The Journal of Veterinary Medical Science

FULL PAPER

Immunology

Poplar bark lipids enhance mouse immunity by inducing T cell proliferation and differentiation

Jinxiu TANG[1,2], Xiuli WEI[2], Youzhi LI[2], Linlin JIANG[1], Tao FENG[2], Hongwei ZHU[1], Meng LI[1], Guozhong CHEN[1], Xin YU[1], Jianlong ZHANG[1,3]* and Xingxiao ZHANG[1,3]*

[1)]College of Life Science, Ludong University, Yantai 264000, Shandong, China
[2)]Shandong Provincial Key Laboratory of Quality Safety Monitoring and Risk Assessment for Animal Products, Ji'nan 250022, Shandong, China
[3)]Yantai Key Laboratory of Animal Pathogenetic Microbiology and Immunology, Yantai 264000, Shandong, China

ABSTRACT. Research on the composition and application of immune enhancers in livestock and poultry breeding has been gaining interest in recent years. Poplar bark lipids (PBLs), which are extracted from poplar tree bark, are natural substances known to efficiently enhance the immune response. To understand the chemical makeup of PBLs and their underlying mechanism for enhancing the immune system, we extracted PBLs from poplar bark using petroleum ether and subjected these extracts to chemical analysis. To evaluate PBLs effect on the immune system mice were treated with different doses of PBL via gavage and sacrificed 4 weeks later. PBLs were shown to be rich in vitamin E, unsaturated fatty acids, and other immune-potentiating compounds. Treatment with PBLs increased the spleen index and stimulated spleen and thymus development. In addition, PBLs increased the number of $CD3^+CD4^+$ cells in the peripheral blood and the ratio of $CD4^+/CD8^+$ cells while decreasing the number of $CD3^+CD8^+$ cells. Moreover, PBLs significantly increased IL-4 and IFN-γ levels in mouse serum and TLR4 mRNA and protein expression in the spleen. Taken together these results demonstrate that PBLs exert their immune-potentiating effects by promoting spleen and thymus development, T lymphocyte proliferation and differentiation, and immune factor expression. These immune-potentiating effects may be related to the activation of TLR4. This study provides a theoretical basis for the development of PBLs as an immune adjuvant or feed additive in the future.

KEY WORDS: immune enhancement, immune factors, poplar bark lipids, T lymphocytes, TLR4

J. Vet. Med. Sci.
82(8): 1187–1196, 2020
doi: 10.1292/jvms.19-0571

Received: 6 November 2019
Accepted: 13 June 2020
Advanced Epub:
14 July 2020

Given the growing scale and intensification of animal husbandry, increasing attention has been directed toward research on and the application of immune enhancers in livestock and poultry breeding. Poplar trees belong to the *Populus* genus and are one of the most widely distributed and adaptable species in the world, with wide distributions in Asia, Europe, and North America [35]. China is in the center of the global poplar distribution area and has abundant poplar resources, primarily in northeast, central north, and northwest China and Inner Mongolia, in addition to other regions [6, 17]. Poplar bark is also an historically important medicine and reports of its use can be found even in ancient medical records [33]. Tang Bencao (newly revised pharmacopeia) describes the use of poplar bark as an herbal medicine called white poplar, used to help to dispel wind, dredge collaterals, disperse blood stasis, and relieve pain [6]. There are relatively few studies describing the active compounds in poplar bark. Bae *et al.* used acetone to extract poplar bark compounds and subjected these extracts to chromatographic analysis. They found that the poplar bark extracts contained phenolic compounds, flavonols, salicylic acid derivatives, aescin, coumarin acid, and other compounds [1]. Li *et al.* studied the seasonal dynamics of the phenolic compounds in poplar leaves and bark [14]. While Wu *et al.* deoxygenated and liquefied poplar leaves, bark, and wood, and then analyzed the leaf oil, bark oil, and wood oil using gas chromatography-mass spectrometry (GC-MS). They found that all three poplar oils contained carbon-hydrogen compounds and phenolic substances [40].

Poplar bark lipids (PBLs) are natural substances extracted from poplar bark with a colorless or light yellow oily liquid appearance and high biological activity [53]. PBLs are rich in phospholipids, sterols, glycolipids, vitamin E (VE), carotene, and unsaturated fatty acids and are widely used in Chinese medicine, every day chemical products, health care products, and a variety

*Correspondence to: Zhang, J.: zhangjianlong@ldu.edu.cn, Zhang, X.: zhangxingxiao@ldu.edu.cn

©2020 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)

of other common items used throughout the world [54]. Studies have confirmed that PBLs can be used as an additive in vitamins to promote weight gain in livestock and poultry, and increase egg production rates [47]. In previous studies, we used PBLs to replace traditional chemical immune-potentiators, prepared a dual-lipid vaccine against infectious rhinitis caused by *Mycoplasma gallisepticum* in chickens and a lipid-inactivated vaccine for Newcastle disease, both of which demonstrated good immune efficiency [46, 48]. In addition, when PBLs were used as immune-potentiators in young chickens, researchers were able to identify increased immune responses in the thymus, bursa and spleen [46].

The immune system is the physical machinery of any organism that produces the immune response and primarily includes immune organs, immune cells, and immune effectors. The spleen and thymus are important immune organs, and changes in the spleen and thymus mass indices and tissue structure invariably affect the immune response [10]. While there are a number of immune cells, T lymphocytes are believed to most directly reflect the immune status of the body and function to regulate cellular immunity, while B lymphocytes are primarily associated with the humoral immunity [22]. T lymphocytes can be divided into several subpopulations, including helper T (Th) cells, which can be further classified into two subgroups: Th1 and Th2 cells; Th1 and Th2 cells secrete various cytokines (interleukin 4 (IL-4) and interferon-γ (IFN-γ), respectively and assist in both cellular immunity and humoral immunity [39, 43]. *Astragalus* polysaccharides (APS) extracted from *Astragalus mongholicus*, have been shown to exert a variety of immunomodulatory effects [42], and can be used as a positive control in immune enhancement studies [21]. Studies have shown that polysaccharides induce immune responses in mice through the toll-like receptor 4 (TLR4) pathway [49]. Therefore, in this study, mice were used as an animal model to examine the effects of PBL treatment on the animal immune response and evaluate the effects of PBL mediated immune enhancement at three levels: the immune organs (the thymus and spleen), immune cells (T cells and B cells), and immune effectors (IL-4 and IFN-γ). In addition, activation of the TLR4 signaling pathway in response to PBL stimulation was also investigated in an effort to identify the underlying molecular mechanism of these compounds.

MATERIALS AND METHODS

Materials

IL-4 and IFN-γ ready-to-use sandwich enzyme-linked immunosorbent assay (ELISA) kits were obtained from Excell Biotech Co., Ltd. (Taicang, China). FACS lysing solution was obtained from BD Biosciences (Gliwice, Poland). Fluorescently-labeled anti-mouse monoclonal antibodies (FITC-CD3, PE-CD4, PerCP/Cy5.5-CD8a, and PE-CD19) were obtained from BioLegend (San Diego, CA, USA). APS (>70%) was obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). All chemical reagents were of analytical purity and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All material used in this study was endotoxin-free, including not only the PBLs but also all biological and synthetic substrates.

Preparation of the PBLs

Poplar bark was collected from 30- to 40-year-old poplar trees located in the Jilin Province, China, thoroughly cleaned, and dried. This dried bark was then pulverized into powder using a crusher, and the poplar bark powder was mixed with petroleum ether at a ratio of 1:2.5 (kg: *l*). This mixture was then sealed for 6 hr. PBLs were obtained by filtering the mixture through filter paper, and the filtrates were then processed by rotary distillation.

