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The immune-adjuvant activity and the mechanism of resveratrol on pseudorabies virus vaccine in a mouse model

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ABSTRACT

Resveratrol had shown various properties before, like immunomodulatory, anti-inflammatory and antiviral activities. Based on these properties, the present study was designed to evaluate the effects and mechanism of resveratrol as an immune-adjuvant for pseudorabies virus (PRV) vaccine. We found that oral administration of resveratrol to mice significantly increased the number of T lymphocytes in the spleen, and elevated the concentrations of antibodies and cytokines in the serum. Resveratrol (30 mg/kg) could enhance phagocytic capacity of peritoneal macrophage (PM) by boosting the percentage of phagocytosis, phagocytic index and the level of lysozyme. Resveratrol also enhanced antigen presentation function of PM by upregulating the expressions of CD86 and MHC-II. Further study revealed that resveratrol could increase the protein levels of TLR4, Ikk, IκBα, NF-κB and JNK when compared with non-adjuvant group. These results provide further insight into the mechanism of action in adjuvant activity of resveratrol, and also offer preclinical evidence for development as a PRV vaccine adjuvant.

1. Introduction

Pseudorabies (PR), also known as Aujeszky's disease, is an acute, high-heat, and high-mortality infectious disease caused by Pseudorabies virus (PRV) that can infect livestock and wild animals [1]. Each outbreak of PR usually results in severe economic losses and has a considerable impact on both national and international trade [2–4].

There are no specific therapeutic drugs for treating animals infected with PRV. Immunization with a safe and effective vaccine is the most important method to prevent PR [5]. New generation vaccines such as recombinant, antigen purified and DNA vaccines are poorly immunogenic due to the lack of an innate immune stimulus [6]. Adjuvants can improve the immunogenicity by exerting its immunomodulatory effect on the response to vaccination [7]. It was reported that commonly-used adjuvants like aluminum hydroxide and oil emulsions induced poor immune response [8,9]. Therefore, search of new vaccine adjuvants has become a topic of interest. Growing evidence has proved that natural compounds have a wide range of acceptability as adjuvant to vaccines. Su et al. [10] evaluated the adjuvant effect of ginsenoside Re, and proved that the ginsenoside Re could enhance the antibody titers in mice immunized with rabies vaccine through activation of cellular and humoral immune responses. Astragalus polysaccharide liposome could enhance the immunity on mice immunized subcutaneously with ovalbumin [11].

Resveratrol (trans-3, 4, 5-trihydroxystilbene; Res) is a natural phenolic compound that mainly presents in grapes and peanuts. It is a kind of phytoalexin that plays an important role in plant defense against pathogen attacks such as fungi and bacteria in nature [12,13]. In the previous study, we found that resveratrol had antiviral, anti-inflammatory and immunomodulatory properties. Our study showed that resveratrol could inhibit the multiplication of PRV in PRV-infected piglets and play a protective function in Rotavirus-infected piglets by reducing the inflammatory response and enhancing the immune function [14,15]. And resveratrol exhibited anti-inflammatory effect by suppressing the signaling cascades of TLR4/NF-κBp65/MAPKs signaling pathway [16,17]. We also reported that resveratrol could promote recovery of immunologic function in immunosuppressive mice by increasing spleen lymphocyte functions and activating JNK/NF-κB

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signaling pathway [18].

Based on these studies, in the present study, we attempt to assess the adjuvant activity of resveratrol for PRV vaccine and the underlying molecular mechanism in mice.

2. Material and methods

2.1. Chemicals

Resveratrol was purchased from Sigma Co., Ltd. (USA). Astragalus polysaccharide was purchased from Aether Center Biology Co., Ltd. (Beijing, China). PRV vaccine was purchased from Harbin Pharmaceutical Group Biological Vaccine Co., Ltd. (Heilongjiang, China; Lot Number: (2016) 080077018).

2.2. Animals

Female BALB/c mice at 6 weeks of age (18–22 g), were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (License No. SCXK (Sichuan) 2015-030). The mice were kept in an environmentally controlled room (23 ± 2 °C, 50% ± 10% humidity) with a 12 h light-12 h dark cycle and fed pathogen-free food and water. The experiments were conducted after acclimating for 7 d. All procedures were performed according to the internationally accepted principles and the China legislation on the use and care of laboratory animals.

The mice were randomly divided into 6 groups (12 mice for each group). Including blank control group (BL), vaccine group (VC), astragalus polysaccharide-treated group (APS), and resveratrol-treated groups (Res_{60}, Res_{30} and Res_{15}; 60, 30 and 15 mg/kg, respectively). All the groups were orally administered with 0.1 mL of solution per 10 g of body weight before each immunization (the BL and VC groups were administered with physiological saline), once daily for 4 d. The mice were subcutaneously immunized with 0.2 mL PRV vaccine on the 5th day (the BL group was subcutaneously injected with physiological saline), and immunized again on the 21th day. On 7 days post second immunization, all the mice were euthanized with ether anesthesia. The following indices were determined.

2.3. Organ index assay

Thymus and spleen tissues were separated and weighed. Organ index was calculated: organ index = organ weight (mg)/body weight (g).

2.4. Splenic lymphocyte proliferation assay

The spleen lymphocyte suspension (2.5×10^6 cells/mL) was prepared in RPMI-1640 media. The cell suspension was added to 96-well plate and stimulated with ConA (5 μg/mL). The control was added with RPMI-1640 and the blank was added with only CCK-8, respectively. Cells were cultured at 37 °C with 5% CO_2 for 22 h, and then 10 μL of CCK-8 was added to each well and incubation was continued for 2 h. The absorbance of each well was determined at 450 nm on the plate reader (Hercules, USA). The stimulation index (SI) was calculated according to the following equation:

$$SI = (\text{experimental well OD} - \text{blank well OD}) / (\text{control well OD} - \text{blank well OD}) \times 100\%.$$

2.5. T lymphocyte subsets assay

The blood samples were collected into EDTA-preservative tube. Then, CD3+, CD4+ and CD8+ were measured by flow cytometry (Backman, USA).

Table 1
Primer sequences for PCR.

