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Adjuvant activity of PCP-II, a polysaccharide from *Poria cocos*, on a whole killed rabies vaccine

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ABSTRACT

Adjuvants are important components of vaccination strategies because they boost and accelerate the immune response. The aim of this study was to investigate the adjuvant activity of PCP-II, a polysaccharide isolated from *Poria cocos*, together with an inactivated rabies vaccine. The polysaccharide PCP-II was compared with the common veterinary rabies vaccine adjuvant Alhydrogel by co-administration of either adjuvant with the inactivated rabies virus rCVS-11-G to mice via the intramuscular route. Blood samples were collected to determine the virus-neutralizing antibody (VNA) titer and assess activation of B and T lymphocytes. Inguinal lymph node samples were collected, and proliferation of B lymphocytes was measured. Splenocytes were isolated, and antigen-specific cellular immune responses were evaluated by enzyme-linked immunospot and immunosorbent assays (ELISpot assay and ELISA, respectively). The results showed that PCP-II enhanced and promoted an increase in the VNA titer in the mice compared to Alhydrogel. Flow cytometry assays revealed that the polysaccharide activated more B lymphocytes in the lymph nodes and more B and T lymphocytes in the blood. Assessment of antigen-specific cellular immune responses showed that PCP-II strongly induced T lymphocyte proliferation in the spleen and high levels of cytokine secretion from splenocytes. All of these data suggest that PCP-II possesses excellent adjuvant activity and enhances both cellular and humoral immunity in mice.

After examining the adjuvant activities of PCP-II in mice, dogs were immunized with rCVS-11-G together with Alhydrogel or PCP-II as an adjuvant; the control group was injected with a commercial rabies vaccine. Serum samples were collected, and the VNA titers were measured. PCP-II caused increases in the VNA titers in both the booster and single-dose immunization tests when co-administered with rCVS-11-G compared with Alhydrogel. The VNA titer of the commercial vaccine group was also significantly lower than that of the PCP-II group. These data indicate that PCP-II is an excellent candidate adjuvant for inactive rabies vaccines in the veterinary setting.

1. Introduction

Rabies is a fatal, non-treatable encephalomyelitis caused by a single-stranded RNA virus; all warm-blooded animals, including humans, are susceptible to this disease (Fu, 1997; Dietzschold, Schnell et al. 2005; Dietzschold, Li et al. 2008). Rabies has the highest mortality rate of all known diseases (World Health Organization, 2013). According to a report by the World Health Organization, approximately 55,000 people die of rabies each year worldwide (Knobel and Cleaveland et al., 2005), and in China, 1425 people died of rabies in 2012 (Zhou and Li et al., 2015). Among all countries, China has the second greatest number of rabies-related deaths. Because the lethality of rabies is nearly 100% once clinical signs appear, the best way to prevent rabies is inoculation with a vaccine (Dietzschold, Schnell et al. 2005). Rabies is predominantly transmitted via bites and scratches by infected animals, especially dogs (World Health Organization, 2005). Previous studies have shown that domestic dogs are responsible for more than 95% of human rabies cases (Hu and Fooks et al., 2008) and that immunization of > 70% of all domesticated dogs is sufficient for preventing rabies epidemics and transmission of the rabies virus to humans (Charlton, 1992; Davlin and Vonville, 2012). Most of the commercial rabies vaccines for animal use in China are inactivated cell culture vaccines.

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Although these whole killed rabies virus vaccines are safe for use and easy to store, their efficiency is low, and multiple injections are needed to ensure that the virus-neutralizing antibody (VNA) titer reaches above 0.5 IU/ml, which is the most reliable indicator that vaccination will prevent rabies infection (Mittal, 2013; Nguyen and Nguyen et al., 2015). Multiple previous studies showed that more than 30% of the dogs failed to reach this threshold antibody titer after receiving only one dose of commercial inactivated rabies vaccine (Sage and Khawplod et al., 1993; Takayama, 1998; Ondrejkova A, 2002; Minke and Bouvet et al., 2009; Van de Zande and Kaashoek et al., 2009). Therefore, vaccine adjuvants are broadly used to accelerate and boost immune responses, especially for inactivated vaccines, which have low immunogenicity. Aluminum hydroxide, the most commonly used adjuvant for veterinary rabies vaccines, has been used for over 70 years (Lindblad, 2004a); however, its contributions to the early antibody responses and cellular immunity are limited (Aprile and Wardlaw, 1966; Lindblad, 2004a,b; Exley and Siesjo et al., 2010). Thus, an adjuvant that can increase the immunogenicity of inactivated cell culture vaccines and induce high VNA titers is urgently needed to provide immunization coverage to domestic dogs and block the transmission of rabies to humans.