PBL characterization

The PBL extraction rate was calculated using the ratio of extracted weight to dried bark powder weight. The dry weight and VE content were determined using a protocol provided by the People's Republic of China's Forestry Industry and compared to the Poplar Bark Lipids Standard [31]. The purified fraction was characterized using GC-MS. Briefly, 0.3 m*l* of the obtained lipids were mixed with 200-proof ethanol (analytical grade) to a volume of 100 m*l* and ultrasonically vibrated for 10 min. The sample were then subjected to GC-MS with the following analysis conditions: a capillary chromatography column (0.25 mm × 30 m × 0.25 μm), charged high-purity helium as the carrier gas (99.999%), was set to a flow rate of 2.0 m*l*/min, with an inlet sample temperature of 260°C, an injection volume of 3 μl, and a split ratio of 50:1. The initial column temperature was 80°C, which was maintained for 3 min and then increased to 120°C at 5°C/min and then held at 120°C for 10 min, and then further increased to 150°C at 5°C/min, maintained at this temperature for 2 min, then increased to 220°C at 10°C/min and maintained at 220°C for 2 min, before finally being increased to 250°C at 10°C/min and maintained at this temperature for 23 min. The total analysis time was 64 min. Analysis was completed using the electron ionization (EI) method, with an electron energy of 70 eV, an ion source temperature of 210°C, a mass scanning range of m/z 40–550, a mass spectrometry detection time of 1.6–64 min, and a GC-MS interface temperature of 260°C.

Animals and treatments

All animal experiments were approved by the Animal Ethics and Experimentation Committee of Ludong University (license number LDU-IACUC2018007) based on the guidelines set out in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

This study was conducted using 5-week-old female BALB/c mice (18–22 g) obtained from Jinan Pengyue Laboratory Animal Breeding Co., Ltd., Jinan, China. The animals were bred and maintained under standard laboratory conditions, including being kept at a temperature of 25 ± 2°C with a photoperiod of 12 hr. A commercial pellet diet and water were available *ad libitum*. PBLs were

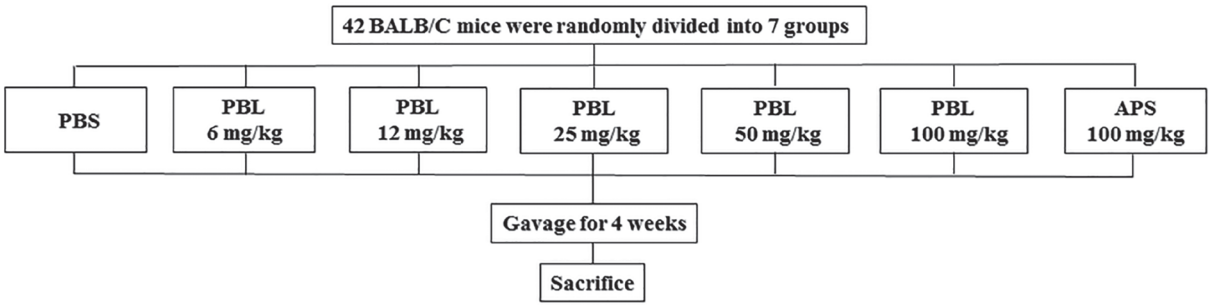

Fig. 1. BALB/c mice were randomly divided into seven groups. Phosphate-buffered saline (PBS): the control group. Poplar bark lipid (PBL): the groups treated with PBL extracts at five doses (6-, 12-, 25-, 50-, or 100-mg/kg). APS: the group treated with APS (100-mg/kg).

orally administered to each animal at a dose of 6-, 12-, 25-, 50-, or 100-mg/kg body weight for 4 weeks; phosphate-buffered saline (PBS) was used as a blank control, and APS (100-mg/kg) was used as a positive control [15, 22]. The animals were randomly divided into seven groups, with six animals in each group, as shown in Fig. 1.

Spleen and thymus indices

Animals were weighed and then sacrificed by cervical dislocation 24 hr after their final dose of treatment. The spleen and thymus were immediately collected and weighed with these values used to establish the spleen and thymus indices, which were calculated according to the following formula:

Index mg/g=weight of the thymus or spleen/body weight.

Histological staining

After the blood samples were collected, mouse spleen and thymus tissues were immediately collected and immersed in 10% formaldehyde. After 24 hr, the tissues were embedded in paraffin and sliced into 5-μm-thick sections for hematoxylin and eosin (HE) staining. Histological changes were observed under an OLYMPUS BX50 microscope (Olympus Optical, Tokyo, Japan).

ELISA

Blood samples were collected after enucleation, allowed to stand at room temperature for 30 min, and then centrifuged at 3,000 rpm for 10 min at 4°C. The serum supernatant was then collected. Cytokine (IL-4 and IFN-γ) levels in these serum samples were then evaluated using commercial ELISA kits according to the manufacturer's instructions.

Flow cytometry

Splenic tissue was ground through a 400-mesh sieve, rinsed thoroughly with PBS, and centrifuged at 1,000 rpm for 6 min. The supernatant was discarded, and red blood cells were lysed. The remaining cells were then blocked with rat serum for 30 min on ice, stained with FITC-CD3, PerCP/Cy5.5-CD8a, and PE-CD4 antibodies (10 g/m*l*) for 30 min at 4°C while protected from light, washed twice with PBS, and then resuspended in 1% paraformaldehyde (PFA). The number of $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes were then determined via flow cytometry (BD, CA, USA), followed by data analysis using FlowJo 7.6.1 software.

Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from the spleen using TRIzol reagent (Invitrogen, Life Technologies Corp., CA, USA), and cDNA was synthesized using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) for qRT-PCR using a ChamQTM SYBR® qPCR Master Mix (Vazyme, Biotech, Nanjing, China) and primers specific for TLR4 (forward 5′-ATGGCATGGCTTACACCACC-3′ and reverse 5′-GAGGCCAATTTTGTCTCCACA-3′) [12] and β-actin (forward 5′-AGAGGGAAATCGTGCGTGAC-3′ and reverse 5′-CAATAGTGATGACCTGGCCGT-3′) [32]. The reaction conditions were as follows: pre-denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing and extension at 60°C for 30 sec. The total reaction volume was 20 μl, and gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method [18].

Western blot

Splenic proteins were collected from each of the groups following sacrifice. These proteins were then determined by a BCA Protein Quantitative Kit from Beijing Solarbio Co., Ltd., Beijing, China and equal concentrations of protein were subjected to SDS-PAGE and then transferred onto a PVDF membrane. These membranes were then blocked with 5% non-fat milk in 0.1% TBST for 2 hr at room temperature and then incubated with primary antibodies against TLR4 (1:1,000, Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:1,000, Cell Signaling Technology) overnight at 4°C and then treated with horseradish peroxidase-labeled secondary antibodies (Proteintech Group, Chicago, IL, USA) for 1 hr at RT. β-actin was used as the loading control. The intensities of the protein bands were determined by densitometry (ImageJ software). Protein expression was assessed relative to β-actin expression.

Statistical analysis

Experimental data are expressed as the mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 6 software for Windows. An unpaired two-tailed Student's *t*-test was used to compare the data. *P*-values <0.01 were considered statistically significant (**P*<0.01, ***P*<0.005, and ****P*<0.001).