Gene	Primer sequences Fragment size (5′ > 3′) (forward/reversed)
IL-2	Forward:5′-CGGCATGTTCTGGATTTGACT-3′ Reverse:5′-CCATCTCCTCAGAAAGTCCACC-3′
IL-4	Forward:5′-TTGAACGAGGTCACAGGAGAAGG-3′ Reverse:5′-CCTTGGAAGCCCTACAGACGAG-3′
IL-10	Forward:5′-GCTCTTACTGACTGGCATGAG-3′ Reverse: 5′-CGCAGCTCTAGGAGCATGTG-3′
IFN-γ	Forward:5′-CAGCAACAGCAAGGCGAA-3′ Reverse:5′-CTGGACCTGTGGGTTGTTGAC-3′
CD80	Forward:5-GCTATGGCTTGCAATTGTCAGT-3′ Reverse:5′-ACGGCAAGGCAGCAATACC-3′
CD86	Forward:5-CCAGTGTGTGTCTCAATGGATGT-3′ Reverse:5′-AAGCCATGAGCTGAAAGAGTCCTA-3′
β-actin	Forward:5′-TGGCATTGTTACCAACTGGGAC-3′ Reverse:5′-TCACGGTTGGCCTTAGGGTTC-3′

Table 2
The indexs of spleen and thymus.

Group	Spleen	Thymus
BL	4.91 ± 1.13	1.57 ± 0.37
VC	5.03 ± 0.78	1.72 ± 0.59
APS	5.12 ± 0.62	2.06 ± 0.55
Res_{15}	5.10 ± 0.39	1.82 ± 0.77
Res_{30}	5.19 ± 0.81	2.63 ± 1.41*
Res_{60}	5.00 ± 0.98	2.03 ± 1.05

BL, blank control group; VC, vaccine group, saline plus pseudorabies vaccine; APS, astragalus polysaccharide plus pseudorabies vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus pseudorabies vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus pseudorabies vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus pseudorabies vaccine treated group.
Data was represented as means ± SD; n = 8.
* $P < 0.05$ vs. BL group.

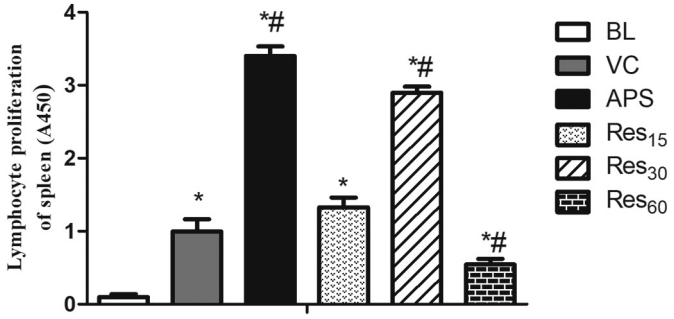

Fig. 1. Effect of resveratrol on proliferation of lymphocyte in spleen (A_{450}).
BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Bars are expressed as mean ± SD, $n = 6$; *$P < 0.05$ vs. BL group; #P < 0.05 vs.VC group.

2.6. Antibodies assay

The blood samples were collected into the tubes without anticoagulant. After coagulation, the serum was separated by centrifugation at 3000 rpm for 10 min after coagulation. The concentrations of IgG, IgG1, IgG2a and IgG2b in serum were detected by ELISA kit (Shanghai Mlbio, China).

2.7. Cytokines assay

The mRNA levels of cytokines were tested by reverse transcription-

Table 3
The T lymphocyte subsets of mice.

Group	CD3 +	CD4 +	CD8 +	CD4 + /CD8 +
BL	28.38 ± 7.16	24.33 ± 9.36	25.64 ± 5.56	0.93 ± 0.16
VC	29.12 ± 6.17	22.90 ± 6.28	20.41 ± 5.34	1.13 ± 0.15
APS	40.42 ± 4.80	32.74 ± 4.71	29.47 ± 3.52	1.11 ± 0.04
Res_{15}	34.99 ± 9.45	29.73 ± 9.70	28.85 ± 8.16	1.02 ± 0.04
Res_{30}	36.57 ± 1.74	32.09 ± 3.90	33.40 ± 1.71$^{\#}$	0.96 ± 0.08
Res_{60}	26.06 ± 9.42	23.30 ± 10.01	21.59 ± 9.65	1.09 ± 0.10

BL, blank control group; VC, vaccine group, saline plus pseudorabies vaccine; APS, astragalus polysaccharide plus pseudorabies vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus pseudorabies vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus pseudorabies vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus pseudorabies vaccine treated group.
Data was represented as means ± SD; n = 3.
$^{\#}$ $P < 0.05$ vs.VC group.

polymerase chain Reaction (RT-PCR). Total RNA from spleen were extracted by the Total RNA Kit (No. R6934-01; OMEGA, USA) according to the manufacturer's protocol. Then, cDNA (1 μL) of each sample was used for RT-PCR with SYBR Green Supermix kit (No. 1725124; Bio-Rad, USA) on a Bio-Rad CFX Connect™ Real-Time PCR System (CA, USA). The relative amount of mRNA was evaluated by $2^{-\Delta\Delta Ct}$ method. Primers were synthesized by Huada genomics technology Co., LTD (Beijing, China). The primers were listed in Table 1.

The levels of IL-2, IL-4, IL-10 and IFN-γ in serum were determined by ELISA kits according to the manufacturer's protocol (Shanghai Mlbio, China).

2.8. Phagocytosis assay

The animals were intraperitoneally injected with 1 mL of 5% chicken red blood cell suspension. Then, a drop of peritoneal fluid was stained with Wright's stain with an oil immersion lens, and the percentage of phagocytic cells (PP) and phagocytic index (PI) were calculated according to the following formula:

PP
= (number of macrophages that engulf chicken red blood cells in 100 macrophages/100 macrophages) × 100%.

PI
= (number of chicken red blood cells phagocytized by macrophages /100 macrophages) × 100%

2.9. Lysosomal enzyme assay

The peritoneal macrophages were extracted from mice, and cultured in 6-well plate in a humid atmosphere with 5% CO_2 at 37.5 °C. After incubation for 4 h, non-adherent cells were removed by washing twice slightly, and then the adherent cells were macrophages. Cell viability determined by trypan blue exclusion assay was not < 95%.

The macrophages were cultured in a 6-well cell plate. After 48 h incubation, the plate was centrifuged at 3000 rpm for 10 min. Then, the supernatant was collected to analyze the levels of lysozyme and acid phosphatase by ELISA kits (Shanghai Mlbio, China).