At present, the majority of human rabies cases are post-exposure cases. The current rabies vaccines for post-exposure prophylaxis (PEP) are whole killed rabies vaccines without adjuvants. A complete PEP schedule requires at least 4 injections to be effective, and treatment with rabies immune globulin is needed in serious cases (Mittal, 2013). The expense of rabies immune globulin is unaffordable for inhabitants in developing countries. Several previous studies reported that aluminum hydroxide can even delay the production of early antibodies, which makes this adjuvant useless to PEP (Lin and Perrin, 1999); furthermore, aluminum hydroxide can produce side effects. Considering these reasons, a record that assents to the use of aluminum hydroxide in combination with inactive rabies vaccines for human use was deleted from the fifth edition of the Chinese Pharmacopoeia. Thus, adjuvants that can rapidly induce antibody responses, cellular immunity and cytokine production during the early stage of anti-rabies therapy are urgently needed to reduce the dose and schedule of PEP.

Some recent studies have shown that certain polysaccharides from plants possess excellent biological activities and can be used as immunopotentiators of inactivated rabies vaccines to enhance both humoral and cellular immunity (Liu and Zhang et al., 2012; Su and Pei et al., 2014). Polysaccharides from Chinese herbs are natural, safe and non-residual.

Poria cocos (Polyporaceae) or Poria, referred to as Fu ling, is a fungus that has been used in clinical medicine for thousands of years in China because of its diuretic, sedative and tonic effects (Rios, 2011; Feng and Zhao et al., 2013). Polysaccharides from Poria have been established to possess many beneficial biological activities, such as antioxidant, anti-inflammatory, anti-cancer and anti-viral activities (Ke and Lin et al., 2010; Sun, 2014). In our previous study, we have isolated the crude polysaccharides from *Poria cocos* which process adjuvant activities and can be used as immunopotentiators. Chemical isolation was performed as described (Wu and Li et al., 2016) and the adjuvant activity of isolated products were tested via H1N1 influenza and HBsAg vaccines (Wu and Li et al., 2016). PCP-II was examined as the active polysaccharide from *Poria cocos*. PCP-II appears in the form of white powder. The HPGPC profile of PCP-II was a single, symmetrically sharp peak. PCP-II has a molecular weight of 29,000 Da according to a calibration curve prepared with standard dextrans. CE analysis of PMP-derivatized PCP-II demonstrated that PCP-II contained fucose, mannose, glucose and galactose at a molar ratio of 1.00:1.63:0.16:6.29.

The present study was performed to evaluate the adjuvant activity of the polysaccharide PCP-II in combination with an inactivated rabies virus in mice and dogs. The effects of PCP-II on rabies-specific humoral and cell-medicate immune responses its protective effect against subsequent challenge with virulent street rabies virus were analyzed.

2. Materials and methods

2.1. Viruses, cells and polysaccharides

The rabies virus wtCVS-11 was provided by the Chinese Center for Disease Control and Prevention. The recombinant virus rCVS-11-G was recovered and stored in our laboratory. rCVS-11-G is gene edited from CVS-11 to express an additional copy of glycoprotein which elicits higher level of antibody titer (Xue and Zheng et al., 2014). A rabies virus street stain, HunPB3, was isolated from a pig in Hunan Province in 2006 and was stored in our laboratory. The rCVS-11-G strain was propagated in BSR cells that were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Baby hamster kidney (BHK)-21 cells were also cultured in DMEM supplemented with 10% FBS. The polysaccharide PCP-II was kindly provided by Professor Shan Junjie of the Beijing Institute of Pharmacology and Toxicology.

2.2. Mice and dogs

BALB/C mice (6- to 8-week-old females) and beagles (3 months old) were purchased from the Changchun Institute of Biological Products (Changchun, China). Beagles that had been immunized with a commercial rabies vaccine two years previously were kindly provided by the Changchun Institute of Biological Products. All animal studies were conducted with prior approval from the Animal Welfare and Ethics Committee of the Military Veterinary Research Institute of the Academy of Military Medical Sciences under permit number SCXK-2014-022. The environment and housing facilities satisfied the National Standards of Laboratory Animal Requirements (GB 14925-2001) of China.

2.3. Preparation of immunogens

rCVS-11-G was applied at an MOI of 0.1 to infect BSR cells. The titer of the collected virus was 10^8 $TCID_{50}$/ml. rCVS-11-G was inactivated by mixing the virus with 0.03% β-propiolactone and incubating this mixture overnight at 4 °C followed by 2 h at 37 °C. Inactivated rCVS-11-G was mixed with the Chinese herbal polysaccharide PCP-II and incubated overnight at 4 °C. Aluminum hydroxide was completely mixed with inactivated rCVS-11-G at a volume ratio of 1:4. All the vaccine was adjusted to the same volume (0.1 ml per dose for mice and 1.25 ml per dose for dogs) with sterilized phosphate buffer saline (PBS).

