International Immunopharmacology 70 (2019) 56–66

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Immunological modulation effects of an acid Epimedium polysaccharide on immune response in chickens

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ARTICLE INFO

Keywords:
Acid Epimedium polysaccharide
Immunological modulation effect
Immune response

ABSTRACT

The purpose of the present study is to investigate the immunological activities of EPS-1 in the non-specific immune response and specific immune response of chickens. In vitro, the results showed that EPS-1 could increase the proliferation and cytokine secretion (IL-2, IL-4, IFN-γ and TNF-α) of spleen lymphocytes, expression of key surface molecules (MHC II, CD11c, CD40 and CD86) and cytokine secretion (TNF-α and IL-10) of matured chBM-DCs, phagocytic rate of matured chBM-DCs, and enhance the maturation and stimulating capacity of chBM-DCs. In vivo, EPS-1 could also prompt the HI antibody titer, boost the peripheral lymphocyte proliferation, enhance the release of cytokine products in blood (IFN-γ, IL-4 and IL-2) and duodenum (IL-17 and sIgA) of chickens. These results indicated that EPS-1 may have the potential as a powerful immune adjuvant in the treatment of chicken diseases.

1. Introduction

Polysaccharide is one of the basic ingredients which consists the creatures in the world [1]. Until recently, oceans of references have reported the bioactivities of different polysaccharides from plants, animals and microbes, including immunomodulation, antiviral, antitumor, antimicrobial, anti-oxidation and anti-inflammation [2]. Among these effects, the immune modulating abilities have attracted more and more scientists' attentions [3]. Polysaccharides from traditional Chinese medicines (TCM) can activate the immune cells such T cells, B cells, NK cells, macrophages and dendritic cells, promote the level of antibodies, activate complement, as well as improve the release of cytokines [4,5]. Moreover, because of their securities in the process of treatment, polysaccharides are widely used as ideal regulators for the immune functions of bodies in clinical application [1].

As known, dendritic cells (DCs) and splenic lymphocytes are both important in the immune systems for birds, mammals and humans. DCs, as a milestone in the history of immunology, are the most powerful antigen-presenting cells (APCs) to process and present antigen to activate T cells [6,7]. They are crucial mediators which can promote the development of cell-mediated immune response [8]. Splenic lymphocytes, containing T cells and B cells, play important roles in the immunological response of animals [9,10]. Up to now, DCs and splenic lymphocytes as therapeutic targets have attracted oceans of researchers' attentions, including those who study in the field of TCM [11,12].

Herba Epimedii (Yin-Yang-Huo in Chinese), the aerial part of some species from genus *Epimedium*, is one of the most important medical herbs which has been used for body-modulating, anti-osteoporosis and anti-aging effects for more than twenty centuries in some countries in East Asia such as China, Korea and Japan [13]. Recently, studies have revealed plenty of bio-effective components from this natural medicine, such as polysaccharide [2], flavonoids [14], and so on. Among them, Epimedium polysaccharides (EPS) have drawn great attention for their special biological abilities. In our previous studies, an acidic EPS (EPS-1) composed of linear chain of 1,4-linked α-D-Gal*p*A, 1,3,4-linked α-D-Gal*p*A, 1,6-linked β-D-Gal*p* and terminal α-L-Rha*p* in a molar ratio of 11.0:1.0:1.0:1.0 with 150 kDa in molecular weight, had been extracted and isolated from *Epimedium acuminatum* Franch., and showed interesting immune modulating effects on peripheral T lymphocyte and immature chicken bone marrow dendritic cells (chBM-DCs) [2]. However, further investigations on the effects of EPS-1 are still needed.

Thus, in the continuous study, the immunological capacities of EPS-1 was carried out by evaluating the effect on the proliferation and cytokine secretion of splenic lymphocytes, the morphology change of

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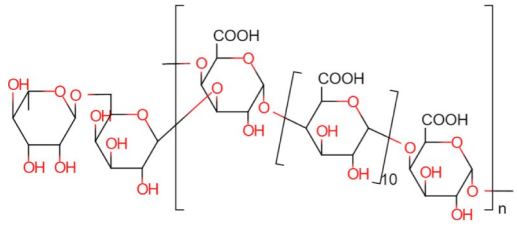
https://doi.org/10.1016/j.intimp.2019.02.009
Received 8 September 2018; Received in revised form 5 February 2019; Accepted 5 February 2019
Available online 20 February 2019

mature DCs, phagocytosis, lymphocyte stimulating and cytokine assays secretion abilities in the non-specific immune response. Additionally, the effects of EPS-1 in the specific immune response were also tested. The results indicated that EPS-1 had immunological modulation abilities in the non-specific and specific immune response, which could be further used as one vaccine adjuvant in future.



The structure of EPS-1

2. Materials and methods

2.1. Animals

Non-immune health White Roman male chickens (One-day-old) were obtained from Tangquan Poultry Farm (Nanjing, China). They were reared in wire cages (40 cm × 60 cm × 100 cm) under specific pathogen-free (SPF) conditions in the Experimental Animal Center at Nanjing Agricultural University (Nanjing, China), at 37 °C and lighted for 23 h at the beginning of pretrial period. The atmosphere was gradually declined to constant room temperature and in a normal 12-h-dark/12-h-light cycle in the following days. Feed and water were supplied ad libitum. The commercial starter diets for chickens were provided by the Tangquan Poultry Farm. All of the animal procedures and care were treated by following the guide for the care and use of laboratory animals of Nanjing Agriculture University.

2.2. Materials

Epimedium polysaccharide (EPS-1, purity of 95%) was obtained in our laboratory according to the reported reference [2]. Roswell Park Memorial Institute 1640 (RPMI-1640, Gibco, USA) with benzylpenicillin (100 IU·mL^{-1}), streptomycin (100 IU·mL^{-1}) and 10% fetal bovine serum (FBS) as the supplement was prepared to wash, re-suspend and culture the cells. Phytohemagglutinin (PHA) as mitogens of T-cell and DCs was purchased from Sigma (USA) and was dissolved into 0.1 mg·mL^{-1} with RPMI-1640. Lipopolysaccharide (LPS) was provided by Sigma (USA) and was dissolved with RPMI-1640 (10 ng·mL^{-1}). 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT, Amresco Co.) was used to test the proliferation assays of lymphocytes and DCs and dissolved into 5 mg·mL^{-1} with calcium and magnesium-free (CMF) phosphate buffered saline (PBS, pH 7.4). All of the reagents were filtered through a 0.22 μm millipore membrane filters. Lymphocyte separation medium (Histopaque 1077, Histopaque 1119) was manufactured by Sigma-Aldrich (Poole, UK). FITC-dextran were obtained from US SIGMA. The other chemicals and reagents with analytical grade were purchased from Nanjing Shoude Chemical Co. Ltd. (Nanjing, China). The used glassware was washed in 50:50 (v/v) ethanol: 1 M sodium hydroxide bath, followed by a 1 M nitric acid bath, rinsed copiously with Milli-Q water, and finally dried in an oven at 250 °C for 1 h for LPS contaminants inactivation. All solution transfers were performed using LPS-free devices. Sterile, disposable plastic ware was used at all times to prevent LPS contamination. The LPS concentration in the Milli-Q water was below 0.05 EU/mL, based on the author's test results.