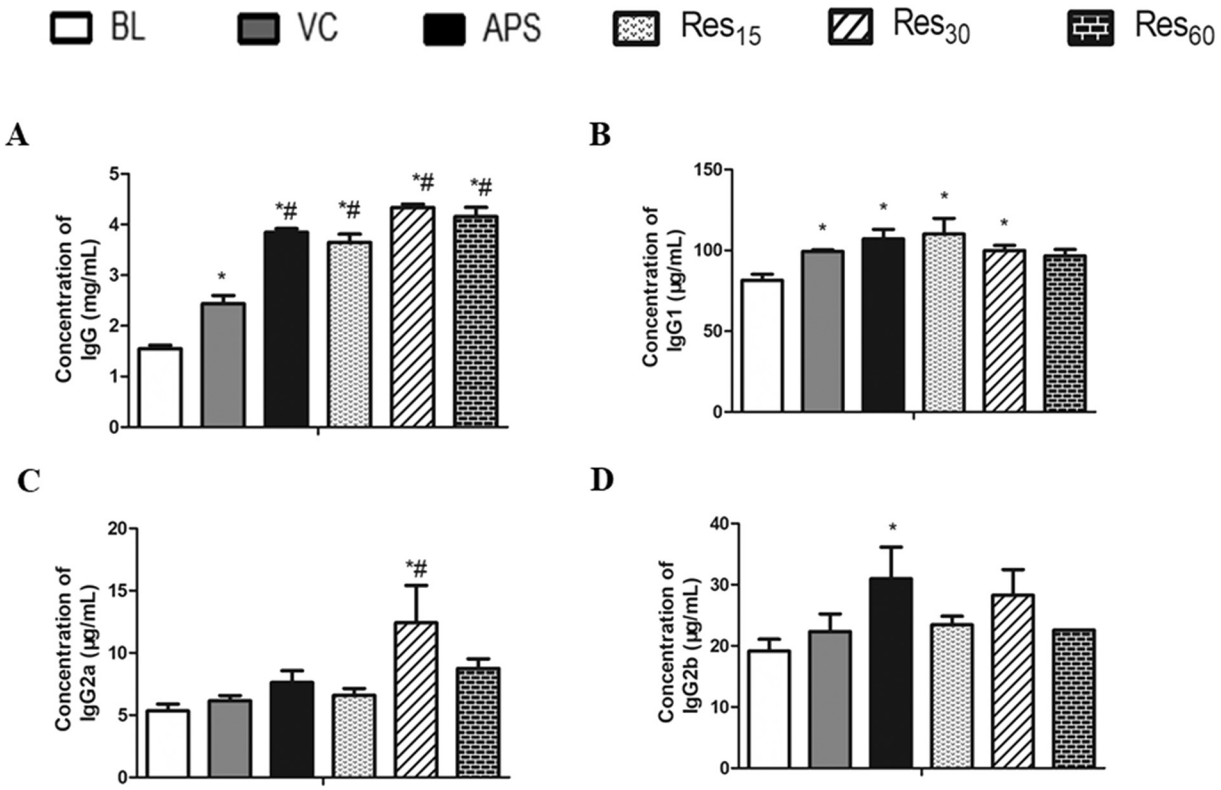

Fig. 2. Effects of resveratrol on the changes in IgG, IgG1, IgG2a and IgG2b concentration. (A) Concentration of IgG; (B) Concentration of IgG1; (C) Concentration of IgG2a; (D) Concentration of IgG2b. BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Bars are expressed as mean ± SD, n = 6; *$P < 0.05$ vs. BL group; $^{\#}P < 0.05$ vs.VC group.

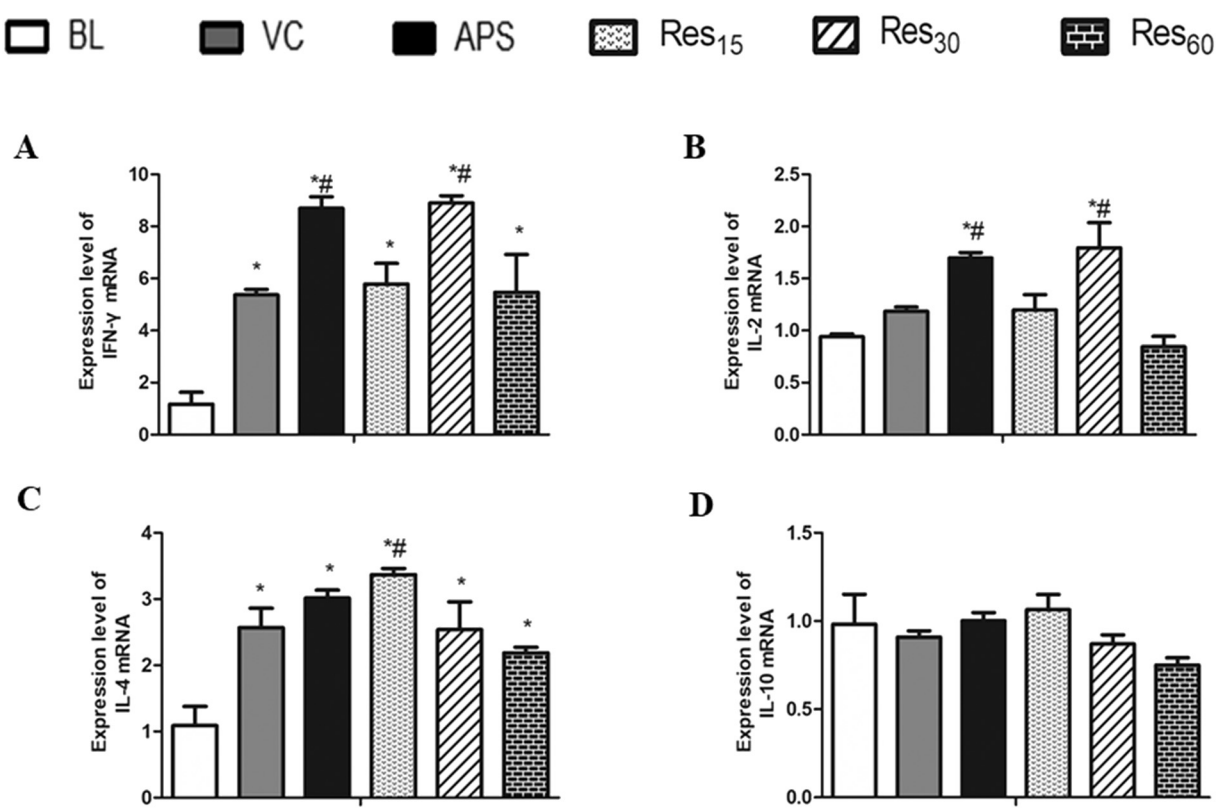

Fig. 3. mRNA levels of IFN-γ, IL-2, IL-4 and IL-10 in spleen by RT-PCR. (A) Level of IFN-γ; (B) Level of IL-2; (C) Level of IL-4; (D) Level of IL-10. BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Bars are expressed as mean ± SD, n = 6; *P < 0.05 vs. BL group; #P < 0.05 vs.VC group.

