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The regulatory effect of edible bird's nest glycoproteins on immune function and intestinal flora in cyclophosphamide-induced immunosuppressed mice

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ABSTRACT: This study investigated the immunomodulatory effect of edible bird's nest glycoprotein (EBNG) regarding physiological and biochemical indices, transcriptomics, and intestinal flora using immunosuppressive mice. The results showed that EBNG could relieve body weight loss, repair the damage to the spleen and thymus, promote the proliferation of spleen T and B lymphocytes, and promote the secretion of cytokines and immunoglobulins in mice. Furthermore, EBNG alleviated the imbalance of gut microbiota by decreasing the ratio of Firmicutes/Bacteroidetes and increasing the abundance of intestinal probiotics such as *Odoribacter*. Transcriptomic analyses indicated that the mechanism of immunomodulatory effects might be triggered by the upregulation of the CD4, CD19, PIK3CD, and IFNG genes expressed by T and B cells, and participated in the immune-related signalling pathway, such as the intestinal immune network for IgA production and Th1 and Th2 cell differentiation. This study provides scientific support for understanding the immunomodulatory mechanism of EBNG.

Keywords: edible bird's nest (EBN), glycoprotein, immunomodulatory, intestinal flora, transcriptomics

1. Introduction

The immune system is a crucial barrier of the human organism that identifies and eliminates invading pathogens, including bacteria, viruses, and fungi, while surveilling and eradicating aberrant cells, such as tumor cells [1]. Due to the repercussions of the post-pandemic, the world population's immune systems may be compromised to differing extents [2]. Furthermore, additional categories of immunocompromised individuals include cancer patients, HIV-positive individuals, organ transplant recipients, and those on long-term drugs that may inhibit immune function [1]. An escalating number of individuals may be encountering diminished autoimmunity, which warrants attention.

In the field of modern medicine, immunomodulators play a vital role in maintaining and regulating the body's immune balance [3]. However, the therapeutic effects of these immunomodulators are often accompanied by a series of potential side effects. For example, they may cause gastrointestinal discomfort and

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liver and kidney impairment, and long-term use may also lead to the development of drug resistance. Given the drawbacks of those immunomodulators, the search for safe and effective natural immunomodulators has become a hot research topic in the field of immunology and pharmacology. Natural immunomodulators are usually derived from natural components of plants, animals, or microorganisms, which have the advantages of low toxicity, low side effects, and diverse biological activities. Consequently, it is essential to identify safe and dependable immune enhancers that may bolster the body's immunity to prevent diseases and mitigate the harm inflicted by immunosuppressive medications.

Natural biochemicals as immunomodulators exhibit significant therapeutic potential with minimal toxicity and adverse consequences. Glycoproteins are regarded as valuable macromolecules and natural immunological enhancers [4]. An increasing number of studies indicated that glycoproteins derived from animals [5], plants [6], and microorganisms [7] exhibit beneficial immunoreactive properties. In contrast to standard proteins, glycoproteins are a category of proteins covalently attached to carbohydrates, and their covalently connected glycan structures have demonstrated an active function in immunomodulation [8]. Concurrently, glycans are vital for cellular recognition as signalling molecules. Research indicates that pattern recognition receptors on macrophages identify specific oligosaccharides, which activate macrophages and enable their involvement in immunomodulatory processes [9]. Li et al. isolated N-glycosylated modified glycoproteins from yam named 30CYGP, which found that the glycosylated 30CYGP had significantly more immunoactivity than deglycosylated 30CYGP [10]. A separate report indicated that the immunomodulatory effect of water-soluble glycoprotein extracted from *Ganoderma lucidum* was predominantly attributed to its glycopeptide fraction, which had crucial terminal fucose residues with α -1,2-fucosidic connections [4]. The dynamic balance between intestinal microecology and the body's immune system is the core barrier to maintaining health. As a key molecular link connecting intestinal flora and immune organs, glycoproteins' unique glycan structure and protein skeleton characteristics directly determine the specificity and functionality of the interaction of the three. From a molecular perspective, the modification of the N/O-glycan (sialic acid, fucose residues) on glycoproteins serves as a targeted binding site for the surface adhesion structure of intestinal flora.

Edible Bird's Nest (EBN) has been eaten since the Tang Dynasty. The "Compendium of Materia Medica" states that EBN can hydrate the lungs, alleviate cough, and reduce phlegm. It predominantly consists of protein (about 60 %), carbohydrates (30 %), and various other nutrients [11]. Recent studies indicate that the protein in EBN is a glycoprotein covalently bonded to carbohydrates, comprising up to 90 % of its content [12]. Choong et al. have evidenced through in vitro cellular tests that EBN extract can augment human immunity by stimulating the synthesis of particular cytokines and immunoglobulin isoforms via accelerated B-cell proliferation [13]. Hanisch et al. observed that the EBNG possesses N-linked and O-linked glycan modifications [14, 15]. Previous studies by our team found that a total of 127 N-glycoproteins were identified in EBN, with a relatively high content of end-silylated and core fucosylated N-glycans. Bioinformatics analysis revealed that EBN N-glycoprotein is mainly involved in immune-related signaling pathways, such as

cell adhesion molecules, TNF signaling, and TGF-beta signaling pathways. Increasing evidence indicated that the immunomodulatory function of glycoproteins was associated with their structural characteristics, particularly the glycan structure [16]. Consequently, it is hypothesized that the immunomodulatory properties of EBN may be related to its unique glycoprotein composition. Nonetheless, there is a lack of research on the immunomodulatory effects of EBNG and its molecular mechanisms, many studies assess immunological effectiveness without a comprehensive investigation of the underlying molecular mechanism.

In this study, a cyclophosphamide (CTX)-induced immunosuppression mice model was established to investigate the immunomodulatory activity of EBNG. CTX is a frequently utilized chemotherapeutic agent for the management of malignant neoplasms. However, while killing tumour cells, it also destroys self-renewing cells. Research indicates that a particular dosage of CTX administered in two brief intervals markedly diminishes immunological function in mice [17]. Several aspects of immune organs, immune cells, and immune factors were explored in this study. The core immune-related genes in the action of EBNG immune response and the molecular mechanism of its immunomodulatory effects were identified through transcriptomics. In addition, the immunomodulatory role of EBNG in the changes of intestinal flora was also confirmed. This study elucidated the immunoregulatory role of EBNG in the changes of intestinal flora, which can be used as a scientific basis for the development and utilization of EBN resources.

2. Materials and methods

2.1 Materials and Reagents

EBNs were obtained from artificial swiftlet habitats in Indonesia and are categorized as house-EBN. CTX was purchased from Sigma-Aldrich (Shanghai, China). Levamisole hydrochloride (LH, 99 %) was obtained from Shanghai Maclean Biochemical Technology Co, Ltd. (Shanghai, China). ELISA kits (IL-2, IL-4, IL-6, IL-10, TNF- α , INF- γ) were purchased from Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China). Bicinchoninic acid (BCA) assay kit was provided by Innochem, (Beijing, China). Fluorescein Allophycocyanin (APC)-labeled Cluster of Differentiation 19 (CD19⁺) Monoclonal Antibody and Fluorescein Isothiocyanate (FITC)-labeled CD3⁺ Monoclonal Antibody were provided by Thermo Fisher, USA.