2.3. Effects of EPS-1 on the spleen lymphocytes proliferation assay

At 6 weeks of age, one chicken in good health were sacrificed. After laparotomy, spleen samples of chickens were removed from the experimental animal. Spleen lymphocytes were isolated by given method according to previous report [15], using Histopaque 1077 (Sigma-Aldrich, Poole, UK) as lymphocyte isolation. The obtained cells were adjusted to 5 × 10^6 cells·mL^{-1}, divided into two groups and incubated in 96-well culture plates. One group was added with PHA (final concentrations of 10 μg·mL^{-1}), 100 μL per well. Then EPS-1 at a range of concentrations (1.25, 2.5, 5, 10 and 20 μg·mL^{-1}) were added into each group respectively, four wells each concentration, 100 μL per well. Cell control group (RPMI-1640 medium) and PHA (final concentration of 10 μg·mL^{-1}) control group were also prepared. All plates were incubated at 37 °C in 5% CO_2 atmosphere (Thermo), and the spleen lymphocytes proliferation assay was determined by MTT method as described by Wu [2]. The absorbance at 570 nm (A_{570} value) measured by microliter enzyme-linked immunosorbent assay (ELISA) reader (MULTISKAN FC, Thermo) was used to compare the strength of proliferation in each well. The average splenocyte proliferation rates of all groups were calculated on the basis of the giving formula: The average proliferation rate (%) = ($\bar{A}_{\text{test group}}$ - $\bar{A}_{\text{control group}}$)/$\bar{A}_{\text{control group}}$ × 100%.

2.4. Effects of EPS-1 on cytokine productions of spleen lymphocyte

Cells harvested in 2.3. were treated synergistically with EPS-1 (final concentrations of 5, 10 and 20 μg·mL^{-1}, respectively) and PHA (final concentration of 10 μg·mL^{-1}) at 37 °C in 5% CO_2 atmosphere (Thermo) for 2 days, together with cell control group (RPMI-1640 medium) and PHA group. Culture supernatants were collected then the concentrations of interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-γ (IFN-γ) and Tumor Necrosis Factor α (TNF-α) were tested by ELISA kits (R&D, Co., USA).

2.5. Morphology of chBM-DCs treated with EPS-1

At 6 weeks of age, one chicken in good health were sacrificed and the femurs and tibias were isolated from the surrounding muscle tissue. The immature chicken bone marrow dendritic cells (chBM-DCs) were collected following our reported method [2]. The obtained cells were adjusted to 2.5 × 10^6 cells·mL^{-1} by DMEM with 10% fetal bovine serum, 100 U·mL^{-1} penicillin, 100 U·mL^{-1} streptomycin and chGM-CSF (50 ng·mL^{-1}, Abcam, USA) then seeded to 12-well cell culture plates (2 mL each well) and incubated at 37 °C in 5% CO_2. The medium with suspended cells was replaced by equal volume of fresh medium after 24 h. On the 3rd and 5th day, half of the cultivate medium was removed and replenished with fresh medium. On the 6th day, 10 μg·mL^{-1} of EPS-1 or serum-free RPMI-1640 were added then continued to cultivate for 24 h.

On the 7th day of incubation, cells were collected and detected for morphology study by scanning electronic microscopy (SEM, JEOL, JSM-7300). Samples were prepared using the given method [16]. The intracellular phagosomes of cells were also analyzed by transmission electronic microscopy (TEM, JEOL, JSM-7300) for morphology study as reported [16].

2.6. Analysis of key surface molecules by flow cytometry (FCM) and confocal microscope (CM)

The cultivated immature chBM-DCs on the 7th day in 2.5. were divided into two parts: EPS-1 at series of concentrations (final concentrations of 5, 10 and 20 μg·mL^{-1}) was added to one part as the EPS group, while the other group was treated with RPMI 1640 as the blank

group. Both of them were incubated at 37 °C in 5% CO_2 for 48 h. After incubation, cells were collected and washed by PBS twice. Both the EPS-1 group and the blank group were stained with anti-CD11c (eBioscience, USA) anti-MHC II (Abcam, USA), anti-CD40 (Abd, UK) and anti-CD86 antibodies (Abd, UK) for 20 min at 4 °C, rinsed by PBS twice and analyzed with FACS Calibur flow cytometry (FCM, Becton Dickinson, San Diego, CA) to evaluate the purity of chBM-DCs.

Cells treated with EPS-1 (final concentrations of 20 μg·mL^{-1}) or RPMI 1640 were centrifuged and suspended in PBS, then incubated with anti-CD40 and anti-CD86 antibodies (Abd, UK) for 20 min at 4 °C. After cultivation, cells were fixed in 4% PFA (Sigma-Aldrich, St. Louis, MO) and observed using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss AG, Germany).

2.7. Cytokine assay of chBM-DCs

Cells were treated following the method in 2.5. in 12-well cell culture plates. On the 6th day of incubation, cells were stimulated with EPS-1 (final concentrations of 5, 10 and 20 μg·mL^{-1}, EPS group) and LPS (final concentration of 10 ng·mL^{-1}, LPS group) at 37 °C in 5% CO_2 for 48 h, together with the RPMI 1640 as the cell control group (CC group). After incubation, the supernatants were collected and the concentrations of interleukin 1β (IL-1β), interleukin 10 (IL-10), interleukin 12p70 (IL-12p70), interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and regulated upon activation normal T-cell expressed and secreted (RANTES) were tested by ELISA kit (R&D, Co., USA). The concentration of nitric oxide (NO) was analyzed by Nitric Oxide Assay Kit (Beyotime, Nantong, Jiangsu).

2.8. Allogeneic mixed lymphocyte reaction

Spleen lymphocytes obtained in 2.3. were cultivated in 96-well culture plates, 100 μL per well. The immature chBM-DCs were stimulated following the method in 2.7. After incubation, cells were stimulated by 50 μg·mL^{-1} mitomycin C for 30 min to get matured, then washed by PBS twice and re-suspended to 5 × 10^5 cells·mL^{-1} by RPMI 1640 at 37 °C as the stimulating cells. The stimulated cells were added into the spleen lymphocytes with the ratio of 1:1, 1:5, 1:10 and 1:20 (DCs: spleen lymphocytes), four wells four wells each concentration. Then, the mixed cells were cultivated at 37 °C in 5% CO_2 for 72 h. The cell proliferation was evaluated using MTT method as 2.3. The A_{570} values were used to evaluate the proliferation of T cells stimulated by chBM-DCs.

2.9. Analysis on phagocytosis ability of matured chBM-DCs

The matured chBM-DCs were obtained following the method in 2.8. On the 6th day of incubation, cells were divided into two parts, one part was stimulated with EPS-1 (final concentration of 20 μg·mL^{-1}, EPS group), LPS (final concentration of 0.5 μg·mL^{-1}, LPS group) or RPMI 1640 (pH 7.2, CC group) at 37 °C in 5% CO_2 for 48 h. At the end of cultivation, cells were stimulated by 50 μg·mL^{-1} mitomycin C for 30 min, then collected and washed by PBS twice. Cells were stained by FITC-dextran in 37 °C avoid light effect for 30 min and the phagocytosis ability of mature chBM-DCs was detected by flow cytometry.

2.10. Immune activities of EPS-1 on specific immune response

2.10.1. Vaccinations

One hundred and fifty non-immune chickens (14-day-old) were randomly divided into five groups, which were EPS high dosage (EPS-H) group, EPS middle dosage (EPS-M) group, EPS low dosage (EPS-L) group, vaccine control (VC) group and blank control (BC) group respectively, thirty chickens per group. Except BC group, chickens were vaccinated with ND vaccine (La Sota train, No. S140901, Bio-drug Company of Veterinary, Beijing City), and repeated vaccination at 28-day-old. At the beginning of the first vaccine, chickens were orally administered with EPS-1 (1 mg·mL^{-1}) at high dosage (0.4 mg·kg^{-1}), middle dosage (0.2 mg·kg^{-1}) and low dosage (0.1 mg·kg^{-1}) respectively for three consecutive days, one time per day, while the VC group and BC group were orally administered with physiological saline (1.0 mg·kg^{-1}).