2.10. Antigen presentation function assay

The macrophages of each group were collected as described above. Then, the expressions of CD80 and CD86 were detected by RT-PCR. The primers were listed in Table 1.

The levels of CD80, CD86 and MHC-II were measured by flow cytometry (Backman, USA).

2.11. Western blotting assay

The visceral protein was extracted by the mammalian tissue protein extraction kit (No. AR0101; Boster, China) and the mixture was boiled at 97 °C for 6 min. The concentrations were determined by the BCA protein assay kit (No. PC0020; Solarbio, China). Equivalent amounts of protein (30 μg) from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Protein blots were blocked with 5% skim milk at room temperature for 1 h, and then incubated with primary antibodies against TLR4, IKK, IκBα, NF-κBp65 and JNK overnight at 4 °C. The protein blots were exposed to the relative peroxidase-conjugated secondary antibody (Goat anti-mouse or goat anti-rabbit, CST, USA) for 1 h at room temperature. Immunoreactive bands were visualized with enzymatic chemiluminescence (ECL prime, Pierce Chemical, USA) and quantified relative to β-actin (CST, USA) using Image J software.

2.12. Statistical analysis

The data were presented as the mean ± standard deviation, and all the statistical analyses were performed using SPSS 22.0 software. All the histograms experiments were plotted by GraphPad Prism 5.0 software. Statistical significance of the data from the control and experimental groups was compared by one-way analysis of variance (ANOVA) and the Least Significant Difference test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Organ index

The effect of resveratrol on the organ index was listed in Table 2. Compared with the blank control and vaccine groups, the spleen and thymus indices were increased in the treated groups. Especially the thymus index was significantly increased in the Res_{30} group as compared to the blank control group ($P < 0.05$).

3.2. Splenic lymphocyte proliferation assays

The lymphocyte proliferation response to treatment with ConA was shown in Fig. 1. Compared with the blank control group, lymphocyte proliferation was significantly increased in all vaccinated groups ($P < 0.05$).

Compared with the vaccine group, lymphocyte proliferation was significantly increased in the APS and Res_{30} groups ($P < 0.05$).

3.3. Percentage of T lymphocyte subsets

The percentage of T lymphocyte was shown in Table 3. Compared with the blank control group, the percentages of T lymphocytes, including CD3+, CD4+, CD8+ and CD4+/CD8+ did not show any differences in all vaccinated groups ($P > 0.05$).

The percentages of CD4+ and CD8+ had the varying degree of

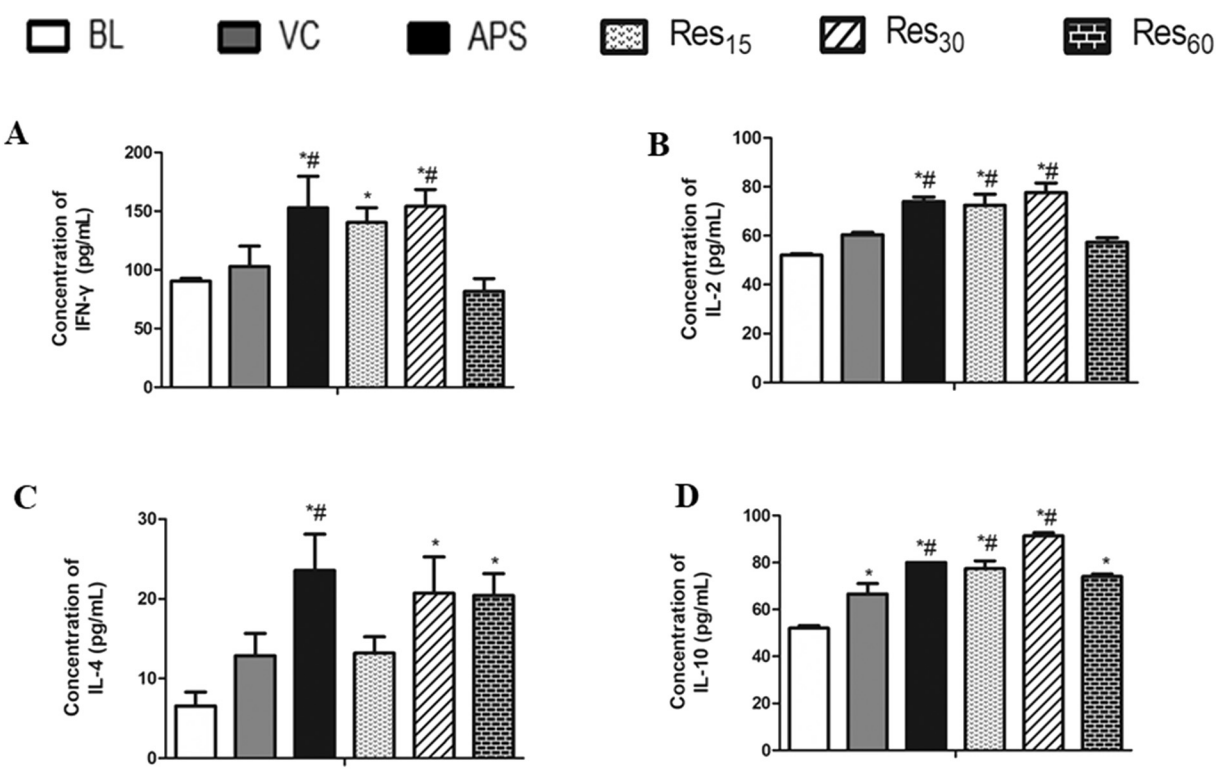

Fig. 4. Effects of resveratrol on the changes in IFN-γ, IL-2, IL-4 and IL-10 concentrations. (A) Concentration of IFN-γ; (B) Concentration of IL-2; (C) Concentration of IL-4; (D) Concentration of IL-10. BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Bars are expressed as mean ± SD, n = 6; *P < 0.05 vs. BL group; #P < 0.05 vs.VC group.