RESULTS

PBL composition

The PBL yield, dry weight, and chemical composition were all evaluated in this study. The PBL yield was 4.1%, the dry weight was 97.41%, and the VE content was 1,325 mg/kg. PBL compounds were characterized using GC-MS, and 62 peaks were observed in the spectrum. Table 1 lists the top 20 PBL compounds based on the GC-MS results. We found that unsaturated fatty acids, including oleic acid and linoleic acid, were the predominant type of biologically active compound in these PBLs.

Table 1. Top 20 compounds isolated from the poplar bark lipid (PBL) extracts

Peak#	Area%	Name
62	75.82	9,12-Octadecadienoic acid (Z,Z)-
52	7.19	n-Hexadecanoic acid
59	2.92	Oleic Acid
60	1.97	Henicosanal
51	1.26	1-Heptacosanol
50	0.94	Eicosanal-
58	0.74	Octadecanoic acid
17	0.51	trans-. alpha. -Bergamotene
46	0.50	Hexatriacontane
29	0.45	Naphthacene,1-methyl
57	0.43	Squalene
43	0.39	Eicosane
41	0.39	Benzoic acid
53	0.32	Henicosanal
45	0.27	Dibutyl phthalate
55	0.24	Heptadecanoic acid
16	0.24	cis-.alpha.-Bergamotene
49	0.23	Pentadecanoic acid
54	0.22	1-Docosanol,methyl ether
4	0.19	Cyclohexasiloxane,dodecamethyl
31	0.18	Hexanoic acid
	4.60	Others
	100.00	Total

PBL treatment promotes spleen and thymus development

Immune organ indices can reflect the developmental status of immune organs in the body and can be used to determine if there has been any immune activation in the study subjects [28]. The spleen index increased in each group (except for the 100-mg/kg group) (*P*<0.05), and the 50-mg/kg PBL group and APS group exhibited significant differences when compared to the control (*P*<0.01) (Fig. 2A). However, no significant differences in the thymus index were observed between any of the groups (Fig. 2B), suggesting that PBL treatment had a smaller effect on thymus development.

Staining of these tissues showed that the experimental group and positive control group demonstrated an obvious boundary between the white pulp and the red pulp of the spleen, with an increased amount of white pulp and an increased number of lymphocyte germinal centers (Fig. 3A) when compared to the control. In addition, the cortical area of the thymus was significantly thickened, and a distinct boundary was observed between the cortex and medulla (Fig. 3B). These results suggest that PBL treatment promotes the development and immune functions of the spleen and thymus in mice.

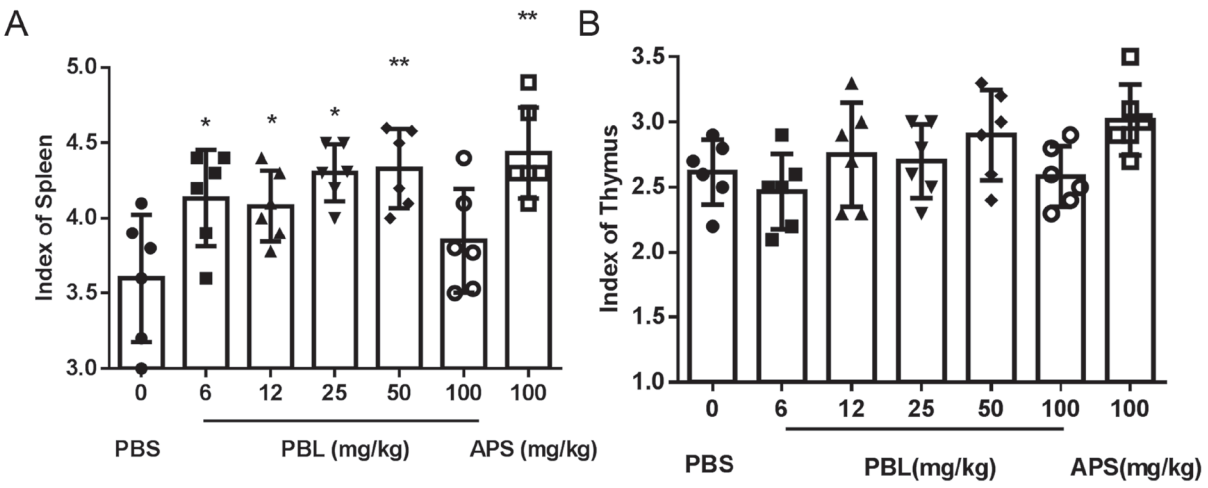

Fig. 2. The effect of poplar bark lipid (PBL) treatment on the immune organ indices in mice. Spleen indices (A). Thymus indices (B). The values shown represent the mean ± SD (n=6). **P*<0.05, ***P*<0.01 when compared to the control group. Abbreviations and symbols are the same as those in Fig. 1.