2.10.2. Serum hemagglutination inhibition (HI) antibody titer

On the 7th (D_7), 14th (D_{14}), 21th (D_{21}), 28th (D_{28}) and 35th (D_{35}) day after the first vaccine, six chickens of each group were selected randomly, and the blood were collected to analyze the serum hemagglutination inhibition (HI) by micro-method as reported [1]. The HI values were shown by the mean titers which were displayed by the reciprocal log2 values of the highest dilution.

2.10.3. Lymphocytes proliferation assay

On the 14th and 28th day after the first vaccination, four chickens of each group were chosen randomly, then the peripheral blood were harvested for testing peripheral lymphocyte proliferation by MTT method, following the reported method [2].

2.10.4. Cytokine productions of peripheral serum

Blood harvested in 2.10.2 were congealed and centrifuged at 2500 rpm. The concentrations of cytokine productions of IL-2, IFN-γ and IL-4 in the supernatants were detected by ELISA kits (Calvin Biological Technology Co., Ltd., Suzhou, China).

2.10.5. Cytokine productions in irrigating solution of duodenum

On the 14th and 28th day after the first vaccination, six chickens of each group were sacrificed randomly. The duodenums were removed and the intestinal cavities were washed by PBS carefully. The solution was centrifuged to give the supernatants. The concentrations of sIgA and IL-17 were determined by ELISA kits (Calvin Biological Technology Co., Ltd., Suzhou, China).

2.11. Statistical analysis

The obtained data in this study were expressed as mean ± SD from at least three independent experiments, each of which was repeated for more than one time. SPSS Statistics 20.0 for Windows was used and $P < 0.05$ was considered to be significant, while $P < 0.01$ was considered as extremely significance.

3. Results

3.1. The effects of EPS-1 on spleen lymphocytes proliferation

As illustrated in Fig. 1A, in single stimulation group, the A_{570} values of 5 and 20 μg·mL^{-1} were significantly higher than the control group ($P < 0.05$). Fig. 1A also exhibited that while synergistical stimulation with PHA, the A_{570} value of each EPS group was significantly higher than the other groups ($P < 0.05$).

3.2. The effects of EPS-1 on secretions of cytokine productions of spleen lymphocyte

The effect of EPS-1 on the secretions of cytokine productions such as IL-2, IL-4, IFN-γ and TNF-α were shown in Fig. 1(B to C). IL-2 and IL-4 at three concentrations, IFN-γ at 10 and 20 μg·mL^{-1}, as well as TNF-α at 5 μg·mL^{-1} were significantly higher than those of control groups

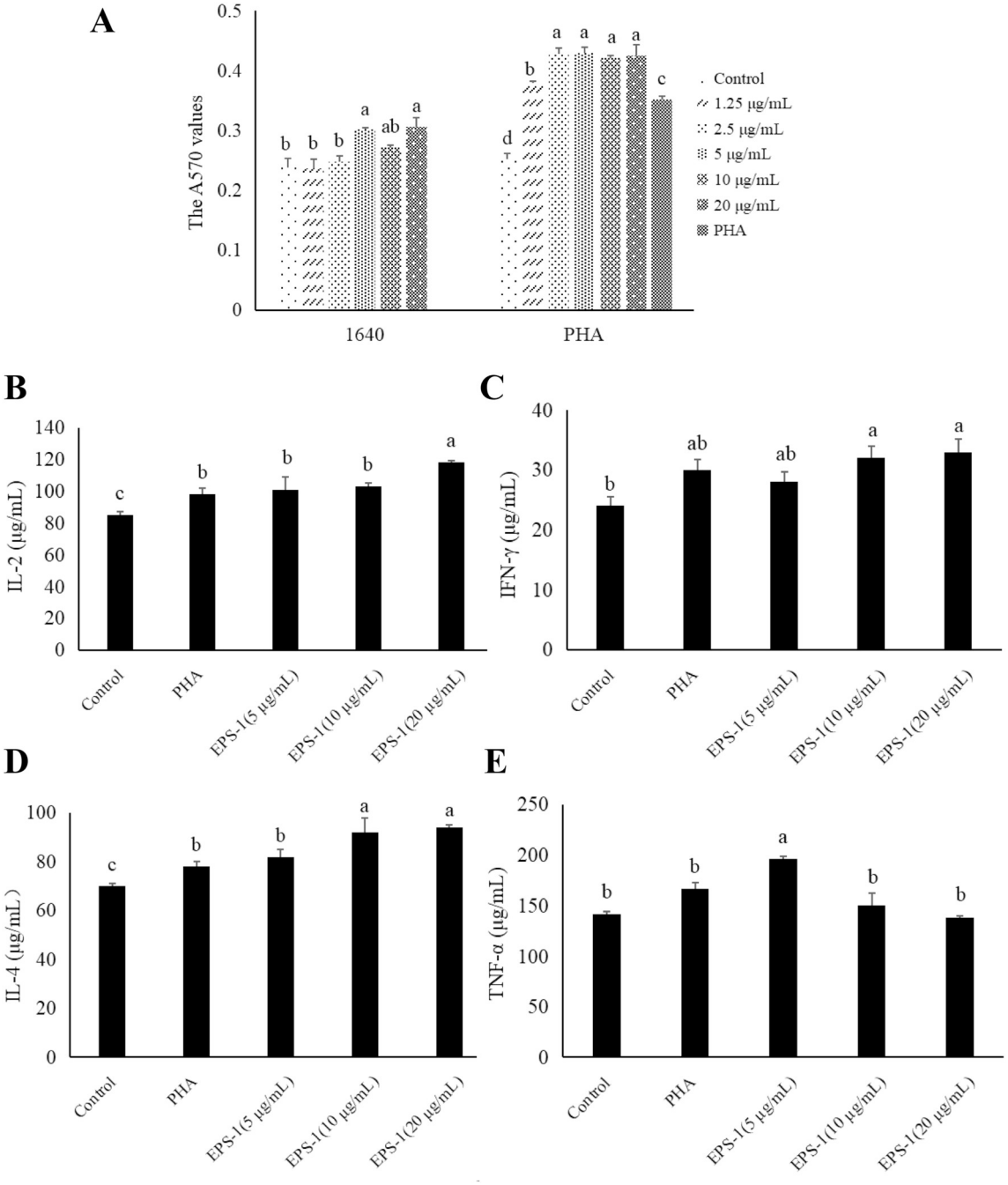


Fig. 1. Effects of EPS-1 on the proliferation of spleen lymphocytes in vitro (A), cytokines of spleen lymphocytes in vitro (B to E) represented IL-2, IFN-γ, IL-4 and TNF-α, respectively. [a-e] Bars without the same superscripts differ significantly ($P < 0.05$).

($P < 0.05$). Moreover, IL-2 at 20 μg·mL^{-1}, IL-4 at 10 and 20 μg·mL^{-1}, together with TNF-α at 5 μg·mL^{-1} were higher than those of PHA groups ($P < 0.05$) (Fig. 1B to C).