Table 4
The effect of resveratrol on phagocytic function of macrophages.

Group	PP	PI
BL	20.33 ± 2.52	6.33 ± 1.53
VC	36.00 ± 1.73*	12.33 ± 2.52*
APS	53.67 ± 1.53*,#	23.67 ± 3.21*,#
Res_{15}	37.00 ± 3.61*	13.00 ± 2.65*
Res_{30}	62.67 ± 3.06*,#	26.00 ± 2.00*,#
Res_{60}	23.00 ± 4.36#	7.00 ± 3.46#

PP, percentage of phagocytosis; PI, phagocytic index; BL, blank control group; VC, vaccine group, saline plus pseudorabies vaccine; APS, astragalus polysaccharide plus pseudorabies vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus pseudorabies vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus pseudorabies vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus pseudorabies vaccine treated group.
Data was represented as means ± SD; n = 4.
* P < 0.05 vs. BL group.
# P < 0.05 vs. VC group.

increase in the treated groups, especially the CD8+ was significantly increased in the Res_{30} group as compared to the vaccine group ($P < 0.05$).

3.4. Effects of resveratrol on specific IgG antibodies and its subclasses (IgG1, IgG2a, IgG2b)

As shown in Fig. 2, compared with the blank control group, the IgG and IgG1 concentrations were significantly increased in all vaccinated groups ($P < 0.05$), except the IgG1 in Res_{60} group (Fig. 2A and B). The IgG2a and IgG2b concentrations were increased, especially the IgG2a in the Res30 group and IgG2b in the APS group ($P < 0.05$; Fig. 2C and D).

Compared with the vaccine group, The IgG concentration was significantly higher in all treated groups (P < 0.05; Fig. 2A). The IgG1 and IgG2b concentrations showed no significant differences in all treated groups ($P > 0.05$; Fig. 2B and D). IgG2a concentration was significantly increased in the Res_{30} group ($P < 0.05$), but no significant difference was observed among other treated groups as compared to the vaccine group (P > 0.05; Fig. 2C).

3.5. Cytokine assay

The mRNA expressions of cytokines in spleen were shown in Fig. 3. The mRNA levels of IFN-γ and IL-4 were significantly increased in all vaccinated groups as compared to the blank control group ($P < 0.05$; Fig. 3A and C). The mRNA levels of IFN-γ and IL-2 were significantly increased in the APS and Res_{30} groups in comparison with vaccine group ($P < 0.05$; Fig. 3A and B). The mRNA level of IL-4 was considerably increased in the Res_{15} group ($P < 0.05$), but no significant differences were observed in the APS, Res_{30} and Res_{60} groups in comparison with the vaccine group ($P > 0.05$); Fig. 3C). The mRNA level of IL-10 showed no significant difference in all groups (P > 0.05; Fig. 3D).

The concentrations of cytokines in serum were test by ELISA, and the results were shown in Fig. 4. Compared to the blank control group, the IFN-γ concentration was significantly increased in the APS, Res_{15} and Res_{30} groups ($P < 0.05$; Fig. 4A) and the IL-4 concentration was significantly increased in the APS, Res_{30} and Res_{60} groups ($P < 0.05$; Fig. 4C). The secretion of IFN-γ was significantly increased in the APS and Res_{30} groups in comparison with the vaccine group ($P < 0.05$; Fig. 4A). Compared with the blank control and vaccine groups, the secretion of IL-2 was significantly increased in the APS, Res_{15} and Res_{30} groups (P < 0.05; Fig. 4B). The IL-10 concentration was significantly increased in all vaccinated groups as compared to the blank control group (P < 0.05), and it was considerably upregulated in the APS,

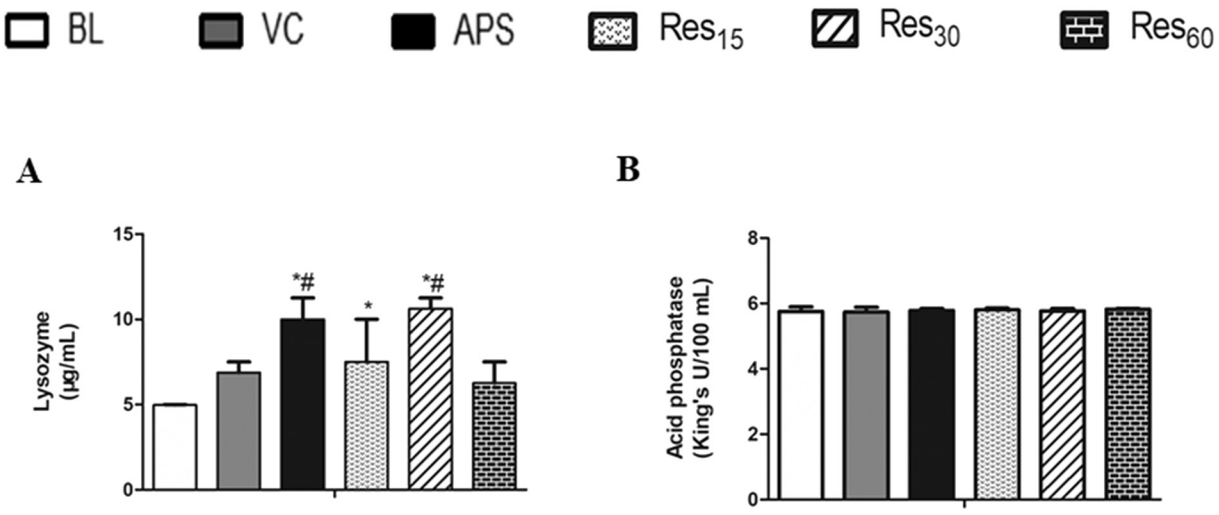

Fig. 5. Effects of resveratrol on release of lysosomal enzymes by macrophages. (A) Level of lysozyme; (B) Level of acid phosphatase. BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Bars are expressed as mean ± SD, n = 6; $^{*}P < 0.05$ vs. BL group; $^{\#}P < 0.05$ vs.VC group.