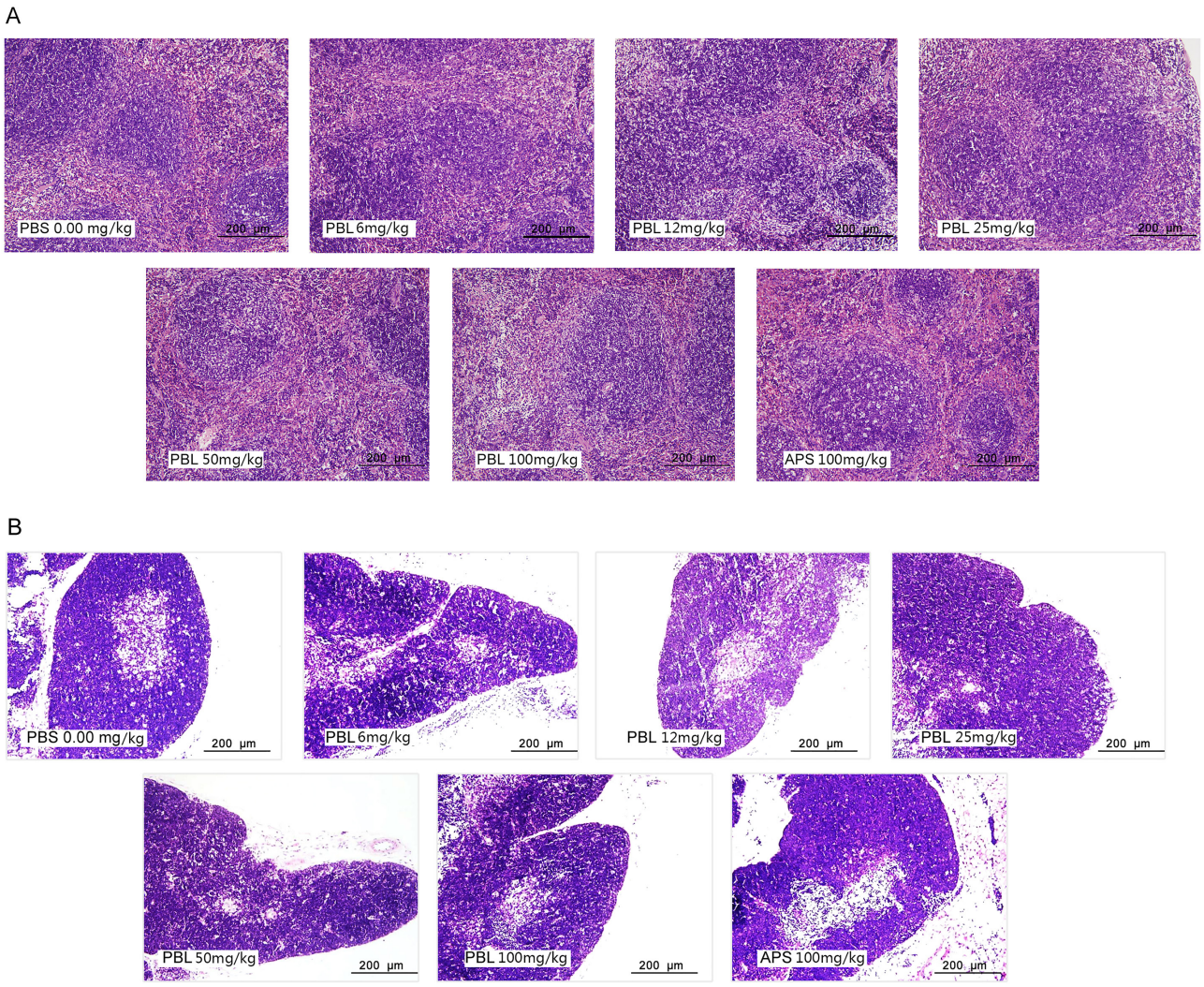

Fig. 3. The effect of poplar bark lipid (PBL) treatment on the structures of the immune organs. The structures of the immune organs were visualized using hematoxylin and eosin staining. The spleen (A). The thymus (B). Images were acquired using an OLYMPUS BX50 microscope (magnification 200×). Scale bars=200 μm. Abbreviations and symbols are the same as those in Fig. 1.

PBL treatment promoted lymphocyte proliferation and differentiation in peripheral blood

The effects of PBL treatment on immune cell proliferation in mouse peripheral blood were examined using flow cytometry (Fig. 4). The proportion of $CD3^+CD4^+$ cells ($P<0.05$) and the $CD4^+/CD8^+$ cell ratio ($P<0.05$) in the blood of the mice in each PBL group increased to different extents, with the 50-mg/kg group showing the most significant effects ($P<0.001$) when compared to the control. The proportion of $CD3^+CD8^+$ cells in each of these groups (with the exception of the 6- and 12-mg/kg treatment groups) decreased, with the 50-mg/kg group demonstrating the most significant decrease ($P<0.001$) when compared to the control. However, no significant effect on B lymphocytes was observed in the peripheral blood for any of the groups.

PBL treatment increased IFN-γ and IL-4 levels in the blood

Serum IFN-γ and IL-4 content was measured for each group using ELISA, and the results are shown in Fig. 5. Figure 5A shows that the IFN-γ content significantly increased in all the PBL groups except for the 50- and 100-mg/kg PBL groups, with the 12-mg/kg group demonstrating the most significant effect ($P<0.01$). All the PBL groups, with the exception of the 100-mg/kg group, showed increases in IL-4 expression, with the effect on IL-4 being most significant in the 25-mg/kg group ($P<0.001$).

PBLs increase the expression levels of TLR4 mRNA and protein

The mechanism by which the PBLs regulate the immune response has not been reported. Studies have shown that APS enhances immunity by promoting the expression of TLR4 [8]. Therefore, we hypothesized that PBLs may also activate the splenic immune system via the TLR4 pathway. We analyzed the expression levels of TLR4 mRNA and protein in the spleens of mice after PBL treatment using qRT-PCR and western blot. We found that PBLs significantly upregulated TLR4 expression in spleen cells (Fig. 6), with the 25-mg/kg group showing the most significant effect ($P<0.01$).

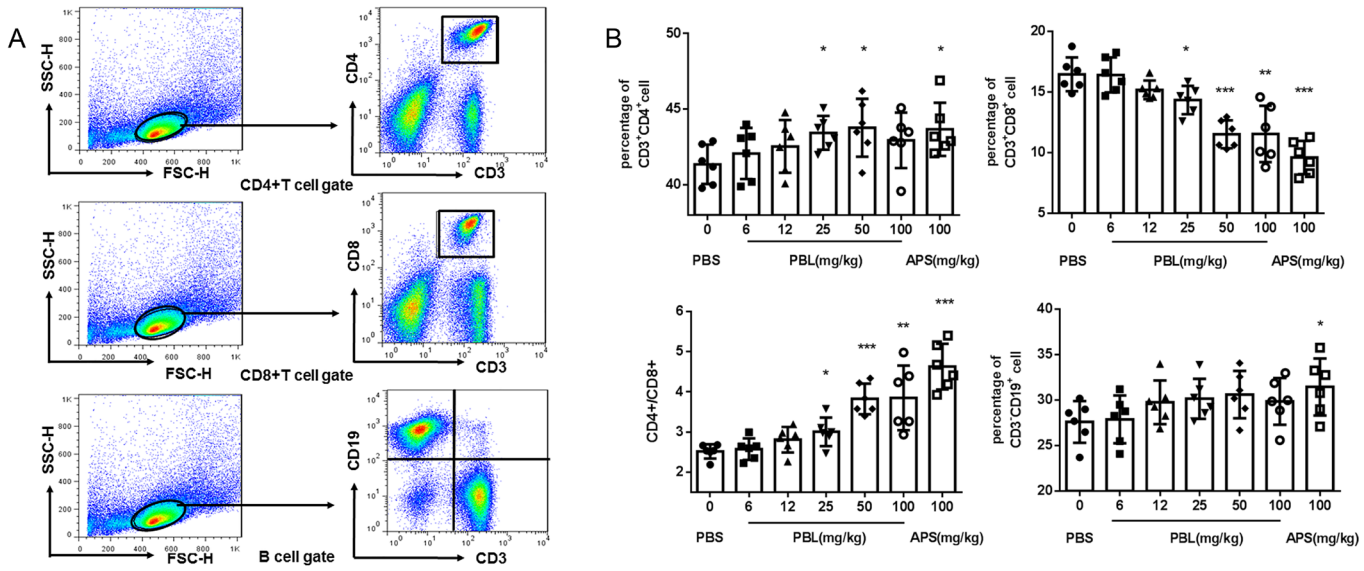

Fig. 4. Flow cytometric evaluation of the effect of PBL treatment on peripheral blood lymphocyte populations (%). Cells were labeled with monoclonal antibodies targeting CD3 (FITC-conjugated monoclonal antibody), CD4 (PE-conjugated monoclonal antibody), CD8 (PerCP/Cy5.5-conjugated monoclonal antibody), and CD19 (PE-conjugated monoclonal antibody) (A) and analyzed via flow cytometry using Cell Quest software. The content and proportion of lymphocytes (B). The values shown represent the mean ± SD (n=6). **P*<0.05, ***P*<0.01, ****P*<0.001 when compared with the control group. Abbreviations and symbols are the same as those in Fig. 1.