2.2 Extraction and Quantification of EBNG

EBNG was obtained following a prior study with particular alterations [18]. Thereafter, EBN powder and distilled water were amalgamated in a 1:46 (w/v) ratio and extracted for 2 h and 40 mins at 80 °C. After attaining room temperature, the EBN mixture was centrifuged at 12,000 rpm for 20 mins at 4 °C, and the supernatant was harvested as the EBN crude extracts. 1 mL of EBN crude extracts was mixed with pre-cooled acetone at -20 °C at a volume ratio of 1:6 and then frozen overnight. On the next day, the mixture was subjected to centrifugation at 5000 rpm for 10 mins at 4 °C. The EP tube was carefully inverted to decant the acetone, retaining the white residue, which was then dried for 2-3 mins. The residue was re-dissolved with 500 μ L of aqueous solution, and free protein was removed using the Sevage method to obtain EBNG. The protein

content was quantified at 562 nm using a BCA assay. A standard curve was established, and the EBNG concentration was quantified.

2.3 Experimental Animals and Intervention

Sixty 4-6 weeks of age SPF BALB/c, half male and half female mice (20 ± 2 g) were purchased from the SPF (Beijing) Biotechnology Co., Ltd. In the tests, mice live in an environment with optimal temperature & humidity (24 ± 1 °C & 50 % - 60 %) in which they are exposed to light half of the time and darkness half of the time for 24 hours per day. The Ethics Committee approved the study for Laboratory Animal Care of the Chinese Academy of Inspection and Quarantine (No.2023-FD-013-1).

Sixty mice were randomly allocated into six groups (Fig. 1A). The normal control (NC) and model control (MC) groups received a gavage of saline at 10 mL/kg·bw. The positive control (PC) group was administered LH at 40 mg/kg·bw. The EBNG-L, M, H doses were categorized into groups of 6.25 mg/kg·bw, 12.5 mg/kg·bw, and 25 mg/kg·bw. Except for the NC group, the other five groups of mice received intraperitoneal injections of CTX at a dosage of 80 mg/kg·bw on days 1-3 and 17-18 to create immunosuppressive mice. After three days, three mice were randomly chosen from each group for weight and hematological assessments. The requirements for effective mouse modeling included substantial weight loss and a marked decrease in leukocyte count, a standard hematological parameter. The experimental period was 28 d.

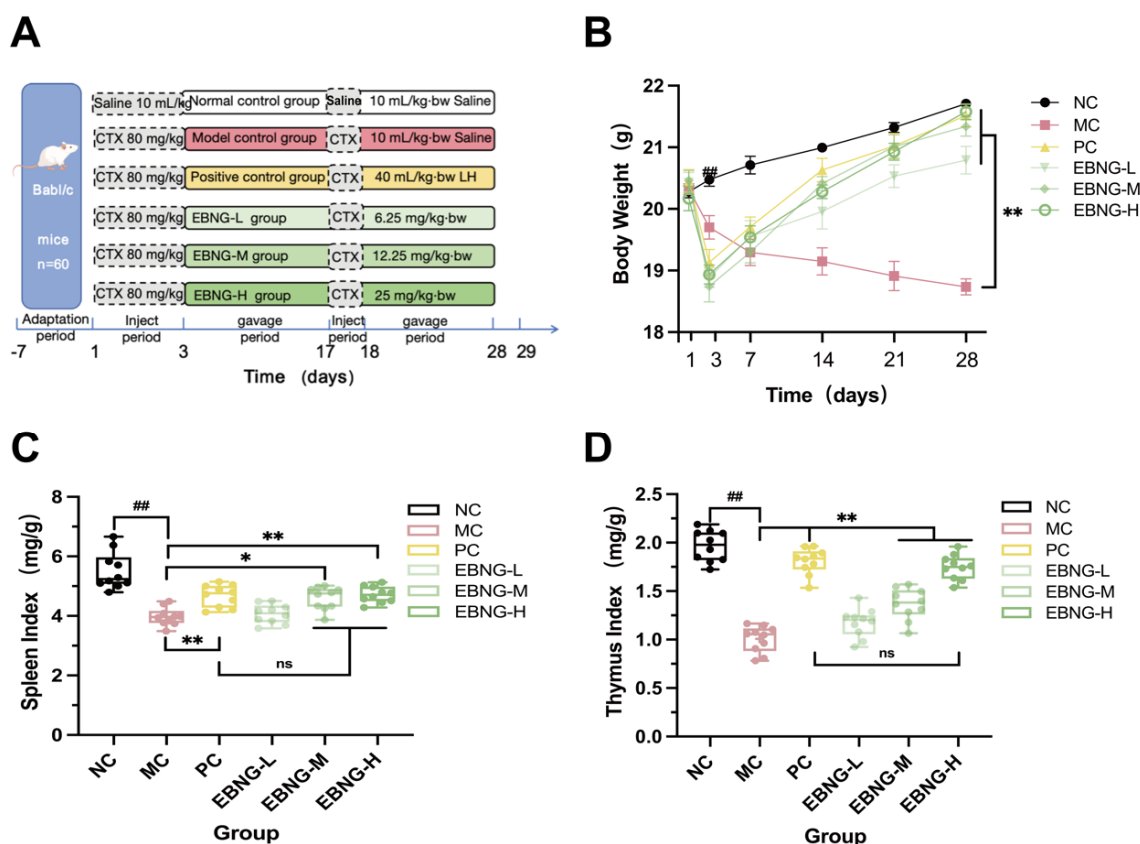


Fig. 1. Effect of EBNG on body weight and organ index in mice. A. Animal experiment design; B. The effect of EBNG on the body weight of mice (n=10); C. Effect of EBNG on the index of spleen in mice (n=10); D. Effect of EBNG on the index of thymus in mice (n=10); Data are presented as the mean $\pm$ SE. Compared with the NC group: $^{\#}P \leq 0.05$ and $^{\#\#}P \leq 0.01$; compared with the MC group: $^*P < 0.05$ and $^{**}P < 0.01$; $P > 0.05$ is marked as ns.

2.4 Body weight and organ index

The mice were weighed weekly. After the treatment, the mice had a 12 h fasting period. The mice were later surgically dislocated and euthanized, after which organs such as the spleen and thymus were excised and weighed. The visceral index was computed using the subsequent formula [19].

$$\text{Organ index}(\%) = \frac{\text{organ weight}}{\text{body weight}}$$

2.5 Histomorphological observations

Spleen and thymus were preserved in 4% paraformaldehyde and then embedded in paraffin, cut into 5 μm thick sections, and stained with hematoxylin and eosin (H&E) [20]. Imaged using a Digital Slide Scanner (PROSCANNER APPro 5, AMES Company, USA).

2.6 Percentage of T and B Cells in Splenic Lymphocytes

The ratios of T and B cells in spleen lymphocytes were assessed by flow cytometry (S3e, Bio-Rad, USA) [21]. Initially, 100 μL of each mouse spleen lymphocyte suspension (concentration 2×10^6 cells/mL) was mixed with 5 μL of FITC CD3⁺ antibody (label T lymphocytes) and APC CD19⁺ antibody (label B lymphocytes) and incubated for 30 mins at 4 °C under light. The reaction was terminated by the addition of 1 mL of PBS containing 1 % FBS, and the cells were washed twice with PBS to remove residual antibodies. The cells were then resuspended in 0.5 mL of PBS. Finally, the cells were detected by flow cytometry, and the results were analyzed using FlowJov.10 software.

2.7 Quantification of cytokine and immunoglobulin concentrations in serum

Whole blood from the executed mice was taken and centrifuged at 3000 rpm for 10 min; then, the upper layer was collected. The levels of IL-2, IL-4, interferon- γ , IgA, and IgG in the serum of mice were measured by enzyme-linked immunosorbent assay [22].