2.4. Mouse immunization and challenge

Mice were randomly divided into 4 groups. The mice were immunized twice with 50 μl of inactivated rCVS-11 alone as a control or together with various adjuvants at 2-week intervals. The mock group was injected twice with PBS at the same time points as the other groups were immunized. Each dose of PCP-II was 200 μg. Then, the mice were challenged with 100 × the mouse intramuscular lethal dose 50% (IMLD50) of street rabies virus strain HuNPB3 via injection into the muscle of the forelimb at the sixth week after the first immunization. Then, the mice were observed for 21 days. During the observation period, all of the mice that developed clinical signs of rabies were humanely euthanized via cervical dislocation under isoflurane anesthesia.

2.5. Antibody response assay

Blood samples were collected from the mice on days 3, 7, 14, 21, 28, 42 via retro-orbital plexus puncture after the first immunization. VNA titers were determined using the fluorescent antibody virus neutralization (FAVN) test (Cliquet, Aubert et al. 1998).

2.6. IFN-γ and IL-4 enzyme-linked immunospot (ELISpot) assays

Spleens were collected from mice at 14 days after the second vaccination, and splenocytes were isolated and suspended at a density of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS. Three mice from each group were sacrificed at that time point. The splenocytes were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml in the suspension and incubated at 37 °C for 48 h. The levels of IFN-γ and IL-4 produced by splenocytes were measured via ELISpot assays (mouse IFN-γ and IL-4 ELISpot kit, Mabtech AB, Stockholm, Sweden) according to the manufacturer's instructions. The number of spot-forming cells (SFCs) was counted using an automated ELISpot reader (AID GmbH, Strassberg, Germany).

2.7. Flow cytometry assays for intracellular cytokine staining (ICS)

Splenocytes were isolated from mice at 2 weeks after the second immunization, and splenocyte suspensions (1×10^6 cells/ml) were prepared in RPMI 1640 medium supplemented with 10% FBS. The splenocyte suspensions were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml and incubated in a protein transport inhibitor solution (containing monensin) (BD Biosciences, Franklin, TN, USA) at 37 °C. The cell suspensions were collected at 6 h after stimulation and surface-stained with anti-mouse CD4 and CD8 monoclonal antibodies (BD Biosciences) for 30 min at 4 °C. The cell suspensions were permeabilized for 30 min with Cytofix/Cytoperm (BD Biosciences) at 4 °C, and the cells were stained with anti-mouse IFN-γ and IL-4 antibodies (BD Biosciences) for 30 min at 4 °C. The stained cells were analyzed using a flow cytometer.

2.8. Flow cytometry assays for B cells and T cells in lymph node and blood samples from mice

Inguinal lymph node samples were harvested from mice at 6 days after the first immunization. Cells were isolated and single-cell suspensions were prepared in PBS; then, the cells were stained with anti-mouse CD19 and CD40 antibodies (BD Bioscience) at 4 °C for 30 min to label B cells. The stained cells analyzed in a flow cytometer washing twice with PBS.

Whole blood samples were collected from mice at 6 days after the first immunization, and peripheral blood mononuclear cells (PBMCs) were isolated and single-cell suspensions (1×10^6 cells/mL) were prepared in PBS. The cells were stained with anti-mouse CD19 and CD40 antibodies (BD Bioscience) to label B cells and with anti-mouse CD3, CD4 and CD8 monoclonal antibodies (BD Biosciences) to label T cells at 4 °C for 30 min. The labeled cells were washed twice with PBS and then analyzed in a flow cytometer.

2.9. IL-2, IL-4, IL-10 and IFN-γ enzyme-linked immunosorbent assays (ELISAs)

Splenocytes were isolated from mice at 2 weeks after the second vaccination, and splenocytes were suspended at a density of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS. The splenocyte suspensions were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml and incubated at 37 °C for 48 h. The cell supernatants were collected after 48 h of stimulation and used for ELISAs (mouse IL-2, IL-4, IL-10 and IFN-γ ELISA kits, Mabtech AB, Sweden) according to the manufacturer's instructions.

2.10. Post-exposure immune test in mice

Mice were randomly divided into 4 groups with 10 mice per group and were challenged with $10 \times \text{IMLD}_{50}$ of street virus strain HuNPB3 in the muscle of the forelimb. Twenty-four hours and ninety-six hours after this challenge, the mice were immunized twice with 10^7 TCID_{50} of rCVS-11-G mixed with different adjuvants (alhydrogel or PCP-II); the control group was injected with rCVS-11-G alone, and the mock group was injected with PBS. All the mice were injected twice at 24 and 96 h after virus challenge. The mice were observed for 21 days, and all mice that developed clinical signs of rabies during the observation period were humanely euthanized via cervical dislocation under isoflurane anesthesia.