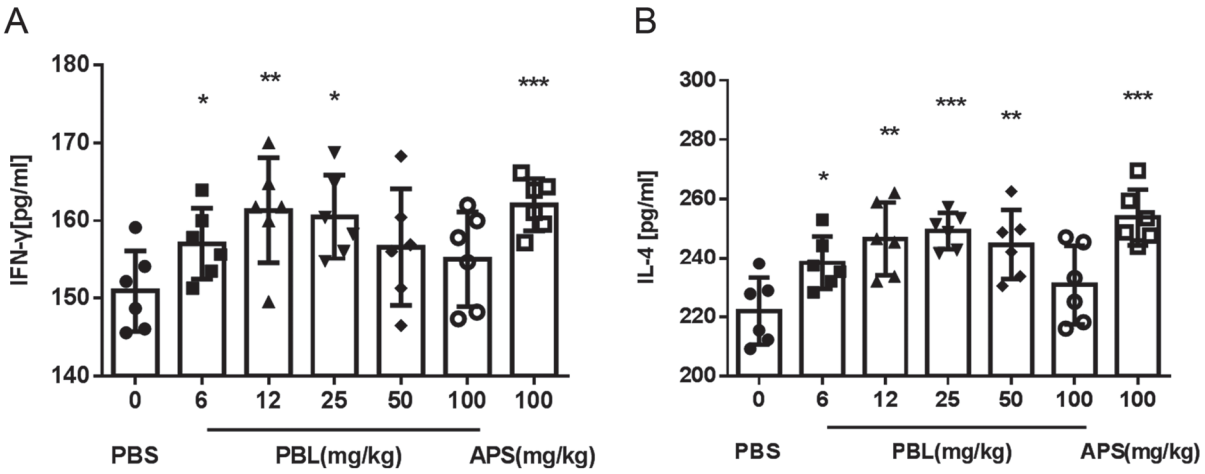

Fig. 5. The effect of PBL treatment on the cytokine levels in mice from different groups. Released IFN-γ (A) or IL-4 (B) levels in peripheral blood were detected using commercial enzyme-linked immunosorbent assay (ELISA), and the results are shown as the mean ± SD (n=6). **P*<0.05, ***P*<0.01, ****P*<0.001 when compared with the control group. Abbreviations and symbols are the same as those in Fig. 1.

DISCUSSION

Given the impact of immunodeficiency on the health and productivity of various animals, the immune induction or suppression effects of various drugs and their underlying mechanisms have remained a priority in animal husbandry and production research [46]. Because of the low toxicity and superior performance of natural products, especially those extracted from plants, these compounds have seen an increase in scrutiny in recent history [2]. PBLs extracted from poplar bark are natural products with high biological activity. When we examined the extraction rate, dry weight, and composition of PBLs extracted using methanol, we found that PBLs are rich in VE and linoleic acid. Satoru Moriguchi *et al.* found that a high-VE diet can activate both rat splenic lymphocytes and lung alveolar macrophages [23]. Wakikawa *et al.* showed that VE treatment resulted in a slight increase in the percentage of T cells in the spleen and enhanced IFN-γ production in mice [34]. Singh *et al.* revealed that VE can exert a regulatory effect on the immune system in buffalo neonates born [30]. Linoleic acid is an essential fatty acid in humans and is

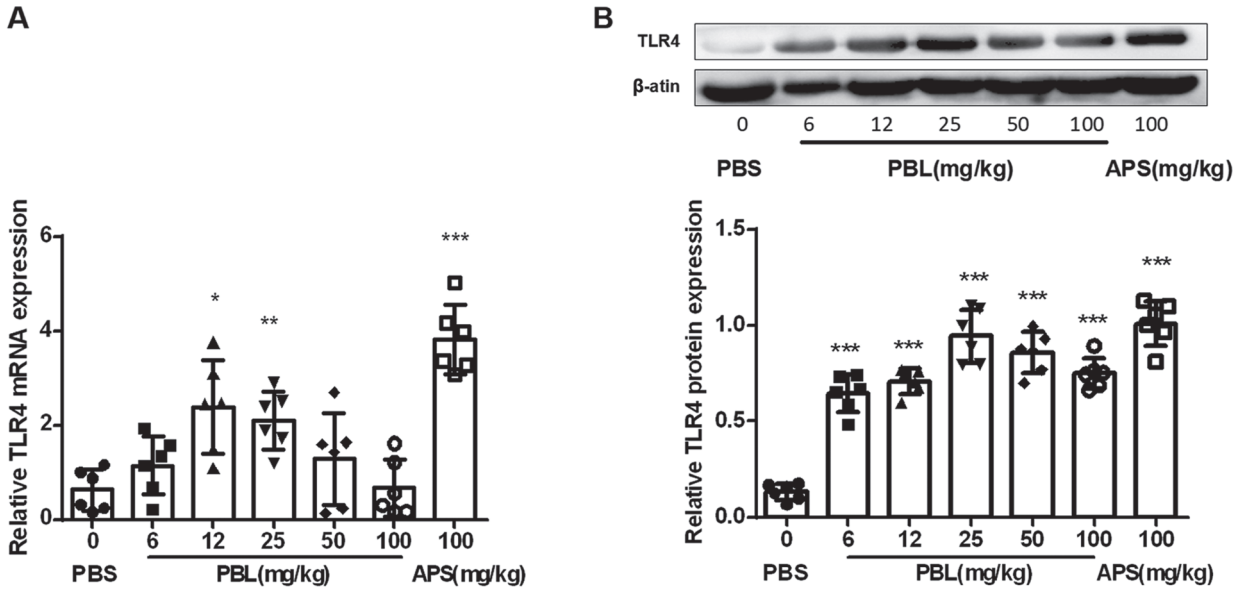

Fig. 6. Poplar bark lipids (PBLs) increase toll-like receptor 4 (TLR4) expression in mouse spleens. (A) mRNA and (B) protein expression levels of TLR4 in the spleen as determined by qRT-PCR and western blot, respectively. The values shown represent the mean ± SD (n=6). **P*<0.05, ***P*<0.01, ****P*<0.001 when compared with the control group. Abbreviations and symbols are the same as those in Fig. 1.

involved in material circulation and immune regulation in the body. Linoleic acid is metabolized to γ-linolenic acid, arachidonic acid (AC), adrenal acid, and docosapentaenoic acid (DPA) in the human body [41]. Nayak *et al.* showed that AC can promote the marbled spinefoot rabbitfish growth, survival, and innate and acquired humoral immune functions [26]. Mudronová *et al.* showed that γ-linolenic acid increases the ratio of $CD4^+/CD8^+$ cells in peripheral blood and improves immunity [25]. Based on these results, we hypothesize that PBLs may exert a stimulating effect on the immune system in mice. To test this hypothesis we treated mice with PBL extracts via oral gavage and performed *in vivo* evaluations on cellular proliferation, lymphocyte profiles and the production of immune signaling molecules.

Both the thymus and spleen are important immune organs which play essential roles in nonspecific immunity, and immune organ indices are good indicators of immune function and activation [20]. Many immune-potentiators, including polysaccharides, phenolic compounds, and saponin, exert immune-potentiating effects by increasing these immune organ indices [7]. We found that PBL treatment did not exert any significant effect on the thymus index in mice but that these compounds did significantly increase the spleen index, giving preliminarily confirmation that PBLs can affect immune function in mice. The degree of tissue development in these organs also directly affects their physiological function. The spleen is the largest peripheral immune organ in the body and is primarily composed of white and red pulp. White pulp is indicative of a highly organized lymphatic region responsible for the induction of adaptive immune responses [19]. The lymphoid nodules in the white pulp are B cell-resident areas, and the nearby periarterial lymphoid sheaths are T cell-resident areas. When the cellular immune response is induced, the periarterial lymphoid sheath thickens, while the size of the splenic nodules increase during the activation of the humoral immune response [37]. The H&E staining results show that treatment with PBLs or APS increase the proportion of white pulp in the spleen, thicken the periarterial lymphoid sheaths, and enlarge the splenic nodules, which effectively enhances the immune functions of the spleen. The thymus is also an important lymphoid organ, acting as the site for T lymphocyte maturation; thus, its functional status is closely related to immunity [52]. The thymus cortex primarily contains T lymphocytes, which means that the proportion of T lymphocytes among all the thymocytes increases with increasing thymus cortex. Treatment with PBLs or APS thickened the mouse thymus cortex, suggesting that PBLs can improve the overall tissue structure of the thymus, delay thymus degeneration in mice, and thus enhance the immune functions of the thymus [37].