2.8 Spleen Transcriptomics analysis

The transcriptome analysis of the spleen was conducted by Lingen Biotechnology Ltd (<https://www.biozeron.com>). Total RNA from the spleen was extracted using Trizol reagent, chloroform, and DEPC water. The concentration and integrity of RNA were assessed using a Nanodrop 2000 (Thermo Fisher Scientific, USA). cDNA was generated and amplified randomly from fragmented mRNA to create multiplexed DNA libraries. The multiplexed DNA libraries were compared to the reference genome using HISAT2, followed by differential expression and enrichment analysis [23].

2.9 Analysis of Gut Microbiota

Fecal samples were cryopreserved in liquid nitrogen for 3 hours and thereafter kept at -80 °C to facilitate the identification of amplified segments of the 16S rRNA gene [24]. The amplified products consisted of forward (5'-ACTCCTACGGGGAGG-CAGCA-3) and reverse (5'-GGACTACHVGGGGTWTCTAAT-3) primers. The amplification products were measured using 2% (w/v) agarose gel electrophoresis and fluorescence. Thereafter, bioinformatics studies were conducted using validated libraries. Computational

evaluations and bioinformatics studies were conducted on the Illumina HiSeq platform using particular sequencing adapters and amplification products. High-throughput sequencing and bioinformatics analysis were conducted by Lingen Biotechnology Ltd (<https://www.biozeron.com>). Valid reads were filtered for fitness, ambiguity, or poor quality from raw sequencing data with Trimomatic version 0.33. Clean data were produced using Flash Memory (version 1.2.7), clustering valid reads at a 97% similarity threshold in the tagged sequences with UCLUST in QIIME (version 1.8.0) to delineate Operational Taxonomic Units (OTUs) and conduct diversity analysis. Samples were evaluated for alpha diversity index with Mothur (v1.30.1) software. The beta diversity of the bacterial population was assessed using QIIME. The OTU sequences of the samples were aligned with microbial databases, and the microbial composition was statistically evaluated across several taxonomic levels. Species abundance analysis was conducted with QIIME. The main gene network was ultimately mapped in R. Effect sizes of differentially enriched genes across several samples were assessed using Linear Dispersion Analysis Effect Size (LEFSE).

2.10 Statistical analysis of data

All statistical analyses were conducted using SPSS version 22.0 software (SPSS Inc.: Chicago, IL, USA). Graphs were generated using Origin v.8.5.1 software. The results were assessed in triplicate and shown as means $\pm$ standard error of the mean (SEM). Statistical differences between groups were evaluated using a one-way ANOVA, followed by Duncan's multiple comparison tests. $P < 0.05$ and $P < 0.01$ denote significant and extremely significant differences, respectively. We express our gratitude for the sketching tools offered by Figdraw (<https://www.figdraw.com/#/>) and ChiPlot (<https://www.chiplot.online/>).

3. Results and discussion

3.1 Effect of EBNG on body weight and organ index in immunosuppressed mice

Mice's body weight visually responds to their physical state, and as their immunity is weakened, they lose weight and become sluggish [25]. As illustrated in Fig.1B, throughout the testing period, the mice in the NC group consistently increased body weight and displayed a lustrous coat, responsiveness, and a typical diet. Compared to the mice in the NC group, the weight of the mice in the experimental group consistently declined. By the third day of modeling, the weight index of mice was calculated from the values of NC group (20.48 ± 0.1079) g was reduced to (18.89 ± 0.1574) g, (18.75 ± 0.2542) g and (18.94 ± 0.1535) g in the low, medium and high dose groups of EBNG group, with the lowest reduction reaching 8.45% ($P < 0.05$) confirming the success of the modelling. After gavage, mice's body weights in each EBNG dosage group exhibited an upward trend, ultimately returning to levels comparable to those of the NC group. EBNG may mitigate CTX-induced weight loss in mice and possesses potential immunomodulatory properties.

The spleen and thymus are the primary immunological organs of the body, significantly contributing to the immune system. The organ index of the spleen and thymus indices can indicate the body's immune health to some degree and are frequently utilized as major metrics for assessing immune function [26]. Fig.1C&D demonstrate that the immune organ indices of the spleen and thymus in the NC group were (5.506 ± 0.1964)

mg/g and (2.013 ± 0.071) mg/g, respectively. Compared with the NC group, the immune organ indices of the spleen and thymus in the MC group were significantly reduced ($P < 0.01$), which were (3.971 ± 0.0951) mg/g and (1.077 ± 0.0443) mg/g, respectively. These suggest that CTX exerted a severely detrimental effect on the spleen and thymus of the mice, hence reaffirming the efficacy of the modelling process. Following administration, compared with the MC group, the immune organ indices of the spleen and thymus in the PC group were significantly increased ($P < 0.01$), which were (4.677 ± 0.1163) mg/g and (1.777 ± 0.0764) mg/g, respectively. The EBNG dose groups demonstrated a gradual recovery correlating with increased dosage, and the immune organ indices rose linearly. It is worth noting that the spleen organ index (4.607 ± 0.1208) mg/g and thymus organ index (1.657 ± 0.1057) mg/g in the EBNG-M group showed a significantly increased concentration effect ($P < 0.05$), while the spleen organ index (4.716 ± 0.2735) mg/g and thymus organ index (1.826 ± 0.1425) mg/g in the EBNG-H group showed a very significantly increased concentration effect ($P < 0.01$). The highest increase in the immune organ index of the spleen and thymus reached 18.76% and 69.55% respectively, indicating that EBNG effectively reduces immune organ damage without toxic side effects.

3.2 Effect of EBNG on histomorphology of immune organs in immunosuppressed mice

The essence of illness in the body is caused by cytopathic changes in tissues or organs [27]. Observing alterations in the cells enables a more precise morphological pathological diagnosis [28]. As illustrated in Fig. 2A, the NC group (Fig. 2A-a) animals exhibited distinct boundaries between the red and white marrow, with structurally preserved splenic vesicles in the white marrow and intact interconnected splenic cords in the red marrow. The MC group mice (Fig. 2A-b) exhibited indistinct red and white marrow and sparse lymphatic sheath structures within the splenic organs, matching the observations detailed by Yu et al [29]. This demonstrated that the modeling was effective. In the EBNG-L group (Fig. 2. A-d), the distinction between the red and white marrow was more distinct; however, the splenic cord structure inside the red marrow was neither entire nor interconnected. The white marrow in the EBNG-M (Fig. 2. A-e), EBNG-H group (Fig. 2. A-f) and PC group (Fig. 2. A-c) exhibited increased prominence, characterised by thickened marginal bands, intact histomorphology of the splenic cysts and a gradual restoration of histomorphology resembling that of the NC and PC groups. This suggests that the EBNG effectively mitigates CTX-induced splenic histomorphology abnormalities.