2.11. Booster immunization in dogs

The dogs had been immunized several years previously, and VNA titers were determined before immunization. The dogs were randomly divided into 4 groups of 5 dogs per group and were injected intramuscularly with 1 ml of inactivated rCVS-11-G at 10^8 TCID_{50} mixed with 2 mg of PCP-II or 0.25 ml of alhydrogel to immunize. Three of the groups were immunized twice with PBS or inactivated rCVS-11-G mixed with PCP-II or alhydrogel. The fourth group was immunized with a commercial vaccine (Merial, Beijing, China) according to the manufacturer's instructions. Blood samples were collected from the front leg vein of the dogs at 1, 2, 3 and 4 weeks after the first immunization. Serum samples were tested for RABV-specific VNA via the FAVN test.

2.12. Single-dose immunization test in dogs

After examining the effect of PCP-II on booster immunization efficacy, we tested the effect of PCP-II on single-dose immunization efficacy. Dogs were randomly divided into 8 groups of 5 dogs in each group. Then, six groups were immunized intramuscularly with 3 different titers (10^6, 10^7 or 10^8 TCID_{50}) of inactivated rCVS-11-G mixed with 2 mg of PCP-II or 0.25 ml of alhydrogel. The seventh group was immunized with a commercial vaccine (Merial, Beijing, China), and the eighth group was immunized with PBS. All of the dogs were immunized only once. Blood samples were collected from the front leg vein of the dogs on weeks 2 and 4 after immunization. Serum samples were tested for RABV-specific VNA via the FAVN test.

2.13. Statistical analysis

Data were expressed as means ± SD. Statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) to determine statistically significant differences in the generated data via analysis of variance (one-way ANOVA). The results were considered significantly different if $p < 0.05$ and very significantly different if $p < 0.01$.

3. Results

3.1. The polysaccharide extract increased the VNA titer in mice

Blood samples were collected, and antibody titers were determined according to the vaccination schedule described in the Materials and Methods. Fig. 1 shows the mean titer of VNA against rabies virus in mice immunized with rCVS-11-G mixed with different adjuvants. None of the VNA titers reached the level of 0.5 IU/ml at 3 days after the first immunization, but the VNA titer for the group of mice that received the polysaccharide extract PCP-II increased to 0.68 IU/ml at 7 days after the first vaccination. The VNA titer of all three vaccinated groups increased to greater than 0.5 IU at 2 weeks after the first immunization, and the antibody titer of all groups that received PCP-II was significantly higher than that of the alhydrogel-receiving group and the control group ($p < 0.05$). The VNA titers for all mice in the mock group were no more than 0.02 IU/ml considering the limit of detection of the FAVN test (data not shown).

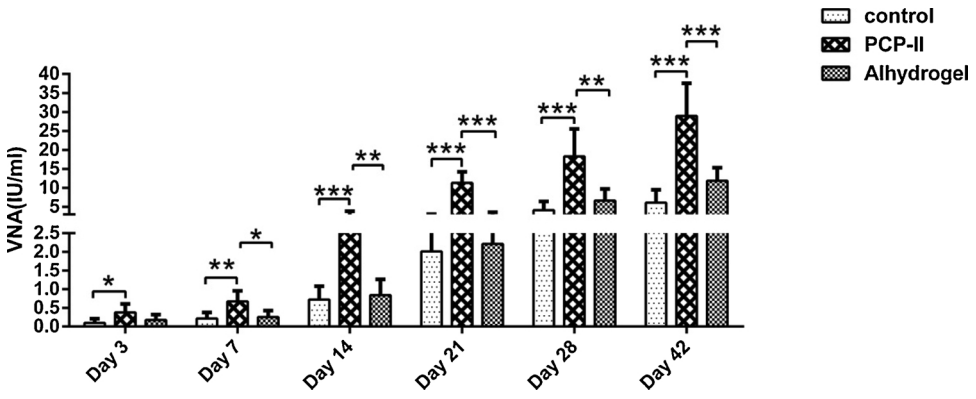


Fig. 1. Specific anti-rabies antibody production in mice following induction with rCVS-11-G mixed with different adjuvants. Mice were immunized twice via intramuscular injection into the hind leg at 2-week intervals. The specific anti-rabies antibody titer was measured via the FAVN test at 3, 7 14, 21, 28 and 42 days after the first immunization. Representative data are presented as the means ± standard deviation (SD) of 8 mice from each group and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

3.2. Polysaccharides induced antigen-specific cellular immune responses

After confirming that the polysaccharide extract PCP-II clearly enhances the VNA response in mice, we used the ELISpot assay to detect antigen-specific IFN-γ and IL-4 activities in splenocytes. As shown in Fig. 2A and B, the number of SFCs in the mice in the PCP-II group was significantly higher than that in the other groups.