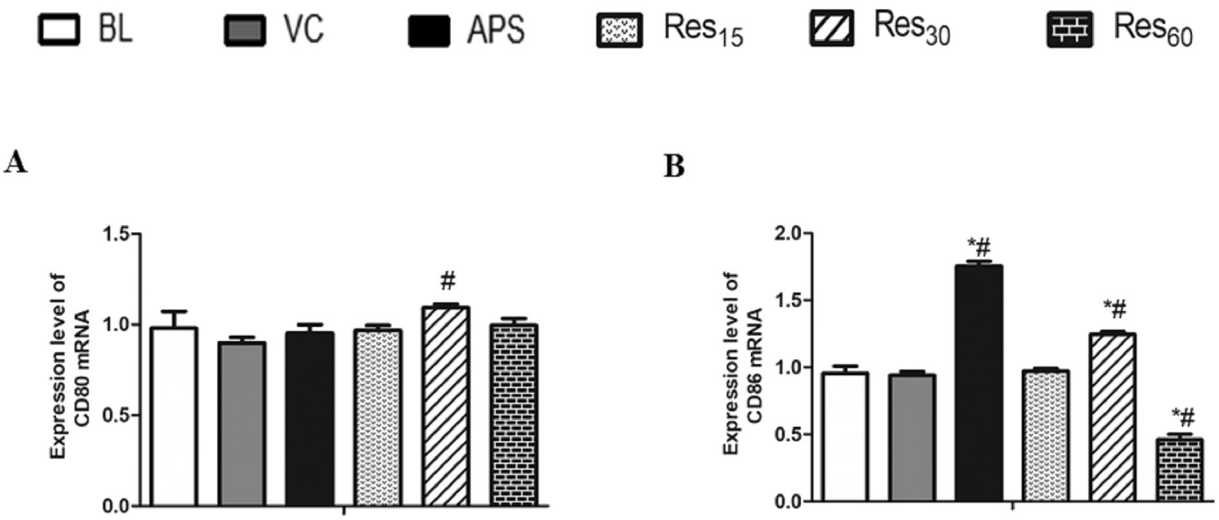

Fig. 6. Effects of resveratrol on mRNA expressions of CD80 and CD86 in macrophage. (A) Level of CD80; (B) Level of CD86. BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Bars are expressed as mean ± SD, n = 6; $^{*}P < 0.05$ vs. BL group; $^{\#}P < 0.05$ vs.VC group.

Table 5
Expressions of CD80, CD86 and MHC-II measured by flow cytometry.

Group	CD80	CD86	MHC-II
BL	16.03 ± 1.20	2.13 ± 1.07	5.10 ± 0.53
VC	39.21 ± 2.22*	7.33 ± 0.92*	15.50 ± 0.35*
APS	42.19 ± 3.26*	12.18 ± 3.41*,#	23.42 ± 1.01*,#
Res_{15}	37.82 ± 1.91*	10.58 ± 2.05*	15.40 ± 1.44*
Res_{30}	24.18 ± 5.92*,#	32.10 ± 2.01*,#	35.52 ± 5.63*,#
Res_{60}	32.13 ± 1.69*,#	16.62 ± 0.61*,#	22.41 ± 3.00*,#

BL, blank control group; VC, vaccine group, saline plus PRV vaccine; APS, astragalus polysaccharide plus PRV vaccine treated group; Res_{15}, resveratrol 15 mg/kg plus PRV vaccine treated group; Res_{30}, resveratrol 30 mg/kg plus PRV vaccine treated group; Res_{60}, resveratrol 60 mg/kg plus PRV vaccine treated group.
Data are represented as means ± SD; n = 3.
* P < 0.05 vs. BL group.
# P < 0.05 vs.VC group.

Res_{15} and Res_{30} groups in comparison with the vaccine group ($P < 0.05$; Fig. 4D).

3.6. Effect of resveratrol on phagocytic function of macrophages

As shown in Table 4, compared with the blank control group, both PP and PI were significantly higher in all groups ($P < 0.05$), except for the Res_{60} group. The PP and PI were significantly increased in the APS and Res_{30} groups in comparison with the vaccine group ($P < 0.05$), but there were no significant differences between the Res_{15} group and Res_{60} group ($P > 0.05$). It is noteworthy that the Res_{30} group showed the highest values of PP and PI as compared to the rest groups.

3.7. Effects of resveratrol on release of lysosomal enzymes by macrophages

The effect of resveratrol on lysozyme release by macrophages was shown in Fig. 5A. Compared with the blank control group, the concentrations of lysozyme were slightly increased in the vaccine and Res_{60}

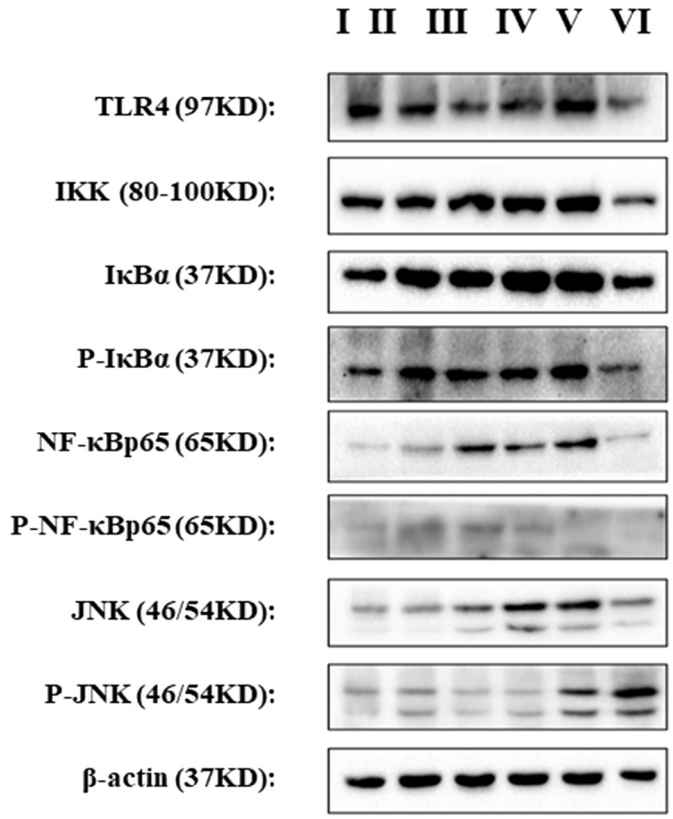

Fig. 7. Protein and phosphorylated protein levels of TLR4, IKK, IκBα, NF-κBp65 and JNK in spleen. I, II, III, IV, V, and VI represent the groups of blank control, vaccine, APS, Res_{15}, Res_{30}, and Res_{60}, respectively.

groups, but it was significantly increased in the APS, Res_{15} and Res_{30} groups ($P < 0.05$). In comparison with the vaccine group, the content of lysozyme was not significantly different in the Res_{15} and Res_{60} groups ($P > 0.05$), but significantly upregulated in the APS and Res_{30} groups ($P < 0.05$).