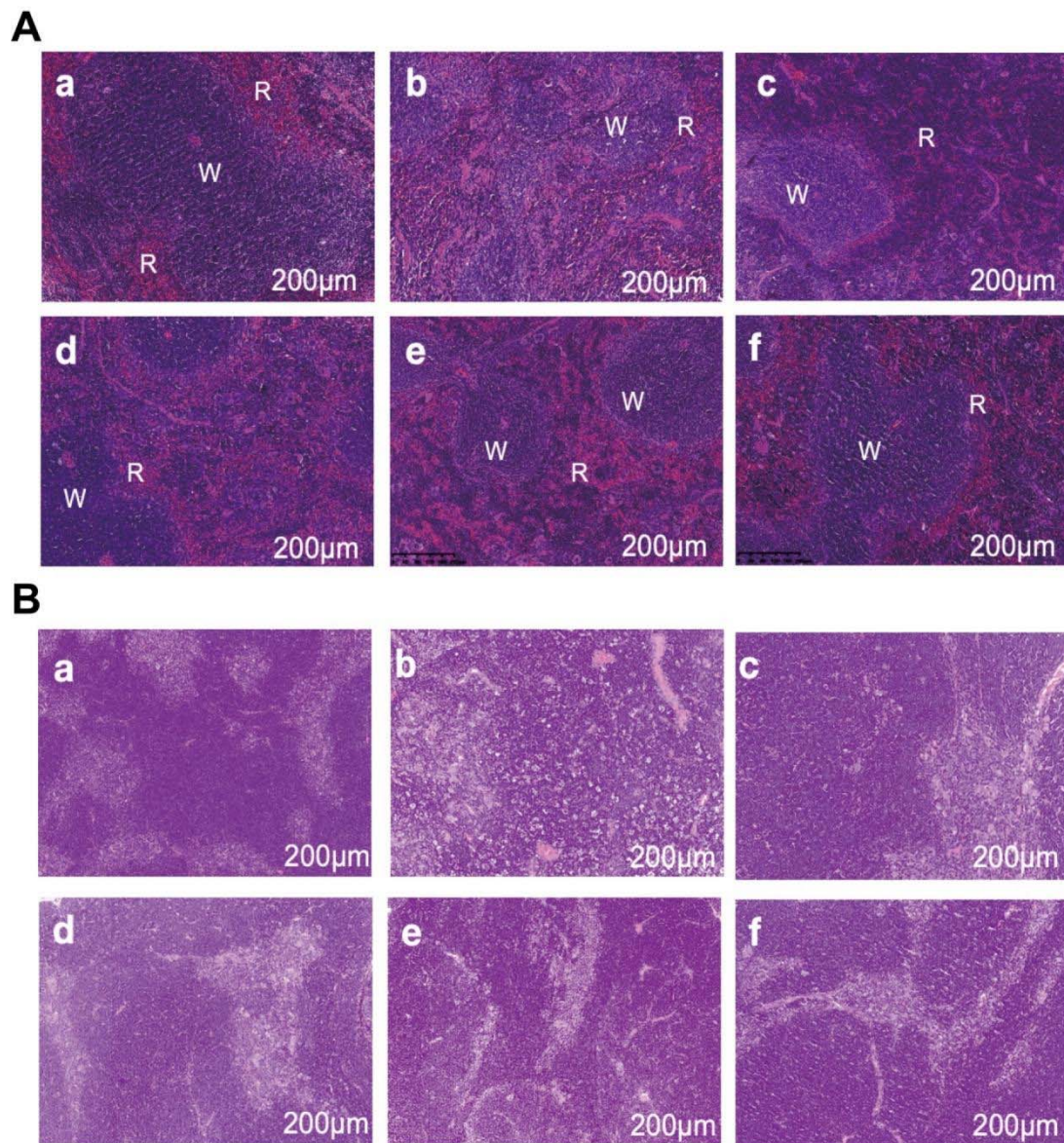


Fig. 2. Tissue section morphology of immune organs in mice in each group ($\times 200$). A. spleen: R represents red marrow and W represents white marrow; B. thymus. (a) Control group, (b) Model group, (c) Positive group, (d) EBNG-L group, (e) EBNG-M group, (f) EBNG-H group. (n=6)

3.3 Effect of EBNG on Splenic Lymphocytes in Immunosuppressed Mice

The spleen can function in an immunomodulatory capacity via T-cell-mediated cellular immunity and B-cell-mediated humoral immunity. The elevation in T- and B-lymphocyte levels may indicate the body's immunological response [27]. To investigate the proliferative effects of EBNG on T and B cells, the proportions of T and B cells in the splenic lymphocytes of mice were analyzed via flow cytometry, utilizing $CD3^+$ and $CD19^+$ as specific surface markers for T and B cells, respectively. $CD3^+$ is a marker on the surface of mature T cells that recognizes the activation of T lymphocytes. B lymphocytes act as antigen-presenting cells and their specific surface marker is the $CD19^+$ molecule. Fig. 3 shows that compared with NC group, the proportion of spleen lymphocytes in mice in MC group was significantly lower, with T cells being 17.5% and B cells being 3.38%. On the contrary, the beneficial drug LH significantly increased the proportion of spleen lymphocytes in mice in the PC group, which was 38.9% for T cells and 9.0% for B cells, respectively,

indicating that LH effectively reduced the damage caused by CTX on T and B lymphocytes in mice, and had a significant impact on T lymphocytes. Compared with the MC group, the ratio of T cells and B cells was significantly increased in mice receiving EBNG, with the EBNG-H group showing the most significant effect. T cells were 43.5%, and B cells were 48.3%, respectively.

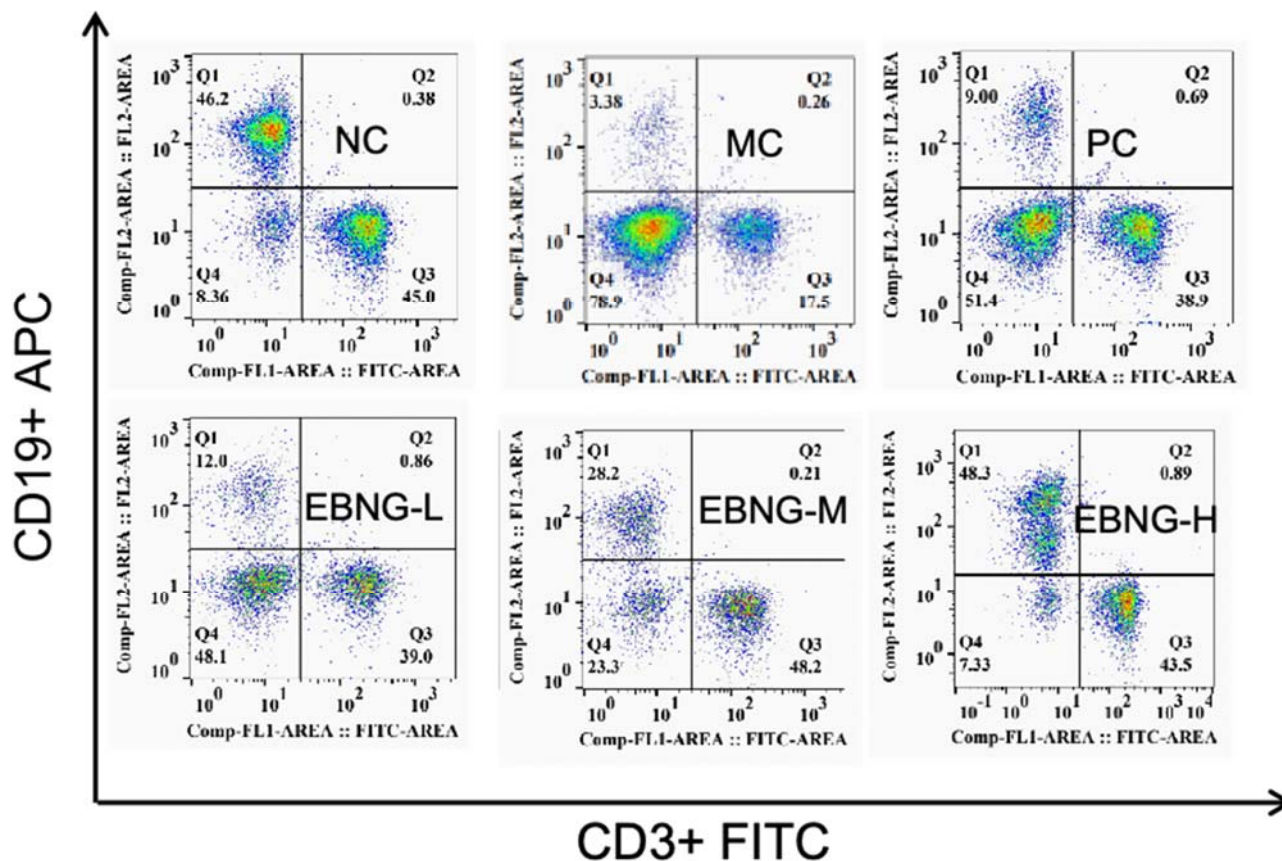


Fig. 3. The effect of EBNG on the proliferation of T and B lymphocytes. The proportions of T and B lymphocytes in mice were detected by flow cytometry. The abscissa is the proportion of CD3⁺-labelled T cells, and the ordinate is the proportion of CD19⁺-labelled B cells. (n=3)