We analyzed the ability of these adjuvants to induce IFN-γ- or IL-4-secreting $CD4^+$ and $CD8^+$ T cells via ICS. As shown in Fig. 2C, the polysaccharide extract PCP-II induced the production of more IL-4-secreting $CD4^+$ T cells than alhydrogel. Identical results were observed for IFN-γ-secreting $CD4^+$ T cells (Fig. 2D). There was no significant difference in the frequency of these cells between the control and alhydrogel groups.

3.3. The polysaccharide extract enhanced the activation of B cells in lymph nodes

To investigate whether the polysaccharide extract can function as an adjuvant to induce B cell production, lymph nodes were collected, and lymph node cells were isolated and examined by flow cytometry analysis. As shown in Fig. 3, significantly more B cells ($CD19^+CD40^+$) in lymph nodes were activated by rCVS-11-G mixed with PCP-II than rCVS-11-G mixed with alhydrogel or the control treatment on the sixth day after the first immunization ($p < 0.05$).

3.4. The polysaccharide extract enhanced the recruitment of T cells and B cells in blood

To further investigate the activity of polysaccharides as adjuvants, blood samples were collected, and cells were isolated from blood and analyzed by flow cytometry. Fig. 4(A—C) shows the recruitment of T cells ($CD3^+CD4^+$ or $CD3^+CD8^+$) and B cells ($CD19^+CD40^+$) in blood. The polysaccharide PCP-II activated the highest percentages of B and T cells among the 3 groups.

3.5. The polysaccharide extract increased the levels of cytokines secreted by splenocytes

The levels of the cytokines IL-2, IL-4, IL-10, IFN-γ secreted by splenocytes were measured using a commercial ELASA kit as described in the Materials and Methods. As shown in Fig. 5A and D, The levels of IL-2 and IFN-γ secreted by splenocytes isolated from the mice in the PCP-II group were significantly higher than those in the other groups ($p < 0.05$). As presented in Fig. 5B and C, the secreted levels of IL-4 and IL-10 in the polysaccharide extract PCP-II group were significantly higher than those in the mock and alhydrogel groups ($p < 0.05$).

3.6. Immunization combined with adjuvant treatment with polysaccharides protected mice against lethal challenge with a street Rabies virus strain

To evaluate whether inactivated rCVS-11-G mixed with the polysaccharide extract PCP-II induced an immune response, the mice were challenged with street rabies virus strain HuNPB3 as described in the Materials and Methods. All mice from the polysaccharide-receiving group survived the test, but one mouse from the aluminum hydroxide group and three mice from the control group failed to survive. None of the mice from the mock group survived the test. The results of the challenge test are shown in Fig. 6A. Analyses of clinical symptoms and the RT-PCR results using total RNA from brain tissues of dead mice showed that these mice died due to rabies infection (data not shown). The results of the challenge test showed that the polysaccharide extract PCP-II provided complete protection against street rabies virus infection.

To determine the protective effect of polysaccharides as adjuvants in post-exposure cases, mice were challenged with HunPB3 24 h before immunization. Only one mouse from the aluminum hydroxide group survived the challenge. In contrast, 7 mice from the PCP-II group survived for the duration of the observation period; all mice in the mock group died (Fig. 6B). There were significant difference between the PCP-II group and aluminum hydroxide group ($p < 0.05$) in the survival rate (Fig. 6B). Analyses of clinical symptoms and the RT-PCR results using total RNA from brain tissues of the dead mice indicated that the mice died due to rabies infection (data not shown).

3.7. Enhancing effects of polysaccharides on the VNA titer in dogs

The results of the booster immunization are shown in Fig. 7. The mean antibody titer of the PCP-II group increased to 11.1 IU/ml within one week after the first vaccination and to 15.204 IU/ml by the second week. In contrast, the antibody titers were only 2.088 IU/ml (week 1) and 2.908 IU/ml (week 2) for the aluminum hydroxide group and 1.516 IU/ml (week 1) and 2.288 IU/ml (week 2) for the commercial vaccine group. After the second vaccination, the antibody titer of the PCP-II group was significantly increased. However, no evident increases in the antibody titers were observed in the aluminum hydroxide or commercial vaccine group.

The results of single-dose immunization in dogs are shown in Fig. 8. The dogs were immunized, and blood samples were collected; then, VNA titers were determined. The mean VNA titers in the three PCP-II groups were above 0.5 IU/ml just 2 weeks after immunization. The VNA titer was significantly different between the PCP-II and aluminum hydroxide groups at all three vaccination doses 4 weeks after the immunization *($P < 0.05$)*. However, at 2 weeks after immunization, only the two high-dose groups showed significant differences in the VNA titer.