The result of resveratrol on acid phosphatase release by macrophages was shown in Fig. 5B. There were no significant changes in all groups ($P > 0.05$).

3.8. Effect of resveratrol on antigen presentation function of macrophages

The mRNA expressions of CD80 and CD86 were shown in Fig. 6. Compared with the blank control group, the expression of CD80 mRNA was not significantly upregulated in all vaccinated groups ($P > 0.05$; Fig. 6A). In comparison with the vaccine group, CD80 expression was significantly increased in the Res_{30} group ($P < 0.05$; Fig. 6A). Compared with the blank control and vaccine groups, CD86 expression was significantly increased in the APS and Res_{30} groups ($P < 0.05$; Fig. 6B).

The expressions of CD80, CD86 and MHC-II measured by flow cytometry were shown in Table 5. Compared with the blank control group, the levels of CD80, CD86 and MHC-II were significantly increased in all vaccinated groups ($P < 0.05$). In comparison with the vaccine group, CD80 expression was significantly down-regulated in the Res_{30} and Res_{60} groups ($P < 0.05$), and no significant differences were observed between the APS and Res_{15} groups ($P > 0.05$). Compared with the vaccine group, the CD86 and MHC-II expressions were significantly increased in the treated groups, except for the Res_{15} group ($P < 0.05$).

3.9. Effects of resveratrol on TLR4/NF-κBp65/JNK signaling pathways

The expressions of related proteins of TLR4/NF-κBp65/JNK signaling pathways in spleen were showed in Figs. 7 and 8. The protein levels of IκBα, NF-κBp65, JNK, and their phosphorylated protein were significantly increased in the vaccine group ($P < 0.05$; Fig. 8C, D and E), while there were no significant differences in TLR4 and IKK protein levels ($P > 0.05$; Fig. 8A and B), when compared with the blank control group.

Compared with the vaccine group, the protein expression of TLR4 in the treated groups were decreased, except the protein level in Res_{30} group was significantly upregulated ($P < 0.05$; Fig. 8A). The expression of IKBα was significantly upregulated in the Res_{15} and Res_{30} groups as well as its phosphorylated protein in the Res_{30} group (Fig. 8C). The expressions of IKK and NF-κBp65 were significantly higher in the APS, Res_{15} and Res_{30} groups ($P < 0.05$; Fig. 8B and D), but with the increasing concentration of resveratrol, the levels of these proteins were significantly decreased ($P < 0.05$). The expression of JNK was significantly increased in the treated groups ($P < 0.05$), and the level of its phosphorylated protein was significantly increased in the Res_{30} and Res_{60} groups (Fig. 8E).

4. Discussion

Based on the immunomodulatory functions of resveratrol, this study systematically evaluated the effect of resveratrol on the immune response to PRV vaccine by various parameters. Astragalus polysaccharide is a commonly used immunopotentiator and has also been reported to possess adjuvant activity [19]. Therefore, it was selected as positive control. In this study, resveratrol could enhance the immune response to PRV vaccine in mice, which was better than APS.

Spleen and thymus are the main immune organs that reflect the growth and development of animals. Spleen is the largest immune organ and thymus is closely related to the development, differentiation and maturation of T lymphocytes [20,21]. In this study, resveratrol had no significant effect on spleen and thymus indices, suggesting that resveratrol does not primarily work through the action of immune organs, which was similar to the previous study [22].

The proliferation of lymphocyte could reflect the cellular immune function [23]. This study showed that the splenic lymphocyte proliferation was increased significantly under ConA stimulation in resveratrol-treated mice (15 and 30 mg/kg), but the proliferation of spleen lymphocyte was decreased with a high dose of resveratrol treatment (60 mg/kg), which was similar to the previous study that resveratrol exhibited bidirectional regulation of splenic lymphocyte proliferation [24].

The CD3+ lymphocytes represent total T lymphocytes, including helper/inducible T lymphocytes (CD3+CD4+) and suppressor/cytotoxic T lymphocytes (CD3+CD8+). Different T lymphocytes act on different cellular immune responses. The normal expression of CD4+ T cells can promote the proliferation and differentiation of T cells, B cells and other immune cells, whereas CD8+ T cells can target and kill cells expressing specific antigens [25,26]. In our study, the percentages of CD4+ and CD8+ T cells were increased to different extents in the treated groups, suggesting that resveratrol is available for enhancing the immune efficacy via upregulating T lymphocyte subsets. These results indicated that resveratrol as an adjuvant could stimulate cellular immune response.

A good adjuvant could stimulate the body's appropriate immune response. Th1 cells induce cellular immunity and Th2 cells induce humoral immunity [27]. In mice, the productions of IgG2a and IgG2b are a prominent feature of the Th1 response, while the productions of IgG and IgG1 are a prominent feature of the Th2 response [28]. Our results showed significant increases in IgG and IgG2a antibodies, suggesting that resveratrol could induce Th1 and Th2 immune responses. Different types of T cells secrete different cytokines [29]. Th1 cells secrete IFN-γ and IL-2, and Th2 cells secrete IL-4 and IL-10. So, analysis of cytokines provided direct valuation of T cell activation. In our study, the levels of IFN-γ, IL-2 and IL-10 were significantly increased with resveratrol-

