = 3).

system (Xie et al. 2016). The mammalian immune system is composed of immune organs, immune cells, and immunoactive substances (Beck and Habicht 1996). The spleen and thymus are the primary organs underlying the immune response, and the indices of the spleen and thymus are considered crucial indicators of immune function (Li et al. 2017). Changes in body weight and immunological organs, which are crucial for non-specific defense in mice, are the main ways that cyclophosphamide affects immune function. Lymphocytes are key cells in the adaptive immune response, and analysis of lymphocyte proliferation is regularly utilized to assess the immune reaction capacity of animals (Singh et al. 2016). Therefore, measuring changes in spleen cell proliferation is an effective method for investigating food-related bioactivity and the mechanisms of action. Lymphocytes comprise T and B lymphocytes, with T lymphocytes playing a direct role in the cellular immune response and controlling peripheral blood lymphocyte subsets (Okutani and Shigeta 1993). B lymphocytes develop into plasma cells under antigenic stimulation and can present antigens to other immune cells (Meng et al. 2019b). Investigations have indicated that polysaccharides from pomegranate peel can delay weight loss in immunosuppressed mice, improve immune organ index, boost the proliferation of splenic lymphocytes, and significantly raise the expression of immunoglobulins as well as cytokine release (Yuan et al. 2019). The administration of buckwheat polysaccharides augments the spleen and thymus indices, demonstrating that buckwheat polysaccharides are potential immunomodulators that activate the immune response (Wang et al. 2022b). Our results are consistent with those previously reported, suggesting that the immunosuppressive model has been adequately developed, and that GBLP reversed cyclophosphamide-induced decay of these organs and has a synergistic effect on splenic cell proliferation when combined with ConA or LPS ($p < 0.05$).

Proteins and small peptides secreted by immune cells are called cytokines (Mei et al. 2013). These modulate the immune response by attaching to the appropriate cell surface receptors and controlling cell proliferation and differentiation (Mei et al. 2013). For instance, interferon (IFN)-γ is an exceptionally powerful antiviral cytokine produced by NK and T helper (Th)1 cells and has extensive immunomodulatory effects (Cai et al. 2012). Meanwhile, interleukin (IL)-4 plays a critical role in regulating acquired and humoral immunity (Junttila 2018). IL-2 can strengthen T cell

activity and promote immunoglobulin production (Liu et al. 2018), whereas IL-10 participates in the separation of B cells into slurry cells (Jia et al. 2014). Natural Cordyceps polysaccharides have been reported to increase intestine IL-4 and IFN-γ mRNA levels in cyclophosphamide-induced immunosuppressed mice (Chen et al. 2019). *Ganoderma lucidum* polysaccharides can promote increased IL-17A, IL-6, and Foxp3 levels in the spleens of cyclophosphamide-treated mice (Xiang et al. 2018). IFN-γ, IL-4, IL-2, and IL-10 mRNA expression and secretion in lymphocytes were investigated in this work. Our outcomes illustrated that the mRNA expression and secretion of these cytokines in the lymphocytes decreased significantly in mice treated with cyclophosphamide (Figs. 2 and 3; $p < 0.05$). Furthermore, the indices were higher in GL, GM, GH, and PC than in SC ($p < 0.05$). These findings are consistent with those previously presented and indicate that GBLP can boost immune function and increase cytokine release to counteract the immunosuppression caused by cyclophosphamide.

Polysaccharides activate immune cells *via* multiple signaling pathways to exert their immunoregulatory activity (Yi et al. 2015). TLRs are a class of proteins present on different cell types. They are an essential component of the innate immune system and identify compounds with conserved structural elements (Zhang et al. 2019). The MyD88 signaling pathway is the main access for TLRs. Via this pathway, TLR2 and TLR4 trigger TRAK4, which controls the production of interferons and associated cytokines (Varshney and Saini 2018). Investigations have demonstrated that TLR2 and TLR4 can activate the MAPK signaling pathway through MyD88 (Kawai and Akira 2010). The MAPK signaling pathway is crucial for activation of cytokines and neurotransmitters. It modulates the immune response by transmitting signals (Shen et al. 2017). In the present study, GBLP significantly upregulated the expression levels of TLR signaling pathway proteins (TLR2, TLR4, MyD88, and IRAK4) and the phosphorylation level of MAPK signaling pathway proteins (JNK, ERK, and p38) ($p < 0.05$), improving immune function after cyclophosphamide treatment. This is consistent with results reported in the literature, which show that GBLP functions modify immune responsiveness in immunosuppressed mice via TLR/MAPK pathway activation.

By activating the TLR/MAPK signaling pathway, GBLP can control immunity by promoting the growth of immune organs, triggering the maturation and proliferation of splenic cells, and enhancing expression of cytokines and their mRNA. There have been limited investigations into how GBLP affects relevant immunological indices, particularly the TLR/MAPK signaling pathway in mice with cyclophosphamide-induced immune suppression. Therefore, through this study, we now have a better understanding of the physiological function of GBLP, which provides further information regarding potential ingredients for dietary supplements for immunosuppressed individuals.

4. Experimental

4.1. Experimental animals

We used healthy male BALB/c mice of the SPF class, aged 6 to 8 weeks, weighing 20±2 g [SCXK (Experimental Animal Center, Jinzhou Medical University, Jinzhou, Liaoning, China) 2014-0004]. The EU Directive (2010/63/EU) for animal research and the ARRIVE guidelines were followed in all animal trials. All animal experimental protocols were authorized by the Animal Ethics Committee of the Experimental Animal Center of Jinzhou Medical University (ethical approval no. 2018008).