The results substantiate that EBNG facilitated cellular immunomodulation primarily by enhancing the proliferation of T and B cells. Zhao et al demonstrated that edible bird nest extraction (EBNE) has reversal influences on CD3⁺ T-cell and CD19⁺ B-cell ratio imbalance in CY-treated mice [30]. Our results further demonstrated that EBNG has a favorable effect on CTX-induced CD3⁺ T-cells and CD19⁺ B-cells, thus, we confirmed that the immunoreactivity of EBN extracts is closely related to glycoproteins, which suggests that EBN is a promising immunomodulator.

3.4 Effect of EBNG on Serum Cytokine and Immunoglobulin Secretion in Immunosuppressed Mice

Cytokines are crucial in modulating the immune response by facilitating the proliferation and differentiation of target cells and promoting the expression of receptors involved in the immune system [31]. T-lymphocytes mostly release Th1 and Th2 cytokines, while B-lymphocytes predominantly secrete immunoglobulins, such as IgG and IgA. Tumor necrosis factor (TNF)- α and Interferon (INF)- γ facilitated the proliferation and development of Th1 cells and performed cellular immunological tasks. Interleukin (IL-4 and IL-6 can facilitate the Th2 cell response and perform humoral immunological functions. As a

chemotherapeutic agent, CTX causes immunological impairment during cancer treatment, reducing levels of several cytokines and immunoglobulins. In the present study, we examined the impact of EBNG on cytokines and immunoglobulins in an immunosuppressed mice model. As shown in Fig.4, cytokines (TNF- α , INF- γ , IL-4 and IL-6) and immunoglobulins (IgG and IgA) release was significantly reduced in the MC group compared to the NC group ($P < 0.05$). In contrast, the cytokine and immunoglobulin levels in the EBNG-L, EBNG-M, and EBNG-H groups were significantly elevated compared to the MC group, approaching the levels observed in the NC group. These indicated that EBNG can mitigate the abnormal secretion of cytokines and immunoglobulins induced by CTX. The secretion level exhibited a dose-dependent relationship with increasing doses of EBNG ($P < 0.01$). These result indicated that EBNG is proposed to facilitate cellular and humoral immunity by promoting the secretion of mice serum cytokine and immunoglobulins.

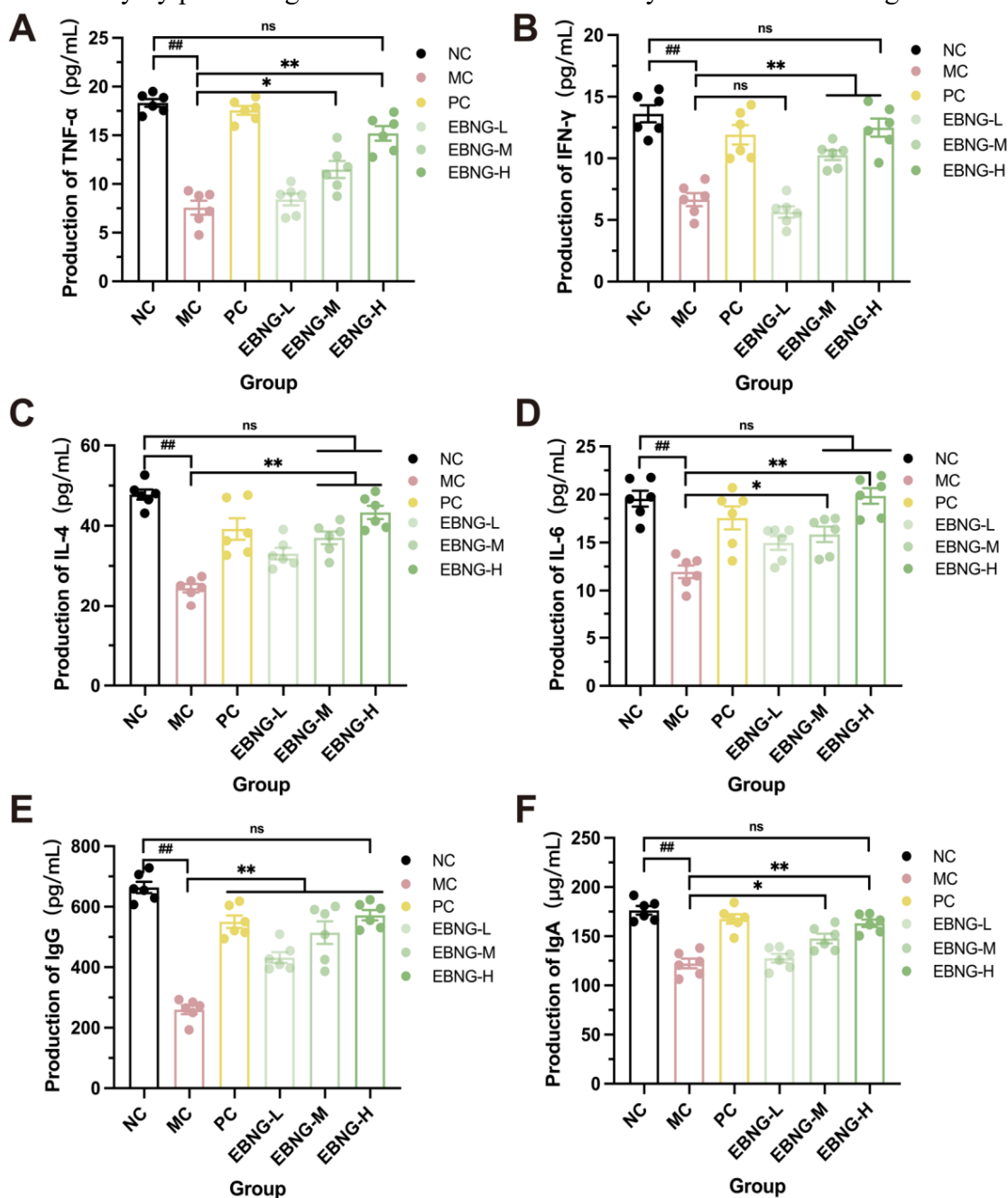


Fig. 4. The effect of EBNG on cytokine and immunoglobulin contents. (n=6) A. Production of TNF- α ; B. Production of IFN- γ ; C. Production of IL-4; D. Production of IL-6; E. Production of IgG; F. Production of IgA; Data are presented as the mean $\pm$ SE. Compared with the NC group: # $P \leq 0.05$ and ## $P \leq 0.01$; compared with the MC group: * $P < 0.05$ and ** $P < 0.01$.