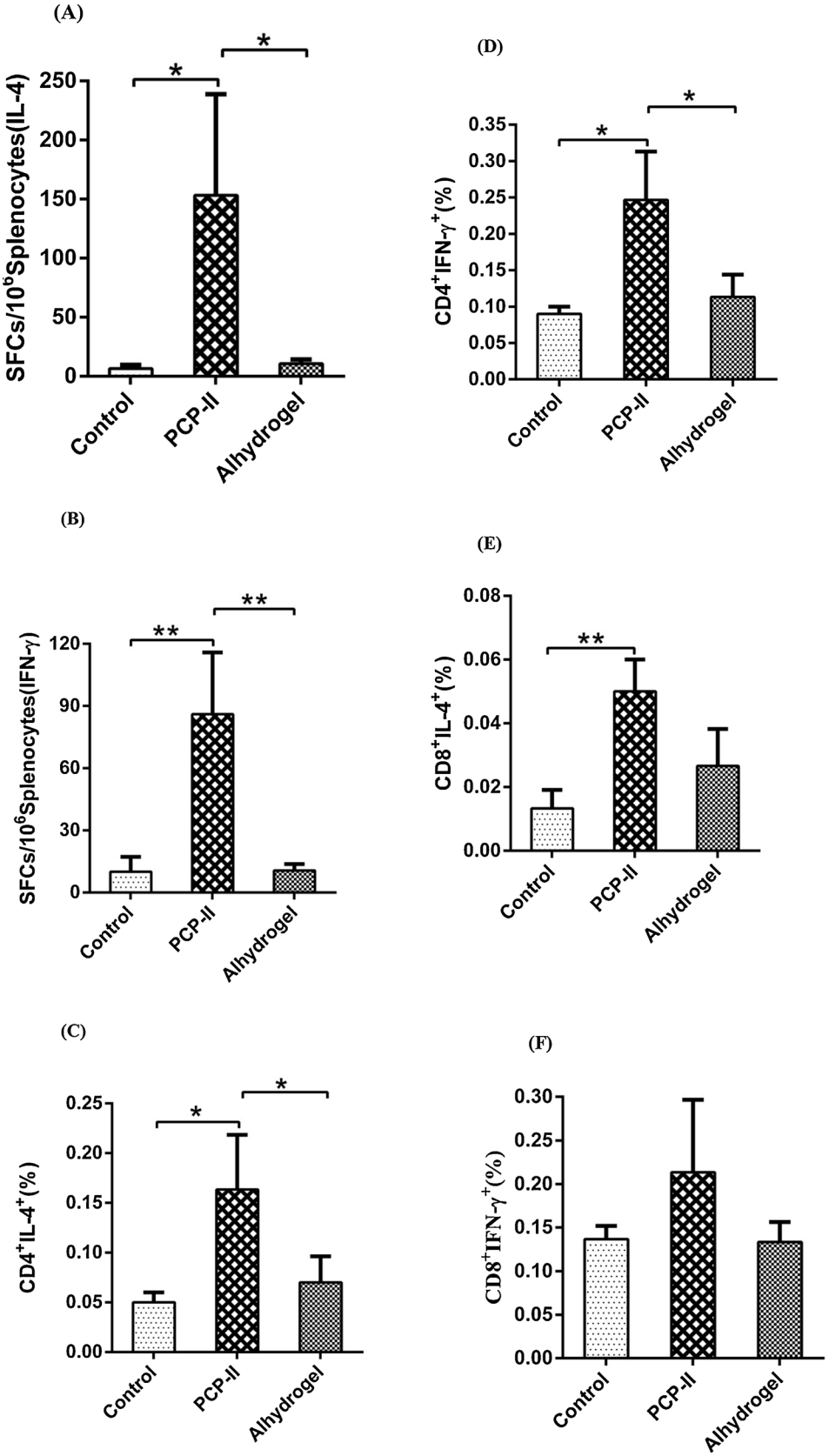


Fig. 2. ELISpot assays of IFN-γ and IL-4 secretion and ICS assays for antigen-specific $CD4^+$ and $CD8^+$ T cells secreting IFN-γ or IL-4 among mouse splenocytes. Spleens were isolated from 3 mice of each group 2 weeks after the second vaccination for the Elispot and ICS assays. The frequency of SFCs secreting IL-4 **(A)** and IFN-γ **(B)** were measured using a commercial Elispot kit. RABV-specific $CD4^+$ and $CD8^+$ T cells were measured via ICS assays. Spleens were isolated from 3 mice of each group 2 weeks after the second vaccination, and splenocytes were stained with mouse monoclonal antibodies against CD4, CD8, IFN-γ, and IL-4. The frequencies of $CD4^+$ cells secreting IL-4 (C) or IFN-γ (D) and $CD8^+$ cells secreting IL-4 (E) or IFN-γ (F) are shown. The data represent the mean relative values ± SD of 3 mice and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