3.3. Morphology of chBM-DCs

When observed under conventional light microscope, the stimulated chBM-DCs (Fig. 2Ab) became irregular, fusiform and large, with large round or oval nuclei, and had more dendrites which were thicker and longer than the control ones (Fig.2A).

In the SEM pictures, compared with RPMI 1640 group (Fig. 2Bc), the cells treated by EPS-1 were larger with more protrusions, and the surfaces became rougher and had more cascading folds (Fig. 2B).

As shown in TEM photos, the cells cultivated with EPS-1 (Fig. 2Cf) displayed larger nuclei, and the surfaces of cells had more dendrites. Moreover, lysosomes inside the cells indicated greater reduction than the control ones (Fig. 2C).

3.4. Effects of EPS-1 on key surface molecules of matured chBM-DCs

In Figs. 3 and 4, the data analyzed by FCM demonstrated the key surface molecules of matured chBM-DCs such as MHC-II, CD11c, CD40

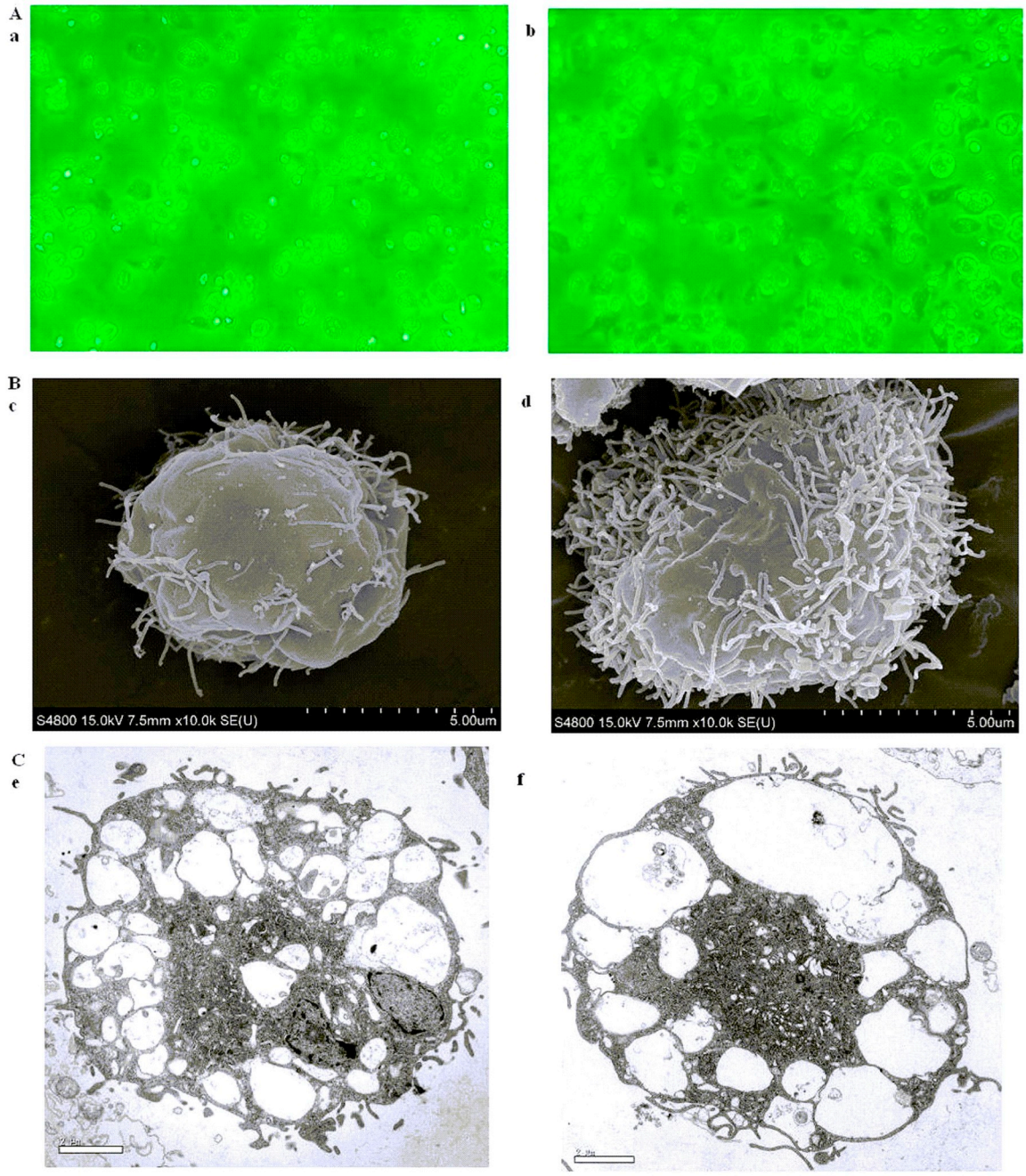


Fig. 2. Morphology of matured chBM-DCs cultured with RPMI 1640 (left) and EPS-1 (right). (A) The morphology (400×) under light microscope; (B) The morphology under scanning electron microscopy (15000×); (C) The morphology under Transmitted electron microscopy (6000×).

and CD86. After being cultivated with EPS-1, the percentage of expression of MHC II and CD11c were 65.3% and 78% respectively, higher than those of 42.4% and 52.6% in the RPMI 1640 group. Meanwhile, under the observation of CM, EPS-1 of final concentrations from 20 to 5 $\mu g \cdot mL^{-1}$ could significantly increase the expression of CD40 and CD86, comparing with the RPMI1640 group (Figs. 3B and 4A and B).

3.5. Effects of EPS-1 on the cytokine secretions of chBM-DCs

Fig. 5(A to F) depicted the cytokines concentrations by matured chBM-DCs. Except TNF-α and IL-10, the excretions of cytokines could be up-regulated by EPS-1, and were significantly higher than the control group at 10 and 20 $\mu g \cdot mL^{-1}$. However, the secretion of TNF-α and IL-10 could be suppressed by EPS-1 significantly at the given concentrations, comparing to the RPMI 1640 group. Moreover, the level of NO was also enhanced by EPS-1 at the concentrations of 10 and 20 $\mu g \cdot mL^{-1}$ (Fig. 4G).

3.6. Effects of EPS-1 in the allogeneic mixed lymphocyte reaction

As exhibited in Fig. 6(A-D), the A_{570} values of EPS-1 at 20 $\mu g \cdot mL^{-1}$ were significantly higher than those of the control groups, which meant that EPS-1 at the highest concentration could enhance the effects of chBM-DCs on stimulating the proliferation of allogenic mixed spleen lymphocytes at all ratios (DCs: lymphocyte).