Immune cells can participate in organism immunity by synthesising and secreting cytokines that can control immune function [32]. Th1 cells are primarily responsible for cellular immunity and the inflammatory response by secreting IFN- γ and TNF- α . Th2 cells are involved in the production of antibodies and the proliferation of B lymphocytes, and they primarily secrete IL-4 and IL-6, which are crucial for boosting humoral immune response. The body's most significant antibody, IgG, also has antiviral, virus-neutralizing, antibacterial, and immunomodulatory properties. IgA, primarily in the form of secretory IgA (sIgA), helps secrete and transport sIgA so that it can perform its immune defence function on mucosal surfaces and shields IgA from protease destruction in mucosal areas, including the intestines. Thus, by regulating the secretion of aberrant Th1-type cytokines (IFN- γ , TNF- α) and Th2-type cytokines (IL-4, IL-6), as well as increasing the secretion of immunoglobulins (IgG and IgA) in B-lymphocytes, we were able to confirm the immunomodulatory effect of EBNG at the level of immunoreactive factors. Consequently, it contributes to humoral and cellular immunity in the body. Zhao et al demonstrated that EBNE has promoted the secretion of sIgA in the small intestine [30]. Our findings further suggest a similar effect in EBNG. Thus, we again speculate that the immunological activity of EBN extracts is closely related to glycoproteins, which also suggests that EBN is a promising immunomodulator.

3.5 Effect of EBNG on gene expression in the spleen of immunosuppressed mice

To explore the underlying molecular mechanisms of dietary EBNG on immunoregulatory effect in CTX-induced immunosuppression mice. Spleen RNA was sequenced in each group of mice by comparing the expression levels and changes at the gene level among the groups. A total of 18 mice spleen samples were detected by the RNA-Seq platform. The average comparison rate of samples was 92.30% for comparative genomes and 71.27% for comparative gene sets. A total of 21,685 genes were detected. As shown in Fig.5, screening for differential expression gene (DEG), $[\log_2FC] > 1$ and $P < 0.05$. 3367 DEGs were identified in the NC and MC groups, of which there were 1593 up-regulated and 1774 down-regulated DEGs, and 2013 DEGs were identified in the MC and EBNG groups, of which there were 1263 up-regulated and 750 down-regulated DEGs were identified. The dietary intervention with the EBNG decreased the number of DEGs in the spleens of immunosuppressed mice. Fig.5E showed that 1072 DEGs were consistently expressed in both comparisons, from which the immune-related DEGs were further extracted and screened for core genes.

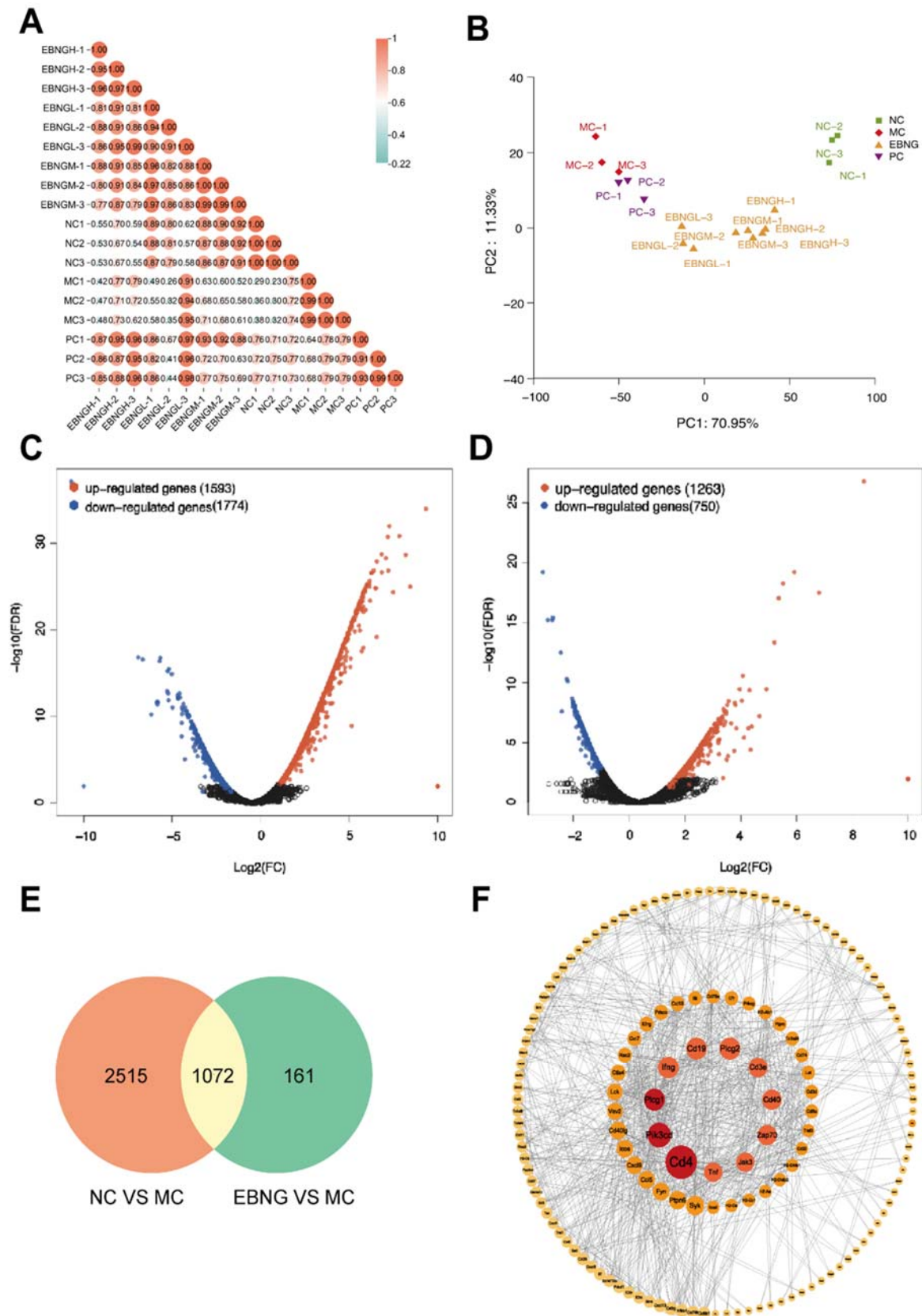


Fig. 5. Overview of mouse spleen transcriptomes. (n=3) A. Pairwise correlation of three biological replicates from mouse spleens of all groups; B. PCA for each sample; C. volcano plot of NC group VS MC group DEGs; D. volcano plot of MC group VS EBNG group DEGs; E. Venn diagrams showing the overlap of DEGs [$|\log_2(\text{fold change})| > 1$] comparing NC group vs MC group and MC group vs EBNG group; F. protein-protein interaction (PPI) analysis.

We explored the immunomodulatory mechanism of EBNG in depth at the molecular level by spleen transcriptomics analysis. The transcriptional expression of CD4, PIK3CD, IFNG, and CD19 was markedly

diminished in the MC group relative to the NC group, while the dietary intervention with EBNG restored and enhanced the transcriptional expression of these genes (Fig. 5F). Numerous data indicated that CD4 serves as a crucial signature molecule on the surface of helper T cells, while CD19 is among the most significant markers on the surface of B lymphocytes. Among them, CD4 is mainly secreted by T cells [33], while CD19 is primarily secreted by B cells [34], which is consistent with the above experimental results, as well as the PIK3CD and IFNG genes co-expressed by T and B cells. The gene IFNG encodes for IFNG protein is a cytokine that plays an important role in the immune system. It is mainly produced by immune cells (e.g., T cells, NK cells, etc.), and can promote the activation and proliferation of immune cells and regulate various immune responses. The PIK3CD gene encodes the p110 delta protein, a component of the enzyme phosphatidylinositol 3-kinase (PI3K). In lymphocytes, PI3K delta signaling is crucial for several cellular functions, including proliferation and differentiation. PI3K delta facilitates the differentiation of B cells and T cells into many kinds, each performing a unique role in the immune system. It is confirmed that EBNG augments the body's immunity by stimulating T and B cells.