4. Discussion

The current rabies vaccines for veterinary and human use are whole killed vaccines. Although these whole killed virus vaccines are safe, they have limited efficacy. The veterinary vaccines currently in use may fail to provide protection against rabies infection after injection (Hu and Fooks et al., 2008; Davlin and Vonville, 2012). Most clinical cases of human rabies exposure are PEPs, and such cases require four or five

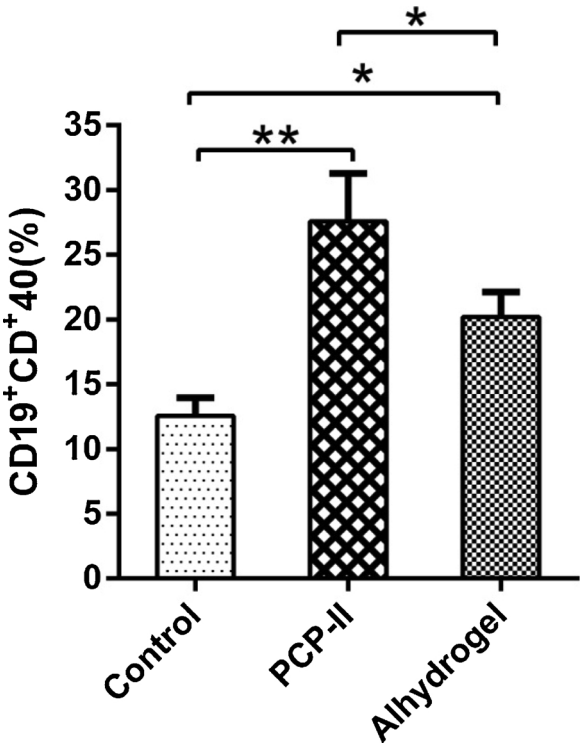
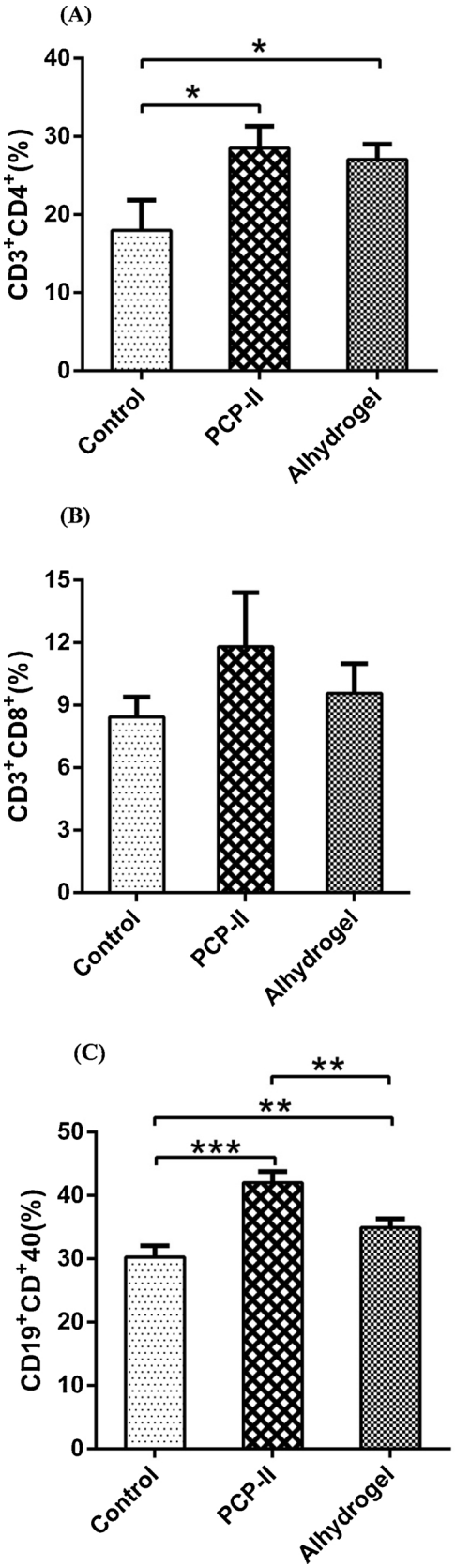