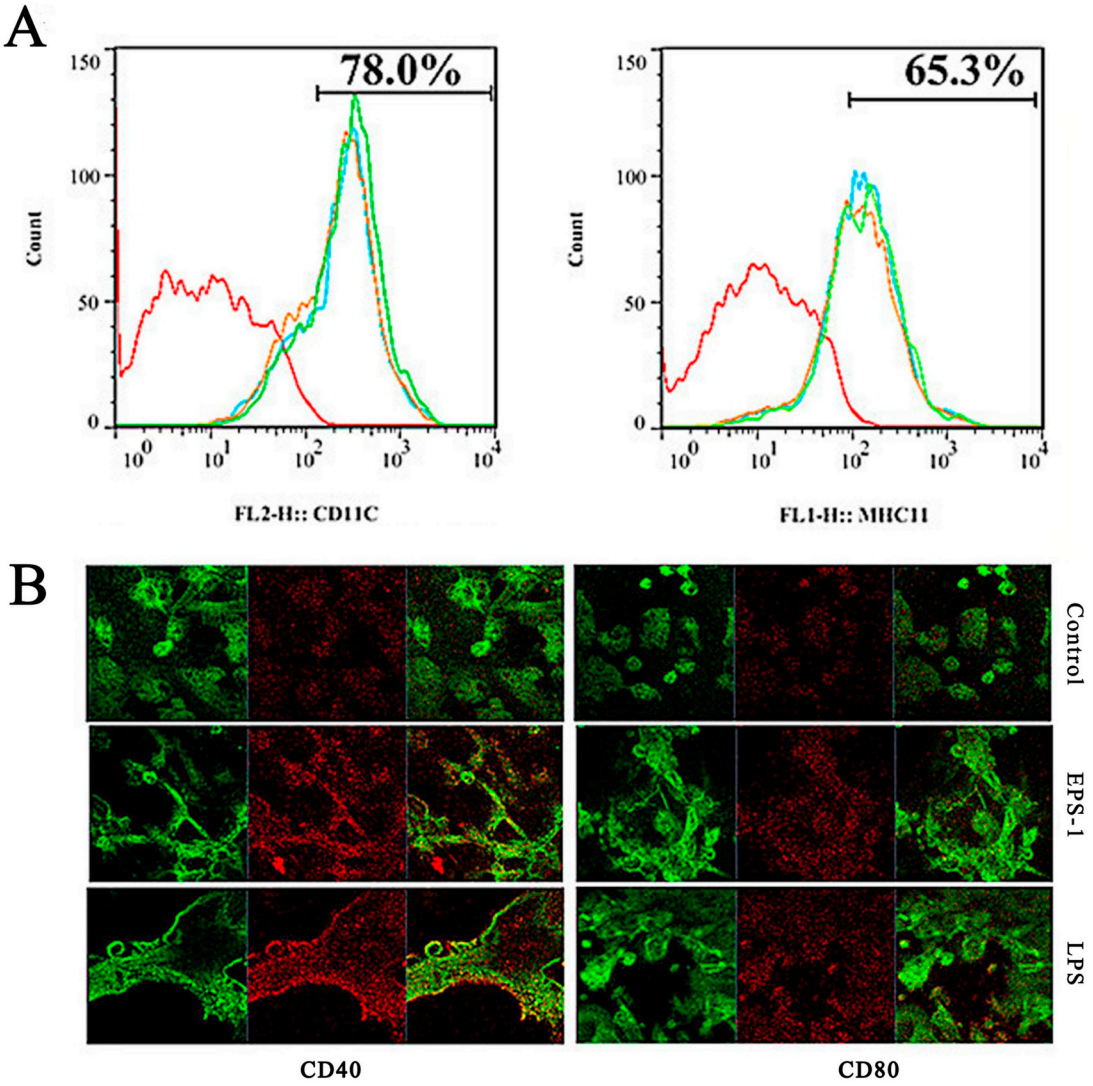


Fig. 3. The variations of key surface molecules of matured chBM-DCs treated by EPS-1, RPMI 1640 and LPS. (A) The percentage of expression of MHC II and CD11c analyzed by FCM (The red line is RPMI 1640 group, the another three colour lines are EPS-1 parallel groups; (B) The expression of CD40 and CD86 detected by CM. Colour in red means expressed molecules, green indicated the cell membranes. [a-d] Bars without the same superscripts differ significantly ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. Effects of EPS-1 on phagocytosis of matured chBM-DCs

According to Fig. 5E, at 30 min and 90 min, both of the percentages of phagocytic rate of matured chBM-DCs of EPS-1 group and LPS group reduced from 39.3% and 35.4% to 29.5% and 25.9%, respectively. However, the data in control group rose from 40.3% to 52.0% simultaneously.

3.8. Immune modulation effects of EPS-1 on chicken in vivo

3.8.1. The variation of HI antibody titer

In Table 1, compared to the VC group and BC group, the titers in each EPS group increased after the first vaccination. Data of EPS-H group at the five time points showed significance ($P < 0.05$), together with those of the EPS-M group on D_7, D_{21}, D_{28} and D_{35} ($P < 0.05$). As for the EPS-L group, data on D_7, D_{21} and D_{35} were significantly higher ($P < 0.05$). Furthermore, the HI value of EPS-H group became highest on D_{14}, and the highest data of EPS-M group and EPS-L group came on D_{21}.

3.8.2. The changes of lymphocytes proliferation in vivo

According to Fig. 7A, compared with the VC group and BC group, the A_{570} values of EPS groups with three dosages were significantly higher on D_{14}, while those of EPS-H and EPS-M groups exhibited significance on D_{28}.

3.8.3. Cytokine productions of peripheral serum and duodenum irrigating solution

As shown in Fig. 7(B, C and D), compared with the VC group and BC group, the concentrations of IFN-γ and IL-4 in EPS-H group and EPS-M group were significantly higher at all testing points after the first vaccination, while the concentrations of IFN-γ of EPS-L group on D_7, D_{21} and D_{28}, as well as concentrations of IL-4 of EPS-L group on D_{14}, D_{21} and D_{28} were significantly higher. As for IL-2, the concentrations of EPS-H group on D_7, D_{21}, D_{28} and D_{35}, those of EPS-M and EPS-L groups on D_{21} and D_{28} were significantly higher.

Fig. 7(E, F) demonstrated the dynamic changes in the duodenum irrigating solution after the first vaccination. On D_{14} and D_{28}, the concentrations of IL-17 and sIgA in three EPS groups were all significantly higher than those in VC group and BC group. Moreover, on

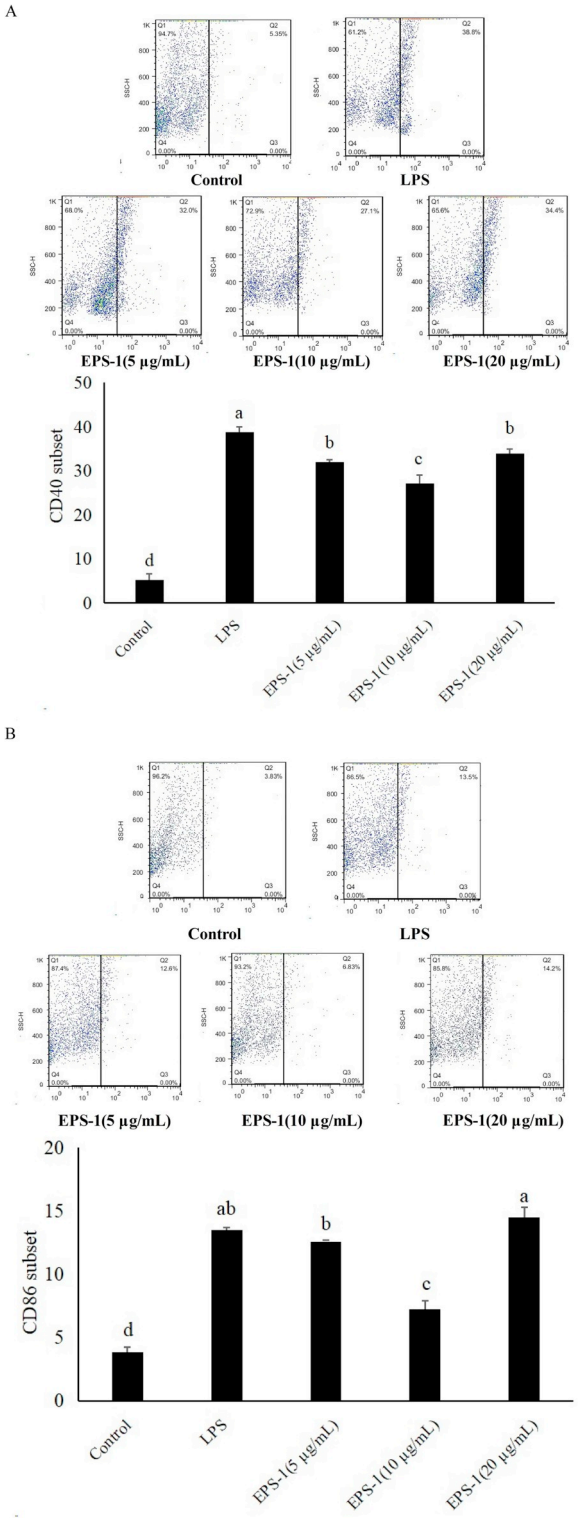


Fig. 4. Effects of EPS-1 on the percentage of expression of CD40 (A) and CD86 (B). [a-d] Bars without the same superscripts differ significantly ($P < 0.05$).