Lymphocytes are the major cell type in the immune system. Lymphocyte proliferation is the most direct indicator of the body's immunity. T lymphocytes function to regulate cellular immunity, and B lymphocytes are the primary effectors of humoral immunity [22]. $CD4^+$ cells are auxiliary cells that play a positive regulatory role in cellular immunity. $CD8^+$ T cells are suppressor cells which play a negative regulatory role in cellular immunity. The $CD4^+/CD8^+$ ratio can be used to reflect the functional status of the immune system [27]. Under normal circumstances, the number of T cells and the ratio of $CD4^+/CD8^+$ T cells in peripheral blood are maintained within a specific range and any increase or decrease in these numbers suggests some underlying change to the status of the immune response. When these values increase, immune responses are typically under activation and any decrease suggests some form of suppression [16]. Studies have shown that when cellular immune function is low, the number of $CD4^+$ T lymphocytes and the $CD4^+/CD8^+$ ratio decrease, while the number of $CD8^+$ T lymphocytes increases [5, 38, 51]. After PBL treatment, the proportion of $CD3^+CD4^+$ cells and the $CD4^+/CD8^+$ cell ratio in mouse peripheral blood samples increased, while the proportion of

$CD3^+CD8^+$ cells decreased, suggesting that PBL treatment elicited some activation of the immune system. Zhang *et al.* showed that polysaccharides can promote splenic cell proliferation [44]. Zhang *et al.* showed that APS significantly increased the proportions of $CD3^+$ and $CD3^+CD4^+$ cells in the peripheral blood of mice, increasing the ratio of $CD4^+/CD8^+$ cells, and decreasing the proportion of $CD3^+CD8^+$ cells [45], which is consistent with our results. These results suggest that PBL treatment positively regulates the immune response by promoting lymphocyte proliferation and differentiation and improving cellular immunity.

The $CD4^+$ subpopulation (Th cells) is further divided into two subgroups, Th1 and Th2 cells, according to their cytokine profile. Th1 and Th2 cells secrete different lymphokines that bind to their corresponding receptors regulating the growth, differentiation, and function of effector cells, thereby regulating immune responses [39, 43]. IFN-γ is secreted by Th1 cells and is an indicator of cellular immunity induced by infection [50]. IL-4 plays an important role in Th2 cell differentiation, promotes B cell proliferation, and is used as an indicator of the humoral immune response [39]. In this study, IFN-γ (Th1 subgroup) and IL-4 (Th2 subgroup) levels were measured, and the effects of PBLs on cellular immunity and humoral immunity were explored. The results showed that the IL-4 concentrations in the 6-, 12-, and 25-mg/kg PBL groups were higher than that in the control group. The concentrations of IFN-γ significantly increased in all the treatment groups, except for the 100 mg/kg PBL group, when compared to the control. These results suggest that PBLs have a stimulating effect on cellular and humoral immunity in mice, with a more significant effect on humoral immunity. Cytokine interactions in the immune system are complex. IFN-γ and IL-4 are antagonists of each other, but some studies have also shown that they may enhance each other's function under specific conditions [13, 24]. Dosage plays an important role in regulating the properties of any therapeutic compound. Studies have shown that some natural medicines exhibit different effects at different doses, i.e., bidirectional dose-dependent effects. The effect of a drug increases or decreases in a dose-dependent manner within a certain range. Outside this range, rather than enhancing the therapeutic effect, further increases in the dose may reduce their effects [36]. *Ganoderma lucidum* polysaccharides have been shown to induce a significant increase in the production of hemolysin antibodies in normal mice at low doses, while they exert an inhibitory effect on these antibodies at high doses [3, 4]. Studies have shown that flavonoids exert differential effects on the humoral immune function in mice with lower doses found to exert more positive effects than high doses [11]. This study showed that treatment with PBL at a dose between 12-mg/kg and 25-mg/kg may exert the best immune enhancement effects. When the concentration of the PBLs was higher than 25-mg/kg, the effect was no longer dose-dependent, and the serum levels of IFN-γ and IL-4 decreased.

Toll like receptors (TLRs) are important pattern recognition with all the described members of the TLR family known to play key roles in the innate and adaptive immune responses [9]. TLR4 is an important signaling molecule and a critical regulator of innate immunity, with most polysaccharides exerting their immunomodulatory effects through alterations in the TLR4 pathway [49]. Shao *et al.* found that APS stimulation activated TLR4 on the surfaces of mast cells *in vitro* [29]. The results of this study show that treatment with PBLs increase the expression levels of TLR4 mRNA and protein in spleen cells, indicating that PBLs may promote activation of the innate immune system by enhancing TLR4 signaling. This aspect will be expanded in future studies.

Here we demonstrated that PBLs are rich in VE and unsaturated fatty acids. PBL treatment promotes both spleen and thymus development in mice, while significantly increasing the proportion of $CD3^+CD4^+$ cells and the $CD4^+/CD8^+$ cell ratio in peripheral blood and decreasing the proportion of $CD3^+CD8^+$ cells, thereby promoting secretion of cytokines IL-4 and IFN-γ. PBL doses of 25- and 50-mg/kg demonstrated the best immune enhancement effects in mice. While increases in TLR4 expression in the spleen suggest that PBLs may promote activation of the innate immune system through the TLR4 signaling pathway. PBL mediated increases in lymphocyte differentiation and IFN-γ secretion may be related to the relative abundance of VE in PBLs [23, 30], while the effects on the $CD4^+/CD8^+$ cell ratio and IL-4 concentration may be associated with the linoleic acid content of these extracts [25, 26, 41].

In summary, PBL treatment can enhance mouse immunity by enhancing T cell proliferation and differentiation. This study provides a theoretical basis for the development of PBLs as an immune adjuvant or feed additive for livestock and poultry in the future. We plan to expand on the findings of this study by evaluating the molecular mechanisms underlying the immune effects described here and examining the potential applications of PBL extracts in other animal models.

ACKNOWLEDGMENTS. This research was supported by the National Key Research and Development Program of China (Grant No. 2017YFD0500806), the Key Research and Development Plan of Shandong Province (No. 2017NC210009), the Major Agricultural Applied Technological Innovation Projects of Shandong Province, the Key Research and Development Plan of Yantai (No. 2018XSCC045), the Innovation Team Project for Modern Agricultural Industrious Technology System of Shandong Province (SDAIT-11-10), and the Shandong Provincial Key Laboratory of Quality Safety Monitoring and Risk Assessment for Animal Products.