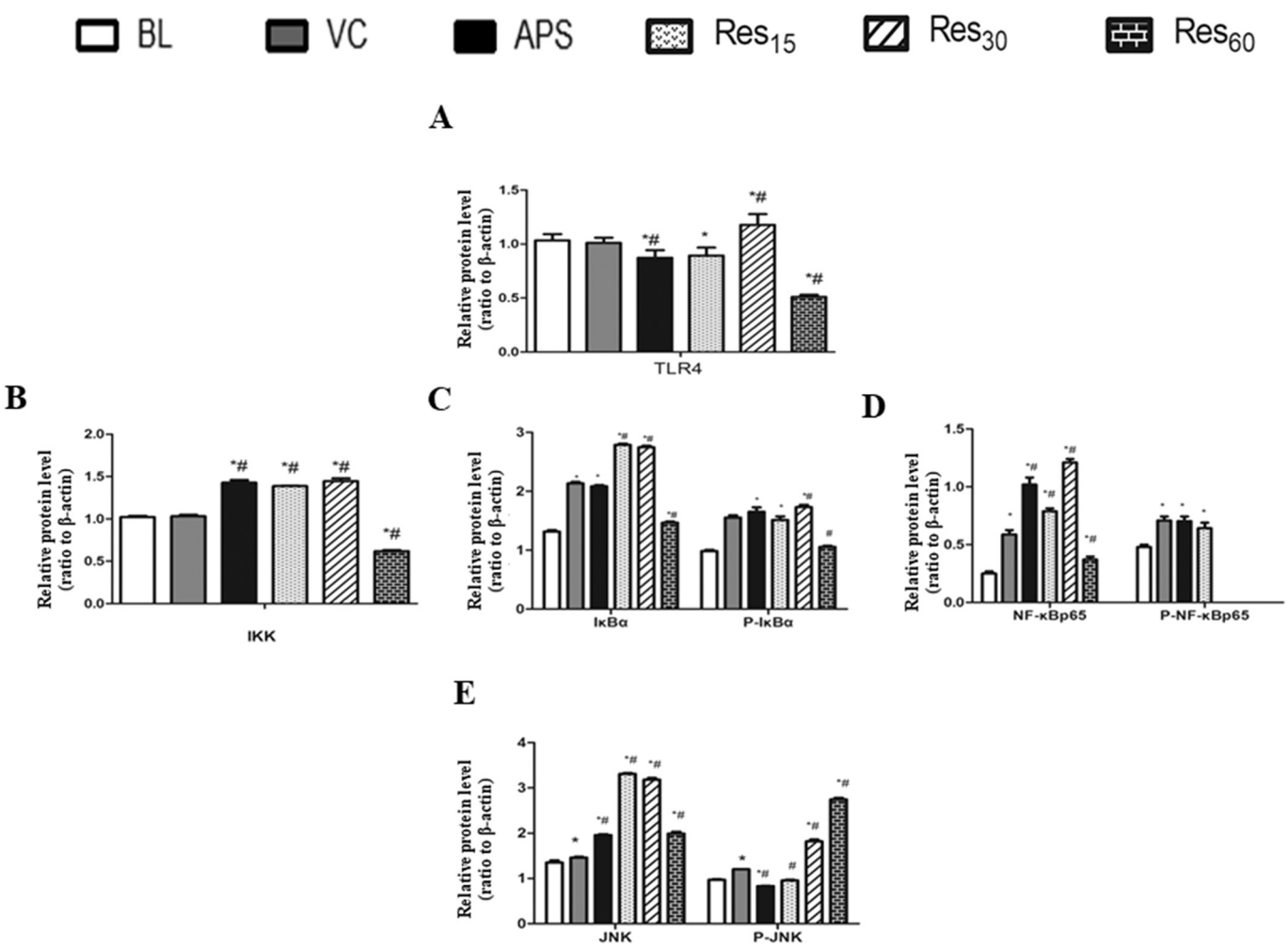

Fig. 8. Densitometric analysis of proteins and phosphorylated proteins in spleen. (A) Relative protein level of TLR4; (B) Relative protein level of IKK; (C) Relative protein and phosphorylated protein levels of IκBα; (D) Relative protein and phosphorylated protein levels of NF-κBp65; (E) Relative protein and phosphorylated protein levels of JNK.
β-actin served as a loading control. Bars are expressed as mean ± SD of three animals; *P < 0.05 vs. BL group; #P < 0.05 vs. VC group.

treatment (30 mg/kg), demonstrating the activation of Th1 and Th2 cells. In conclusion, resveratrol used as an adjuvant could induce mixed Th1/Th2 response.

Macrophages are involved in specific and non-specific immunity and have at least three major functions: phagocytosis, antigen presentation and immunomodulation [30]. Invading pathogens were engulfed by macrophages, and then the intracellular vesicles were formed to protect the cells themselves from being damaged. Then the secondary lysosomes were formed by fusion of intracellular vesicles with lysosomes. Finally, lysosomal enzymes degraded the antigenic proteins into polypeptides under acidic conditions and present the polypeptides on the cell surface [31]. As professional antigen-presenting cell, macrophages present antigens to relevant sites for immune function, and MHC-II, CD80 and CD86 are important in inducing immune responses [32]. In our study, a medium dose of resveratrol (30 mg/kg) can significantly enhance the PP, PI and lysozyme content, suggesting that resveratrol could enhance the phagocytosis function. Further study showed that the expressions of CD86 and MHC-II were increased, suggesting that resveratrol could enhance the ability of antigen presentation in vaccinated mice.

TLR4-mediated signal pathway activates the immune-related signaling pathways, such as NF-κB and MAPKs. JNK pathway is one of the MAPK pathways, which shows an important role in the cellular immune response as well as the stress response [33]. So, the TLR4/NF-κB/JNK signaling pathways are known as pivotal links to immune responses. In this study, significantly increased level of TLR4 was observed, and the protein levels of Ikk, IκBα, NF-κB, and JNK proteins were higher in the resveratrol-treated groups. These results indicated that the adjuvant effect of resveratrol was exerted through activation of TLR4/NF-κB/JNK signaling pathways. In addition, JAK/STAT signaling pathways are essential for the development and function of both innate and adaptive immunity [34]. To fully understand of the mechanism, further study should be conducted to evaluating whether the JAK/STAT pathways were regulated by resveratrol.

Vaccines are the main prophylactic measure against human viral infections, and a vast number of doses will be needed to meet the worldwide demand [35]. So, we should supply sufficient antigen to meet the pandemic demand. The use of adjuvant to improve immunogenicity is a crucial antigen-sparing strategy. It has been demonstrated that resveratrol had immunomodulatory property. The previous study showed that resveratrol could induce stimulation of the human immune cell response [36]. It is revealed that resveratrol significantly increased the number of circulating T cell subsets in healthy volunteers and decreased plasma levels of proinflammatory cytokines, suggesting that it could be used for modulating the response of the immune system in various pathological situations [37–39]. Therefore, with the adjuvant activity for PRV vaccine, resveratrol exhibits the possibility for enhancing immune responses to human vaccines.

5. Conclusion

Resveratrol could enhance immune response to PRV vaccine. The mechanisms may attribute to the enhancement of phagocytic function and antigen presentation function of macrophages and activation of TLR4/NF-κB/JNK signaling pathways. Resveratrol could be considered as an effective adjuvant with promising application prospect.

Declaration of competing interest

There is no conflict of interest.

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