This outcome aligns with our experimental results on the proliferation of T and B lymphocytes in splenic lymphocytes, indicating that EBNG intervention can significantly enhance the immunological function of immunosuppressed mice at the transcriptional level. In further annotating the core differential genes associated with EBNG regulation of organismal immunity, we discovered that contrary to prior research on immune activity, in addition to the two frequently examined immune pathways, Nuclear Factor kappa-B (NF- κ B) and Mitogen-Activated Protein Kinase (MAPK), two other signaling pathways that appeared to be more highly enriched were the intestinal immune network for IgA production and Th1 and Th2 cell differentiation. Of these, the former has not been previously documented in studies of the immunoregulatory mechanisms of EBNG. The main purpose of this pathway is to promote the differentiation of B lymphocytes into IgA-secreting plasma cells that can function in the gut for immune defense.

3.6 Bioinformatics analysis of pathways enriched for different genes in the spleen of EBNG and model group mice

GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses are pivotal in bioinformatics research [35]. They assist researchers in acquiring a more profound comprehension of the functions, pathways, and biological processes of gene sets under particular situations. In Fig.6, the 3,748 differential genes between the MC and EBNG groups were annotated into 816 GO items: 583 biological processes, 88 cellular components, and 144 molecular functions, of which immune system processes, antioxidant activity, and transcription regulator activity were related to immunity. Transcription regulators can bind to the promoter or enhancer regions of immune-related genes, modulating their transcriptional activity and thus influencing the magnitude and nature of the immunological response.

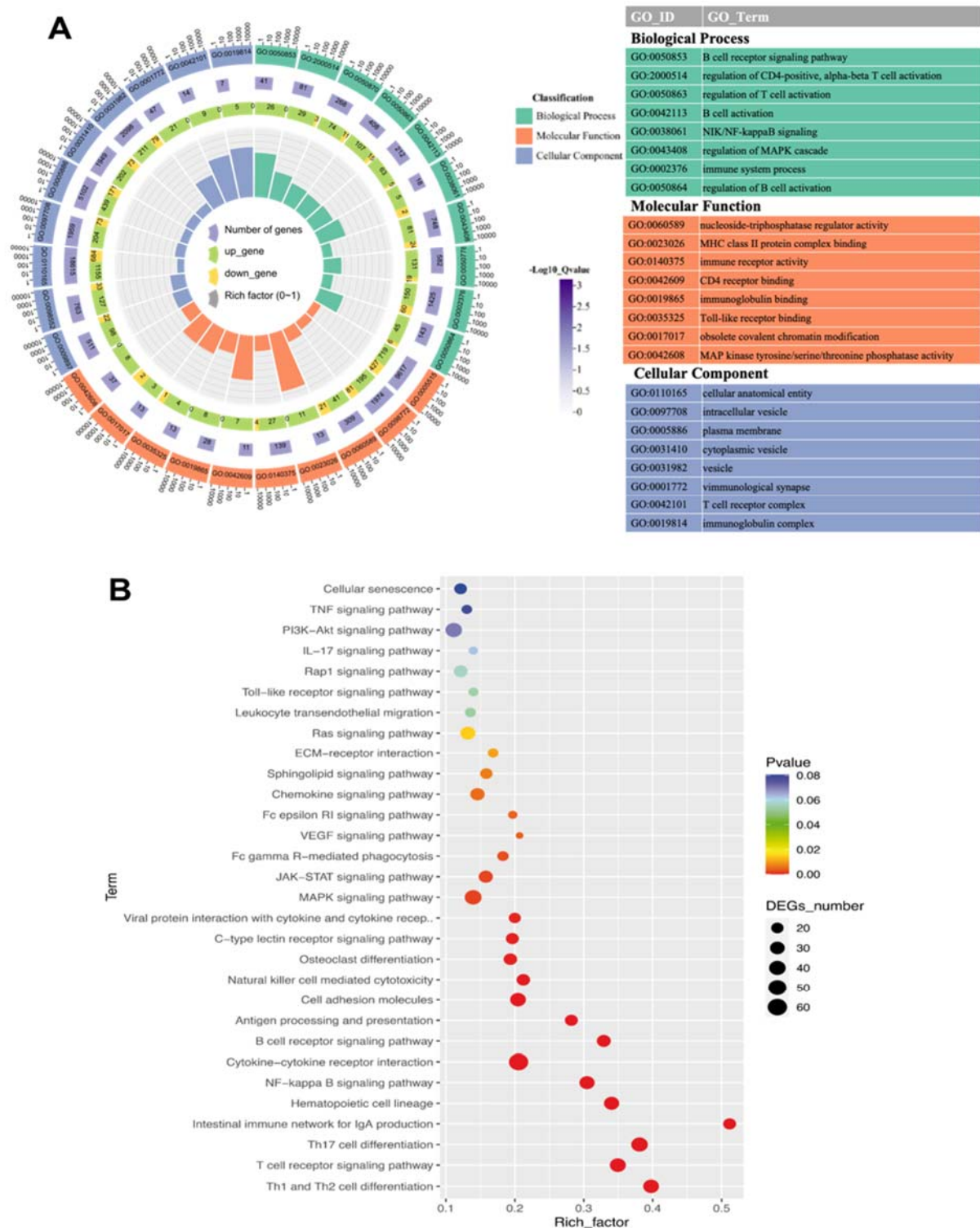


Fig. 6. KEGG and GO analysis of differentiated expression genes in the spleen of mice in the EBNB group and MC group. A. GO enrichment analysis; B. KEGG enrichment analysis.

KEGG enrichment analysis assists researchers in investigating the biological activities of genes [36]. We subjected all differential genes to KEGG functional annotation analysis. We enriched 221 pathways, of which 23 had $P < 0.05$, indicating that the results were statistically significant. Of the 23 signalling pathways, 17 were intimately associated with the immune system, including NF- κ B and MAPK, two commonly examined immune pathways. Moreover, the Rich factor is crucial to KEGG enrichment analysis. A higher value of the Rich factor indicates that there are more DEGs and a higher degree of enrichment in the pathway, which

suggests that the path may be closely related to a specific biological process or disease state. Results showed that, aside from the intestinal immune network for the IgA production signalling pathway, which exhibited the highest Rich factor, the Th1 and Th2 cell differentiation pathways displayed the second highest Rich factor.

Recent studies indicated that high protein diets can increase the levels of secretory IgA by activating Toll-like receptor 4 and increasing the levels of the cytokine A Proliferation-Inducing Ligand through extracellular vesicles secreted by intestinal flora or the metabolite succinate [37]. This pathway was initially discovered in the EBNG. Furthermore, the regular operation of the organism is significantly influenced by the differentiation balance of Th1/Th2 cells. Th1 cells secrete cytokines, such as IFN- γ and TNF- α , which are integral to cellular immunity and the delayed hypersensitivity inflammatory response. Th2 cells secrete cytokines, such as IL-4 and IL-6, which can help B cells differentiate into antibody-secreting cells and participate in the humoral immune response. Therefore, the organism's normal functioning is contingent upon the Th1/Th2 cell differentiation balance.