D_{28}, the concentrations of EPS-H groups were both significantly higher than those of EPS-L groups respectively.

4. Discussion

Polysaccharides could modulate the immune functions in vitro and in vivo [3,17,18]. As an important herbal polysaccharide, EPS could affect chicken peripheral lymphocyte and immature DCs [2]. However, to our knowledge, few literature had reported its effects on the phenotypic and matured chBM-DCs in vitro. Thus, this experiment was performed to investigate the immunomodulatory activities of EPS-1 on chicken from these facets.

Spleen lymphocytes are one kind of important cells for birds and mammals immunological system. Its proliferation and cytokine productions are both significant to the immune responses [9]. Our study showed that EPS-1 could significantly boost the proliferation of spleen lymphocyte in both single and synergistical stimulation with PHA, and EPS-1 had better stimulating effect in synergistical stimulation group. This result was in accord with the reported conclusion [9]. Moreover, our results proved that EPS-1 of 20 $\mu g \cdot mL^{-1}$ could significantly improve the capacity of chBM-DCs on stimulating the proliferation of allogenic mixed spleen lymphocytes. It was reported that the ability of DCs on inducing lymphocyte proliferation was directly depended on the maturation degrees of DCs [19]. Thus, this indicated that EPS-1 could enhance the maturation of chBM-DCs.

Cytokines are potential modulating targets secreted by immune cells such as lymphocytes, DCs or macrophages, which can take part in immunological response [1]. Among these molecules, TH1 cells produce IL-2, IL-12, IFN-γ and TNF-α [18], while TH2 cells express IL-4 [20]. IL-1β and TNF-α can lead to local cell death and promote the abilities of macrophages [21]. IL-10 is usually secreted by macrophages, monocytes and dendritic cells (DCs), and can control the nature and extent of inflammatory responses [22]. And RANTES (regulation upon activation normal T cell expressed and secreted), which are produced by CD8 + T cells, can modulate the biological abilities of T cells, monocytes, dendritic cells and so on [23]. At last, as a reactive free radical, NO has high activities to form various nitrogen oxides which take part in the immune procedures [17]. In our present study, the effects of EPS-1 on cytokine assays of spleen lymphocytes in vitro were studied and the results demonstrated that EPS-1 of relative concentrations could significantly increase the concentrations of IL-2, IL-4 and IFN-γ than the CC groups and PHA groups. The result revealed that EPS-1 inducted both TH1 and TH2 responses simultaneously. As to the matured chBM-DCs, the concentrations of IL-1β, IL-12p70, IFN-γ and RNATES of EPS-1 stimulated groups were significantly higher than the data of control groups. Similarly, after stimulated by EPS-1 of 10 and 20 $\mu g \cdot mL^{-1}$, the concentrations of NO were also significantly higher than that of CC group. These exhibited that the release of the above products were activated by EPS-1. On contrary, for the EPS-1 stimulated chBM-DCs, the concentrations of IL-10 and TNF-α reduced, indicating that EPS-1 could inhibit the secretions of these cytokines. These results could proclaim that EPS-1 may have the ability on modulating the cellular immune via different pathways.

Until now, numerous investigations have confirmed that polysaccharides obtained from medical herbs could affect the maturation of DCs by inducing the morphology alterations, improving the expression of key surface molecules of matured chBM-DCs, and modulate the cellular phagocytosis [3,6,16]. The maturation of chBM-DCs could be observed in our morphology studies. Figures of cells treated by EPS-1 showed significant morphological differences comparing to those of CC group, which exhibited typical characters of matured DCs. These changes may exhibit the effects from EPS-1 on the structural maturation of chBM-DCs. Moreover, our FCM and CM analysis demonstrated that after treated by EPS-1, the expressions of key surface molecules of matured chBM-DCs got upgraded. This phenomenon implied that EPS-1 might activate the cellular immune of DCs by stimulating the cell-membrane receptors. Additionally, the reduction of phagocytic rate of EPS-1 cultivated DCs proclaimed that the phagocytosis of matured chBM-DCs was inhibited by EPS-1, which was similar to the conclusions that polysaccharides could speed the reduction of the phagocytic ability of matured DCs [3,6,16]. These studies provided the conclusion that EPS-1 could enhance the maturation of chBM-DCs.

Humoral immunity plays an important role in the specific immune reaction. The HI antibodies could prevent virus from invading the cells,