REFERENCES

1. Bae, Y. S. and Ham, Y. H. 2000. Extractives of the wood of black locust and the bark of poplar as decay-resistant hardwood tree species. *Mokchae Konghak* **28**: 52–61 (in Korean).
2. Borchers, A. T., Hackman, R. M., Keen, C. L., Stern, J. S. and Gershwin, M. E. 1997. Complementary medicine: a review of immunomodulatory effects of Chinese herbal medicines. *Am. J. Clin. Nutr.* **66**: 1303–1312. [Medline] [CrossRef]
3. Cao, J., Jiang, G. P. and Wang, C. H. 1996. Effect of *Ganoderma lucidum* mycelium extract on humoral immune function of mice. *Tianjin Pharm.* **8**: 9 (in Chinese).
4. Cao, R. H., Hou, G. H., Jiang, Q. H. and Wang, D. F. 1993. Experimental study on immune regulation of *Ganoderma lucidum* polysaccharide. *Chin. J. Exp. Clin. Immunol.* **5**: 43–46 (in Chinese).
5. Cao, Y., Zhao, Z., Zhang, Y. and Yang, Y. 2002. Expression of $CD4^+$ and $CD8^+$ T lymphocytes in mice under ultraviolet irradiation. *Can. J. Appl.*

Physiol. **18**: 403–405 (in Chinese).
6. Chen, P. D. and Liang, J. Y. 2006. A new phenolic glycoside from populus davidiana. *Chin. Tradit. Herbal Drugs* **37**: 1607–1608.
7. Chen, Y., Tang, J., Wang, X., Sun, F. and Liang, S. 2012. An immunostimulatory polysaccharide (SCP-IIa) from the fruit of *Schisandra chinensis* (Turcz.) Baill. *Int. J. Biol. Macromol.* **50**: 844–848. [Medline] [CrossRef]
8. Du, X., Chen, X., Zhao, B., Lv, Y., Zhang, H., Liu, H., Chen, Z., Chen, Y. and Zeng, X. 2011. *Astragalus* polysaccharides enhance the humoral and cellular immune responses of hepatitis B surface antigen vaccination through inhibiting the expression of transforming growth factor β and the frequency of regulatory T cells. *FEMS Immunol. Med. Microbiol.* **63**: 228–235. [Medline] [CrossRef]
9. Feng, H., Du, X., Tang, J., Cao, X., Han, X., Chen, Z., Chen, Y. and Zeng, X. 2013. Enhancement of the immune responses to foot-and-mouth disease vaccination in mice by oral administration of a novel polysaccharide from the roots of *Radix Cyathulae officinalis Kuan* (RC). *Cell. Immunol.* **281**: 111–121. [Medline] [CrossRef]
10. Guo, Y., Pan, D., Li, H., Sun, Y., Zeng, X. and Yan, B. 2013. Antioxidant and immunomodulatory activity of selenium exopolysaccharide produced by *Lactococcus lactis* subsp. lactis. *Food Chem.* **138**: 84–89. [Medline] [CrossRef]
11. Hou, C. Y., Qin, R. and Wang, Y. Z. 1998. Study on the effect of total flavonoids of Fructus Choerospondiatis on humoral immune function in mice. *Chin. J. Ethn. Med.* **4**: 38–39 (in Chinese).
12. Hsieh, J. L., Shen, P. C., Wu, P. T., Jou, I. M., Wu, C. L., Shiau, A. L., Wang, C. R., Chong, H. E., Chuang, S. H., Peng, J. S. and Chen, S. Y. 2017. Knockdown of toll-like receptor 4 signaling pathways ameliorate bone graft rejection in a mouse model of allograft transplantation. *Sci. Rep.* **7**: 46050. [Medline] [CrossRef]
13. Karlsson, M. G. E. and Ludvigsson, J. 2000. The ABBOS-peptide from bovine serum albumin causes an IFN-γ and IL-4 mRNA response in lymphocytes from children with recent onset of type 1 diabetes. *Diabetes Res. Clin. Pract.* **47**: 199–207. [Medline] [CrossRef]
14. Li, C. M., Wang, Y. and Yu, W. X. 2011. Dynamic changes of phenolic compound contents in leaf and bark of poplar during autumn temperature drop. *J. For. Res.* **22**: 481–485. [CrossRef]
15. Li, W., Hu, X., Wang, S., Jiao, Z., Sun, T., Liu, T. and Song, K. 2020. Characterization and anti-tumor bioactivity of *astragalus* polysaccharides by immunomodulation. *Int. J. Biol. Macromol.* **145**: 985–997. [Medline] [CrossRef]
16. Li, Y., Zheng, B., Tian, H., Xu, X., Sun, Y., Mei, Q., Lin, X. and Liu, L. 2017. Yupingfeng Powder relieves the immune suppression induced by dexamethasone in mice. *J. Ethnopharmacol.* **200**: 117–123. [Medline] [CrossRef]
17. Liu, W. G., Zhang, X. D., Huang, L. L., Liu, L. and Zhang, P. 2010. Research progress on physiologic and ecologic characteristics of poplar. *World Forestry Research* **23**: 50–55 (in Chinese).
18. Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ method. *Methods* **25**: 402–408. [Medline] [CrossRef]
19. Mebius, R. E. and Kraal, G. 2005. Structure and function of the spleen. *Nat. Rev. Immunol.* **5**: 606–616. [Medline] [CrossRef]
20. Mei, Y. X., Chen, H. X., Zhang, J., Zhang, X. D. and Liang, Y. X. 2013. Protective effect of chitooligosaccharides against cyclophosphamide-induced immunosuppression in mice. *Int. J. Biol. Macromol.* **62**: 330–335. [Medline] [CrossRef]
21. Meng, F., Xu, P., Wang, X., Huang, Y., Wu, L., Chen, Y., Teng, L. and Wang, D. 2017. Investigation on the immunomodulatory activities of *Sarcodon imbricatus* extracts in a cyclophosphamide (CTX)-induced immunosuppressanted mouse model. *Saudi Pharm. J.* **25**: 460–463. [Medline] [CrossRef]
22. Montomoli, E., Piccirella, S., Khadang, B., Mennitto, E., Camerini, R. and De Rosa, A. 2011. Current adjuvants and new perspectives in vaccine formulation. *Expert Rev. Vaccines* **10**: 1053–1061. [Medline] [CrossRef]
23. Moriguchi, S., Kobayashi, N. and Kishino, Y. 1990. High dietary intakes of vitamin E and cellular immune functions in rats. *J. Nutr.* **120**: 1096–1102. [Medline] [CrossRef]
24. Mu, H. H. and Sewell, W. A. 1994. Regulation of DTH and IgE responses by IL-4 and IFN-γ in immunized mice given pertussis toxin. *Immunology* **83**: 639–645. [Medline]
25. Mudronová, D., Karaffová, V., Koščová, J., Bartkovský, M., Marcincáková, D., Popelka, P., Klempová, T., Certík, M., Mačanga, J. and Marcincák, S. 2018. Effect of fungal gamma-linolenic acid and beta-carotene containing prefermented feed on immunity and gut of broiler chicken. *Poult. Sci.* **97**: 4211–4218. [Medline] [CrossRef]
26. Nayak, S., Koven, W., Meiri, I., Khozin-Goldberg, I., Isakov, N., Zibdeh, M. and Zilberg, D. 2017. Dietary arachidonic acid affects immune function and fatty acid composition in cultured rabbitfish *Siganus rivulatus*. *Fish Shellfish Immunol.* **68**: 46–53. [Medline] [CrossRef]
27. Rosbottom, A., Guy, C. S., Gibney, E. H., Smith, R. F., Valarcher, J. F., Taylor, G. and Williams, D. J. L. 2007. Peripheral immune responses in pregnant cattle following Neospora caninum infection. *Parasite Immunol.* **29**: 219–228. [Medline] [CrossRef]
28. Shang, Y. Z., Gong, M. Y., Zhou, X. X., Li, S. T. and Wang, B. Y. 2001. Improving effects of SSF on memory deficits and pathological changes of neural and immunological systems in senescent mice. *Acta Pharmacol. Sin.* **22**: 1078–1083. [Medline]
29. Shao, B. M., Xu, W., Dai, H., Tu, P., Li, Z. and Gao, X. M. 2004. A study on the immune receptors for polysaccharides from the roots of *Astragalus membranaceus*, a Chinese medicinal herb. *Biochem. Biophys. Res. Commun.* **320**: 1103–1111. [Medline] [CrossRef]
30. Singh, A. K., Pandita, S., Pampoori, Z. A., Huozha, R. and Ashutosh, M. 2013. Effect of pre-partum supplementation of vitamin E to Murrah buffaloes on immune functions and viability of calves. *J. Anim. Physiol. Anim. Nutr. (Berl.)* **97**: 255–262. [Medline] [CrossRef]
31. State Forestry Administration. 2006. Poplar Bark Lipids, 1th ed., Standards Press of China, Beijing (in Chinese).
32. Sun, C., Xue, L., Zhu, Z., Zhang, F., Yang, R., Yuan, X., Jia, Z. and Liu, Q. 2016. Insights from lncRNAs profiling of MIN6 beta cells undergoing inflammation. *Mediators Inflamm.* **2016**: 9275106. [Medline] [CrossRef]
33. Torkaman, J. and Seyam, S. 2010. Measurement of salicylic acid in poplar's bark (*P. deltoides* and *P. euramerican*) by high performance liquid chromatography (HPLC). *Faslnamah-i Giyahan-i Daruyi* **9**: 117–123 (in Russian).
34. Wakikawa, A., Utsuyama, M., Wakabayashi, A., Kitagawa, M. and Hirokawa, K. 1999. Vitamin E enhances the immune functions of young but not old mice under restraint stress. *Exp. Gerontol.* **34**: 853–862. [Medline] [CrossRef]
35. Wang, F., Xiong, Z., Yan, X. D., Dai, X. G., Wang, L. B. and Li, Y. F. 2018. Geographical distribution pattern of species diversity of the genus *Populus L.* in China. *Acta Ecol. Sin.* **38**: 282–290 (in Chinese).
36. Wang, L. 2012. Discussion on the influence of two-way regulation of the dosage of traditional Chinese medicine on the clinical effect. *Contemp. Med. Symp.* **10**: 596–597 (in Chinese).
37. Wang, S. L. 2013. The antagonism of termitomyces albuminosus polysaccharide to cyclophosphamide on immune organ injury in Mice. *Prog. Vet. Med.* **34**: 56–59 (in Chinese).
38. Wang, Y. M., Bian, S. H., Zheng, J. F., Yu, G. Y. and Nie, L. P. 2009. $CD4^+$ and $CD8^+$ T lymphocyte subsets in local lesions and peripheral blood of patients with condyloma acuminatum. *J. Trop. Med.* **9**: 899–900 (in Chinese).
39. Wei, W., Xiao, H. T., Bao, W. R., Ma, D. L., Leung, C. H., Han, X. Q., Ko, C. H., Lau, C. B., Wong, C. K., Fung, K. P., Leung, P. C., Bian, Z. X.