3.7 Effects of EBNG on intestinal flora of immunosuppressed mice

CTX was able to trigger a significant increase in the relative abundance of *Firmicutes* (F) and *Bacteroidetes* (B), and a significant increase in F/B in the MC group. F/B is associated with symptoms such as obesity, colitis, and immunodeficiencies [21]. The decrease in F/B in all groups of the EBNG suggests that EBNG can restore CTX-induced imbalance of the gut microbiota (Fig.7A). *Duncaniella* and *Odoribacter* exhibited significant alterations relative to other bacteria at the genus level (Fig.7B). Elevations in *Duncaniella* may provoke the gut immune system to generate an exaggerated immunological response. When *Duncaniella* proliferation is above the normative range, the gut immune system may recognise it as an exogenous pathogen, triggering inflammatory signalling pathways. Immune cells secrete inflammatory mediators, resulting in gastrointestinal inflammation. *Odoribacter* is a common short-chain fatty acid-producing human gut microorganism. Xing et al. established that *Odoribacter* was adequate to stimulate intestinal Th17 cell development and provide resistance to colitis and colon cancer in mice [38]. Furthermore, Venn diagrams facilitate the enumeration of unique and shared OTUs across several samples, allowing for a visual comparison of their similarity and overlap. As shown in Fig. 7C, 442 OTUs were common across all groups, representing 2.74 % of the total OTUs, indicating the presence of core microflora. In addition, there were 8923, 6514, 6905, 6107, 6972, and 7202 unique OTUs in the NC, MC, PC, EBNG-L, EBNG-M, and EBNG-H groups, respectively. LEFSE analysis (Fig.7D) helps elucidate the disparities in the gut microbiota composition among the groups. The specific bacterial family Lachnospiraceae in the administered group may enhance the formation and function of gut-associated lymphoid tissues and prompt immune cells to generate a suitable immunological response in contrast to the model group. Certain studies indicate that this bacterial group can enhance the production of regulatory T-cells, which reduce overactive immunological responses, hence aiding in maintaining intestinal immune homeostasis and reducing intestinal inflammation.

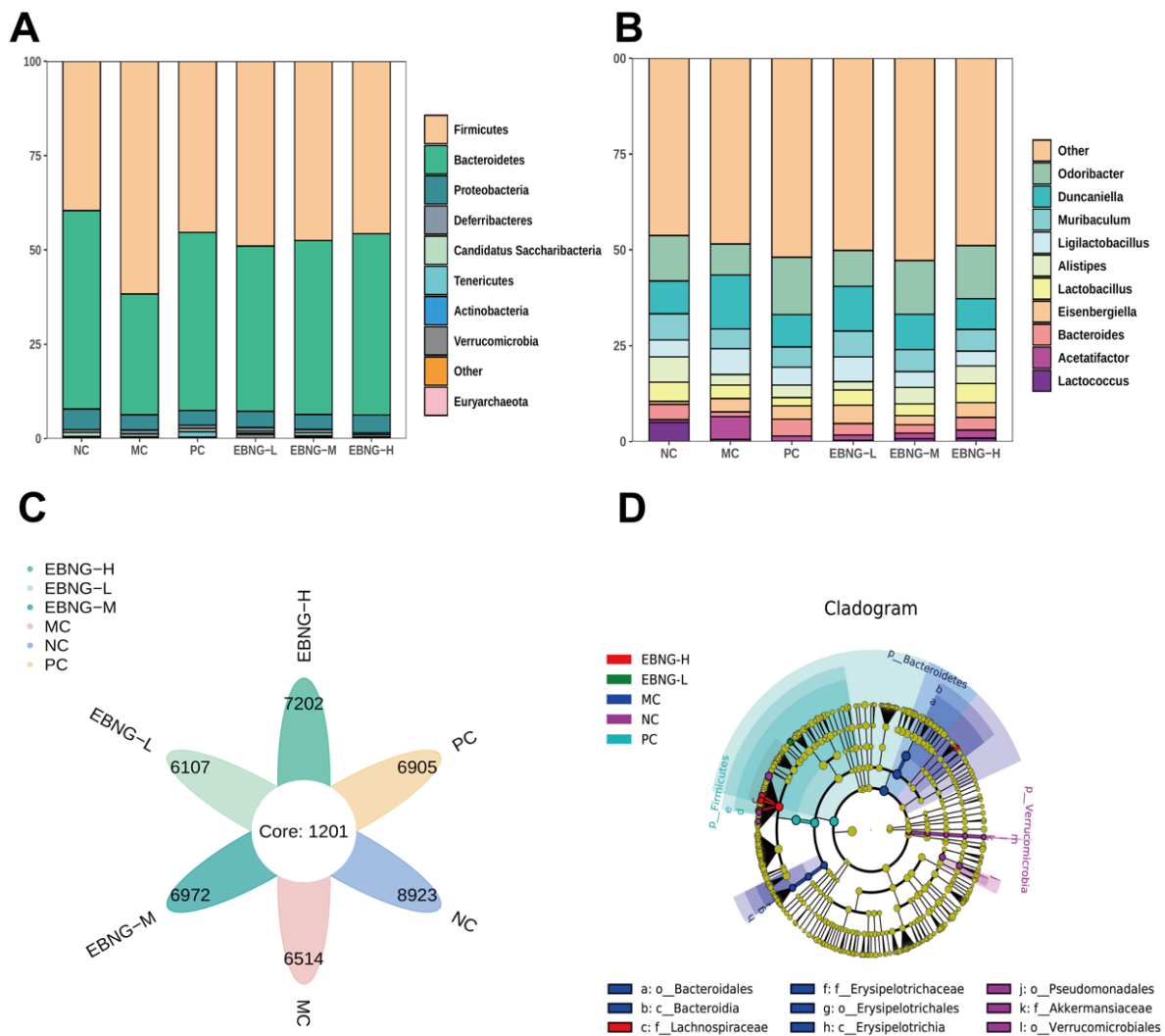


Fig. 7. Effect of EBNG on intestinal flora. A. Relative abundance of all groups at the phylum; B. Relative abundance of all groups at the genus; C. Venn diagram of all groups OTUs; D. LEFSE analysis among different groups of mice intestinal flora

As a key molecular mediator in the intestinal microecological environment, glycoproteins have a close relationship with the intestinal flora of "structure-dependence-functional interaction-maintenance of steady-state". The two jointly affect the intestinal wall barrier function and microecological balance through two-way regulation mechanisms. The core feature of glycoproteins is the diverse sugar chains (e.g., O-sugar chains, N-sugar chains) connected on the peptide backbone. These sugar chains are the "recognition anchors" of the intestinal flora and directly determine the survival and functional status of the intestinal flora. The intestinal flora does not passively rely on glycoproteins, but actively modifies the sugar chain structure of glycoproteins or regulates their expression by secreting sugar metabolic enzymes and releasing signal molecules, thereby affecting the physiological function of glycoproteins [39, 40]. In short, the essence of the correlation between glycoproteins and intestinal flora is a "two-way adaptation of structure and function", which together constitute a key regulatory network for intestinal microecology and host health.

4. Conclusion

In terms of physiological and biochemical parameters, EBNG was able to increase CTX-induced body weight loss and decrease immune organ index (spleen and thymus) in mice, alleviate CTX-induced immune

organ of spleen and thymus damage, and regulate several parameters of immune cells (T and B lymphocytes), immune cytokines (TNF- α , INF- γ , IL-4, IL-6), and immunoglobulins globulins (IgG, IgA) and several other indicators. The underlying molecular mechanisms of EBNG were explored in terms of transcriptomics, which may be mediated by the up-regulated expression of the T-cell-secreted CD4 gene, the B-cell-secreted CD19 gene, and the T- and B-cell-co-secreted PIK3CD and IFNG genes, which are predominantly involved in the production of the intestinal immune network IgA and play an immunomodulatory role in the Th1 and Th2 cell differentiation pathways. More importantly, in terms of intestinal flora, EBNG can repair intestinal immune injury and reduce intestinal inflammation. Thus, EBNG can synergize with each other to exert immunomodulatory effects at multiple levels.

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Conflicts of 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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