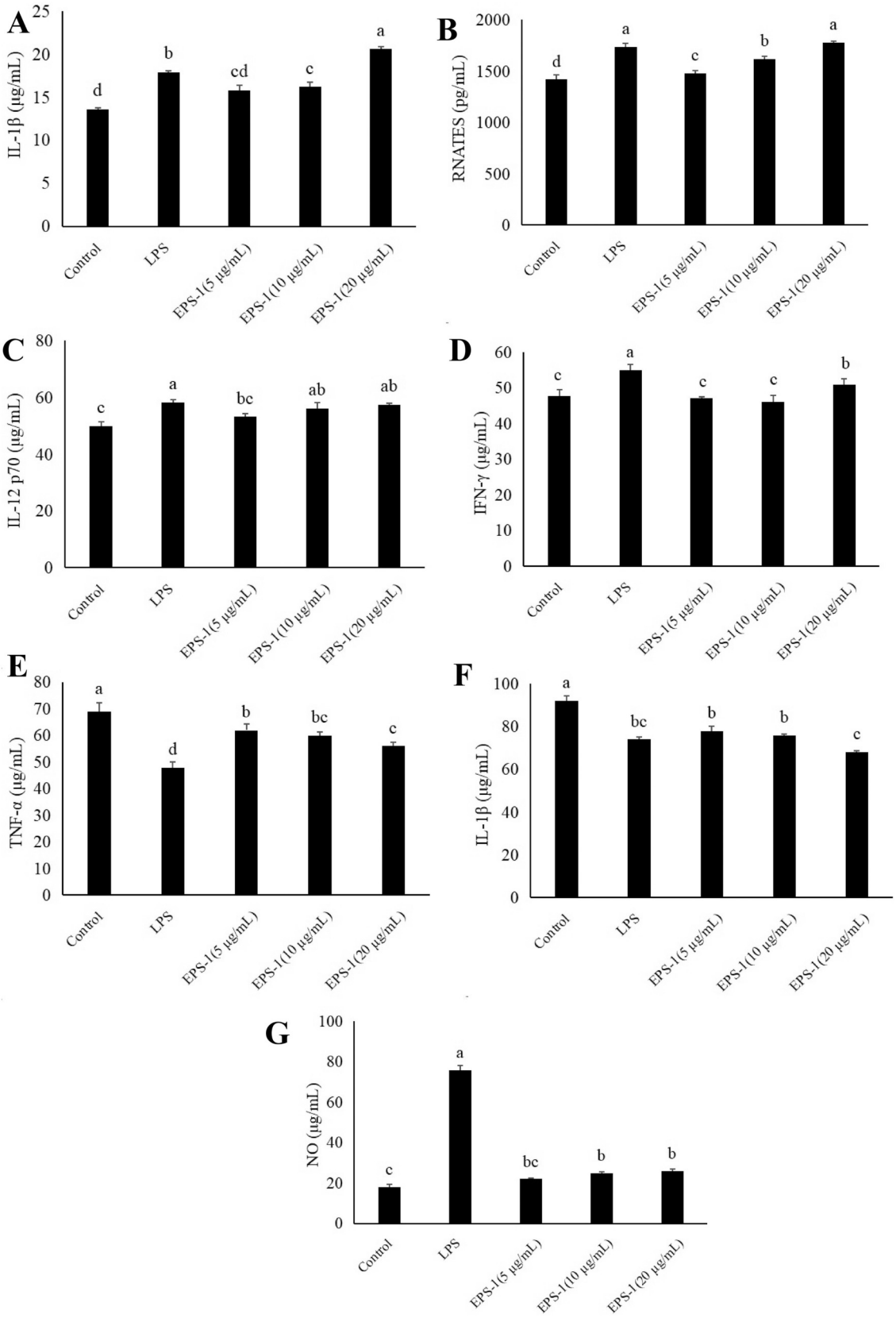


Fig. 5. Effects of EPS-1 on cytokines of matured chBM-DCs (A to F) and the nitric oxide of matured chBM-DCs (G). (A to F) represented IL-1β, RNATES, IL-12p70, IFN-γ, IL-4 TNF-α and IL-10, respectively; (G) indicated the nitric oxide of matured chBM-DCs. $^{a-d}$ Bars without the same superscripts differ significantly ($P < 0.05$).

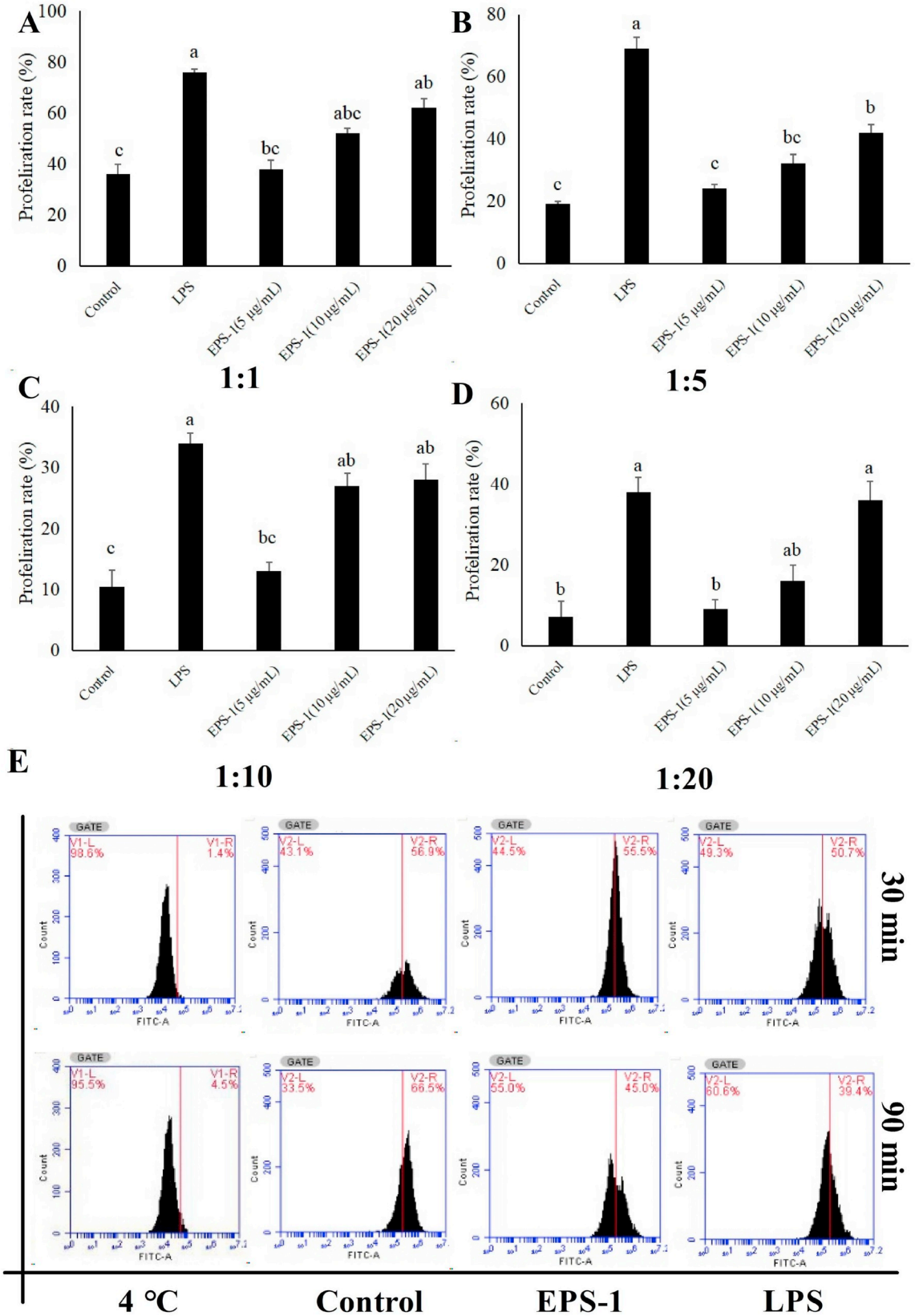


Fig. 6. Effects of EPS-1 on the proliferation of allogeneic mixed lymphocyte reaction (A-D) and phagocytic activity of matured chBM-DCs (E). (A to D) demonstrated the effects of EPS-1 on the proliferation rate with different ratio (DCs: T cells as 1:1, 1:5, 1:10 and 1:20, respectively). [a-c] Bars without the same superscripts differ significantly ($P < 0.05$).

Table 1
The HI antibody titer variation of every group (log2).

Groups	D_7	D_{14}	D_{21}	D_{28}	D_{35}
EPS_H	4.75 ± 0.50 [a]	7.50 ± 0.99 [a]	7.25 ± 0.95 [a]	7.00 ± 0.82 [ab]	7.00 ± 0.01 [a]
EPS_M	4.50 ± 0.58 [a]	6.00 ± 0.01[ab]	6.75 ± 0.50 [ab]	6.50 ± 0.58 [ab]	6.25 ± 0.50 [b]
EPS_L	4.25 ± 0.50 [a]	5.75 ± 0.96 [b]	6.25 ± 0.50 [ab]	5.85 ± 0.50 [bc]	6.00 ± 0.01 [b]
VC	3.50 ± 0.58 [b]	4.50 ± 0.95 [bc]	5.50 ± 0.58 [c]	5.35 ± 0.01[c]	5.00 ± 0.01 [c]
BC	3.25 ± 0.50 [c]	3.33 ± 0.58 [c]	3.25 ± 0.50[d]	2.75 ± 0.50 [d]	2.25 ± 0.50 [d]

[a-d]Data with in a column without the same superscripts differ significantly($P < 0.05$).

tissues and organs, and could help the relative cells to recognize and eliminate the intrusion pathogens [24]. In this study, the antibody titers in three EPS groups at five testing points were higher than VC group and BC group, and EPS_H group showed significance. These results provided evidences that EPS could enhance the humoral response in chickens. Some researchers also proved that compound polysaccharides from Chinese herbal medicine possessed the best effects on enhancing the antibody titers of chickens [25]. This might imply that EPS could strengthen the humoral immunity of chickens singly or synergistically with the vaccine.