4.2. Animal model

The mice were reared at 21±2 °C for one week and received a standard rodent diet and water. The six groups of mice (n = 12 per group) were named NC, SC, PC, GL, GM, and GH. Cyclophosphamide (Jiangsu Hengrui Medicine Co., Lianyungang, Jiangsu, China) (60 mg/kg·bw) was injected into each mouse every three days, with the exception of those in the NC group. Only 0.9% saline was given to treated mice in the NC group. The PC group received 40 mg/kg·bw levamisole (Beijing Solarbio Technology, Beijing, China), and mice in GL, GL, and GH groups were administered 200, 400, and 800 mg/kg·bw GBLP, respectively (Shanxi Chuanjiu Biotechnology, Xi'an, China) by gavage for 28 consecutive days. The mice were then euthanized by cervical disengagement 24 h after the last treatment. Blood samples were collected and processed to obtain a suspension of peripheral blood lymphocytes.

4.3. Organ index determination

After the spleen and thymus were extracted, organ index was calculated.

$$\text{organ index (mg/g)} = \frac{\text{spleen or thymus weight (mg)}}{\text{mouse weight (g)}}$$

4.4. Preparation of peripheral blood lymphocytes

After 28 days of treatment, blood was taken by retro-orbital hemorrhage into anticoagulation tubes. The freshly anticoagulated whole blood sample was combined with an equal volume of lymphocyte separation solution. After centrifugation, the middle white membrane containing the lymphocytes was carefully aspirated and placed into a 15 mL centrifuge tube. Following addition of lymphocyte washing solution (10 mL), the sample was centrifuged at 250 g for 20 min. After discarding the supernatant, complete RPMI-1640 medium was added to adjust the cell concentration to 5×10^6 cells/mL for ensuing trials.

4.5. MTT assay to evaluate cell proliferation

As previously mentioned, the spleen lymphocyte proliferation index was calculated (Huang et al. 2020). Varioskan FlashT multipurpose microplate reader (Thermo Fisher Scientific, Shanghai, China) was used to measure the optical density (OD) value at 570 nm. Lymphocyte proliferation rate of mice was expressed as the proliferation index (PI):

$$PI = \frac{\text{Experimental Group OD Value}}{\text{Blank Group OD Value}} \times 100\%$$

4.6. Evaluation of cytokine levels using ELISA

According to the manufacturer's instructions, the samples were detected using ELISA kits (Shanghai Enzymatic Biotechnology Shanghai, China). The OD values were compared to those of a standard curve to determine the cytokine concentrations.

4.7. Evaluation of mRNA expression levels using q-PCR

Total RNA was extracted from the lymphocytes using TRIzol Reagent (Shanghai Biyuntian Biotechnology Co., LTD, Shanghai, China). RNA concentrations and purity were determined utilizing a spectrophotometer (Thermo Fisher Scientific (China) Co., LTD, Shanghai, China). The experiment was conducted using the reverse transcription kit (TaKaRa, Dalian, China) and TB Green Premix Ex Taq II (Tli RNaseH Plus) Kit (TaKaRa). The primer sequences are as follows: IFN-γ, (forward primer)5′-CGGCACAGTCATTGAAAGCCTA-3′ and (reverse primer)5′-GTTGCTGATGGCCTGATTGTC-3′; IL-4, (forward primer)5′-CCTTGGTGGCCATCATCATTC-3′ and (reverse primer)5′-CCTGGCTTCGGGTCTGCTTA-3′; IL-2, (forward primer)5-CCCAGGATGCTCACCTTCA-3′ and (reverse primer)5′-CCGCAGAGGTCCAAGTTCA-3′; IL-10, (forward primer)5′-GCCAGAGCCACATGCTCCTA-3′ and (reverse primer)5′-GATAAGGCTTGGCAACCCAAGTAA-3′; β-actin, (forward primer)5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and (reverse primer)5′-ATGGAGCCACCGATCCACA-3′. Following amplification, the PCR products were sampled and electrophoresed at 120 V for approximately 40 min. The gel imaging equipment (General Electric Company, Boston, MA, USA) was utilized to quantify images and detect PCR products. The calculated values were computed using the $2^{-\Delta\Delta Ct}$ approach.

4.8. Evaluation of TLR/MAPK pathway-related protein expression levels using western blotting

RIPA lysis buffer was used to extract the protein (Beyotime Biotechnology, Shanghai, China). For quantification, the BCA (Beyotime Biotechnology) method was utilized. The membrane was transferred and blocked after gel electrophoresis. The primary antibodies [β-actin, TLR2, TLR4, MyD88, IRAK4, JNK, P-JNK, ERK, P-ERK, P38, P-P38 (Cell Signaling Technology, Danvers, MA, USA)] were incubated with the films overnight at 4 °C. The corresponding secondary antibodies against Anti-rabbit IgG (Cell Signaling Technology) were then added and incubated for 2 h. The protein bands were observed after the ECL reaction.

4.9. Statistical analysis

Each experiment was repeated thrice. The outcomes are presented as the mean (x) ± standard deviation (SD). Comparisons between groups were made by single factor analysis of variance and the significance test performed using the least significant difference method with the test standard $\alpha = 0.05$. Statistical analysis was carried out utilizing SPSS 22.0. (IBM, Armonk, NY, USA).

Acknowledgements: We appreciate Professor Yu Yang's thorough advice on the planning and writing of this experiment. We thank Mr. Hui Wong for his valuable advice on a preliminary version of the manuscript and Jing Shuang Li, Yi Lun Wang, Ablimit Arzugul, Yue Zhu Zhao, and Yunan Zhang for help with the experiments.

Funding: The Natural Science Foundation of Liaoning Province (Grant No. 20170540369, 20170540357) and Key Research Project of Education Department of Liaoning Province in 2022 (Grant No. LJKZZ20220094) provided funding for this work.

Conflicts of interest: None declared.

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