and Han, Q. B. 2016. TLR-4 may mediate signaling pathways of *Astragalus* polysaccharide RAP induced cytokine expression of RAW264.7 cells. *J. Ethnopharmacol.* **179**: 243–252. [Medline] [CrossRef]
40. Wu, L., Guo, S., Wang, C. and Yang, Z. 2009. Production of alkanes (C_7-C_{29}) from different part of poplar tree via direct deoxy-liquefaction. *Bioresour. Technol.* **100**: 2069–2076. [Medline] [CrossRef]
41. Xu, Y. and Qian, S. Y. 2014. Anti-cancer activities of ω-6 polyunsaturated fatty acids. *Biomed. J.* **37**: 112–119. [Medline]
42. Yuan, C., Pan, X., Gong, Y., Xia, A., Wu, G., Tang, J. and Han, X. 2008. Effects of *Astragalus* polysaccharides (APS) on the expression of immune response genes in head kidney, gill and spleen of the common carp, *Cyprinus carpio* L. *Int. Immunopharmacol.* **8**: 51–58. [Medline] [CrossRef]
43. Yuan, L., Wu, L., Chen, J., Wu, Q. and Hu, S. 2010. Paclitaxel acts as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. *Vaccine* **28**: 4402–4410. [Medline] [CrossRef]
44. Zhang, Q., Yu, J., Zhang, L., Hu, M., Xu, Y. and Su, W. 2016. Extraction, characterization, and biological activity of polysaccharides from *Sophora flavescens* Ait. *Int. J. Biol. Macromol.* **93** Pt A: 459–467. [Medline] [CrossRef]
45. Zhang, R. Q., Chen, Z. L. and Luo, Q. H. 2015. Enhancing effect of *Astragalus* polysaccharide on the immune function in cytoxan-induced immunosuppressed mice. *Acta Lab. Anim. Sci. Sin.* **23**: 389–394 (in Chinese).
46. Zhang, X. X., Guo, X. and Yang, H. C. 2008. Effects of poplar bark lipids on immune function of chickens. *Chin. J. Immunol.* **24**: 630–631 (in Chinese).
47. Zhang, X. X., Liu, M. L., Yang, L. Z., Wang, X. Y. and Han, X. H. 2007. Effects of poplar bark lipid as adjuvants on immune effects of inactivated vaccine against Newcastle disease virus. *China Poultry* **29**: 11–13 (in Chinese).
48. Zhang, X. X., Yang, L. Z., Liu, M. L. and Yang, H. C. 2007. Growth and decline of antibodies and microscopic changes of immune sites in chickens immunized with inactivated Newcastle disease lipid (PBL) vaccine. *Chin. J. Vet. Med.* **43**: 34–36 (in Chinese).
49. Zhao, T., Feng, Y., Li, J., Mao, R., Zou, Y., Feng, W., Zheng, D., Wang, W., Chen, Y., Yang, L. and Wu, X. 2014. *Schisandra* polysaccharide evokes immunomodulatory activity through TLR 4-mediated activation of macrophages. *Int. J. Biol. Macromol.* **65**: 33–40. [Medline] [CrossRef]
50. Zheng, Y. S., Zhao, H. K. and Cui, Z. Z. 2007. Effects of reticuloendotheliosis virus infection on IFN-γ production in the splenocytes of SPF chickens. *J. Agric. Biotechnol.* **15**: 112–114 (in Chinese).
51. Zheng, Z. Y., Yu, X. L., Dai, T. Y., Yin, L. M., Zhao, Y. N., Xu, M., Zhuang, H. F., Chong, B. H. and Gao, R. L. 2019. Panaxdiol saponins component promotes hematopoiesis and modulates T lymphocyte dysregulation in aplastic anemia model mice. *Chin. J. Integr. Med.* **25**: 902–910. [Medline] [CrossRef]
52. Zhou, J. X., Qian, Z. Q., Jing, E. H., Liu, W. J. and Jin, G. M. 2018. Effects of compound traditional Chinese medicine preparation on thymus tissue structure and caspase-3 protein distribution of SD rats. *Acta Agr. Zhejiangensis* **30**: 722–729 (in Chinese).
53. Zhou, W. C. and Wang, J. Q. 1990. Extracting lipid from poplar bark (*Populus Tomentosa*). *Nat. Prod. Res. Dev.* **2**: 74–80 (in Chinese).
54. Zhou, W. C., Jiang, R. Z., Wang, J. Q., Song, Q. and Song, J. B. 2004. Chemical constituents of unsaturated fatty acid ethyl ester from poplar bark and its medicine effectiveness. *Linchan Huaxue Yu Gongye* **24**: 73–76 (in Chinese).