As discussed above, lymphocyte proliferation and cytokine products were crucial events in the cellular immune and humoral immune. Previous researches had reported the modulating capabilities of medicinal polysaccharides on boosting the lymphocyte proliferation and regulating cytokine secretions in vivo [17,24]. Our data showed that the EPS at high and middle doses could significantly increase the

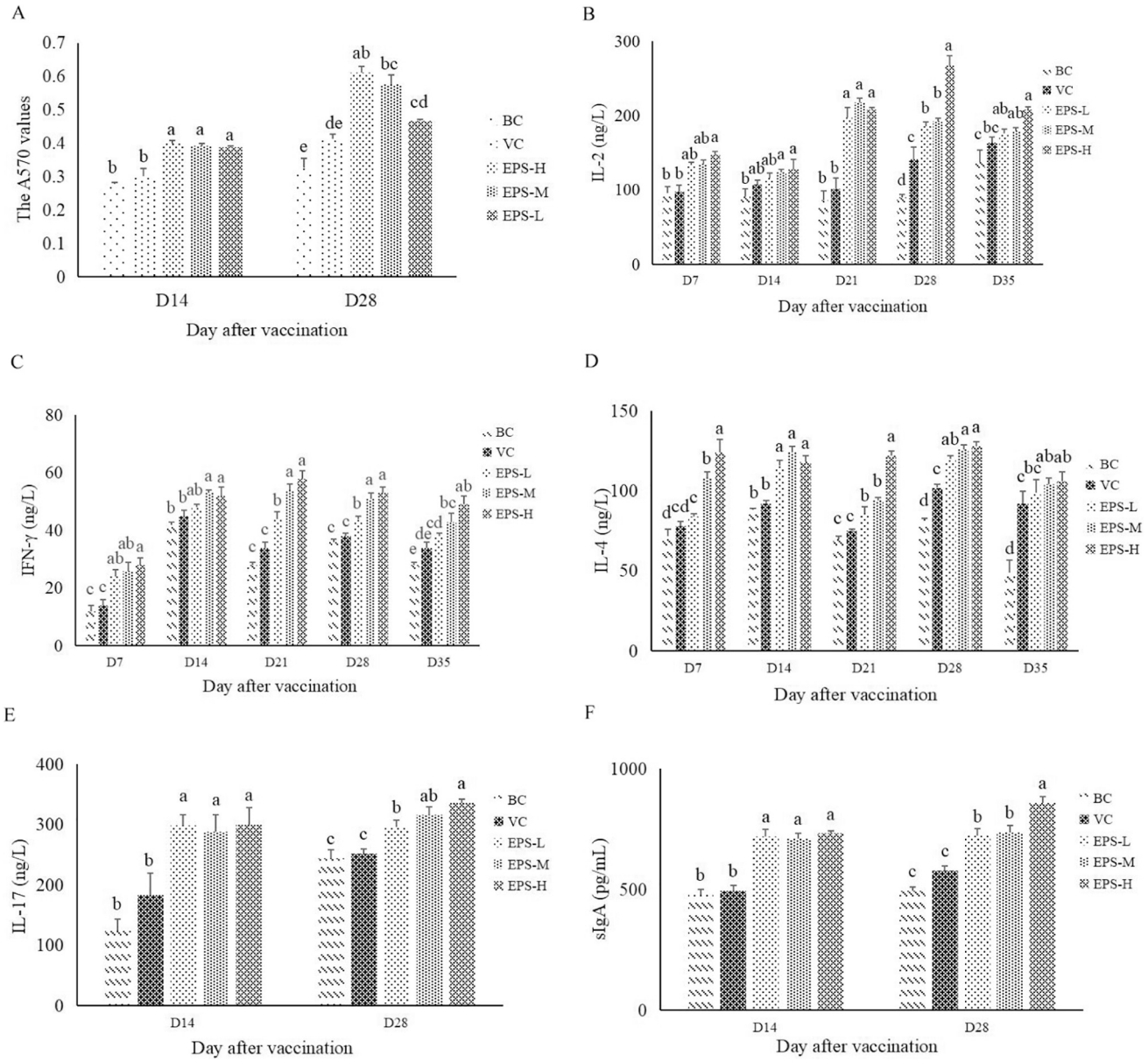


Fig. 7. Effects of EPS-1 on lymphocytes proliferation in vivo (A) cytokines of peripheral serum (B, C and D) and duodenum irrigating solution (E and F). [a-d] Bars without the same superscripts differ significantly ($P < 0.05$).

peripheral lymphocyte proliferation of chickens after the first vaccination. Furthermore, after the first vaccination, EPS of high concentration could significantly prompt the release of IFN-γ and IL-4 at all testing points and enhance the excretion of IL-2 on D_7, D_{21}, D_{28} and D_{35}, meaning that EPS of high dose may have better immune-enhancement effect in vivo. An ideal adjuvant must promote an appropriate cellular and/or humoral immune response [26]. To determine if EPS could allow an ideal adjuvant in chicken, the humoral antibody-mediated immunity as well as the cell-mediated immune response against ND vaccine was evaluated.

Secretory IgA (sIgA) and IL-17 are both important immune modulating products which exist in the duodenum environment. The former has protective functions on the mucous membrane surface, while the latter can induce some immune cells to generate chemotactic factors to prevent infection [11]. This experiment in vivo revealed that orally administered EPS-1 at three doses could significantly improve the immune capacities of duodenum mucous membrane cells. From the experiments in vivo, we can conclude that EPS-1 may have the protective capacity against NDV.

5. Conclusion

To summarize, our study demonstrated that EPS-1 had immune modulating abilities in vitro, such as prompting the proliferation of spleen lymphocytes, regulating the cytokine secretions of spleen lymphocytes and matured chBM-DCs, improving the maturation of chBM-DCs, upgrading the expression of key surface molecules of matured chBM-DCs and reducing the phagocytosis of matured chBM-DCs. Furthermore, EPS-1 could also exhibit immune-enhancement effects in vivo from raising the HI antibody titer, improving the peripheral lymphocyte proliferation, boosting the release of IL-2, IFN-γ and IL-4 in peripheral blood, as well as enhancing the excretion of sIgA and IL-17 in chicken duodenum. Therefore, EPS-1 may have potential prospect in the control of chicken diseases.

Acknowledgements

The work was supported by Discipline Development Grant from College of Animal Sciences FAFU (2018DK007), National Natural Science Foundation of China (NSFC, Grant No. 31402214, 31872514, 31802236), Natural Science Foundation of Fujian Province of China (Grant No. 2016J01697), Scientific research program of outstanding youth of Fujian Agriculture and Forestry University (Grant No. xjq201604), Natural Science Foundation of Jiangsu Province of China (Grant No. BK20130678). We are grateful to all other staff in the Fujian Key Laboratory of Traditional Chinese Veterinary Medicine and Animal Health for their assistance in the experiments.

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