Fig. 3. Flow cytometry analysis of B cells in lymph nodes. Lymph nodes were collected from 3 mice of each group, and isolated cells were cultured as described in the Materials and Methods. The effect of different adjuvants on the frequency of activated $CD19^+CD40^+$ B cells was determined a 6 days after the first immunization. Representative data are presented as the means ± SD of 3 mice from each group and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

injections of whole killed rabies vaccine without adjuvant (Mittal, 2013). Because the incubation period of rabies can be as short as one week, rabies immune globulin is required in serious cases (Gozdas, 2015). The high cost of rabies immune globulin could be unaffordable to the inhabitants of rural areas in developing countries, who are most likely to be at risk of rabies exposure. Approximately 2000 IU of equine rabies immune globulin must be used for each case. For instance, in Cambodia, a developing country in East Asia, the cost of 1000 IU of equine rabies immune globulins is likely more than US$ 30, but the monthly income of farmers in developing countries is always below US$ 80 (Tarantola and Ly et al., 2015). Although human rabies immune globins are more effective, but the cost is even more expensive. PEP failure rarely occurs when rabies immune globin is used, PEP failure is most common in cases of insufficient or improper use of rabies immune globin. Such instances could occur in developing countries because of a lack of skilled doctors (Jain and Gupta et al., 2015). Considering these reasons, adjuvants that can rapidly generate an antibody response and induce cytokine production to promote early anti-viral activities are optimal for inactivated rabies vaccines for human use, especially in developing countries.

The traditional, most widely used adjuvant is aluminum hydroxide. Although the mechanisms by which aluminum hydroxide stimulates immune response may involve several pathways, indicating a Th2 response, prolonging exposure of the antigen to the immune system and delaying antigen clearance from the immunization site are considered to be the major mechanisms (Brewer, Conacher et al., 1999). However, these mechanisms provide minor contributions to early antibody responses and the activation of the production of Th1 type cytokines that participate in early anti-viral activities, such as tumor necrosis factor-α and IFN-γ (Nair and Melnick et al., 2006). Furthermore, side effects, such as severe inflammatory and nerve issues, may appear in certain



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cases (Lindblad, 2004a,b). In contrast, herbal polysaccharides can serve as an emerging, novel, and valuable type of vaccine adjuvant to stimulate immune responses and activate cellular responses.

Fig. 4. Blood samples were collected from mice at 6 days after the first immunization. The frequencies of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells and $CD19^+CD40^+$ B cells produced in the different adjuvant groups were quantified via flow cytometry analysis. (A) and (B) show the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, respectively, and (C) shows the B cells following activation in blood by different adjuvants for 3 mice in each group. The data represent the mean relative values ± SD of 3 mice and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

Several reports have indicated that polysaccharides from fungi can enhance immune responses via activation of immune cells such as monocytes, granulocytes, macrophages and nature killer (NK) cells by interacting with immune cell receptors and triggering cytokine secretion (Ladanyi and Timar et al., 1993; Lin and Zhang et al., 2004; Lavi and Friesem et al., 2006; Zhu and Chen et al., 2007; Li and Li et al., 2015). The reports on immunostimulating polysaccharides isolated from Poria are mostly glucans and its derivatives. A (1→3)-β-D-glucan was isolated from *Poria cocos* and was termed PCS3-II. Its carboxymethylated-sulfated derivative, CS-PCS3-II, was shown to possess excellent immunopotentiative and antitumor effects. The immunopotentiative effects of CS-PCS3-II have been confirmed by macrophage phagocytosis, thymus and spleen indexes, humoral antibody production, and delayed-type hypersensitivity responses (Chen and Zhang et al., 2010). The immunopotentiative effects of other components isolated from *Poria cocos* were also examined in some reports. In a previous study, a protein was purified from *Poria cocos*, and the gene corresponding to that protein was cloned. The purified protein displayed the ability to activate Th1 responses and increase IgE production (Lu and Kuan et al., 2014).

Although there have been only seldom reports that polysaccharides isolated from *Poria cocos* can be used as adjuvants, but the anti-cancer and anti-inflammatory activities of polysaccharides always results from their immunopotentiative effects (Hsieh and Chien et al., 2008, Togola and Inngjerdingen2008). Polysaccharides isolated from *Poria cocos* such as wc-PCM1, wc-PCM2 and ac-PCM2 have frequently been reported to exert anti-cancer and anti-inflammatory effects (Sun, 2014). These three polysaccharides are similar to PCP-II because they are also a mixture of fucose, mannose, glucose and galactose and are also isolates of *Poria cocos.* Their anti-tumor and immunostimulating effects were measured *in vivo* (Jin and Zhang et al., 2003a,b). In the current study, PCP-II, a mixture of fucose, mannose, glucose and galactose, with a molecular weight of 29,000 Da, was isolated from *Poria cocos*. The immunostimulatory effects of PCP-II were evaluated by co-administering this adjuvant with H1N1 influenza and HBsAg vaccines in the previous studies (Wu and Li et al., 2016). In this study, the potential use of PCP-II is confirmed by co-administering with a whole killed rabies vaccine, rCVS-11-G, to mice and assessing the antigen-specific humoral and cellular immune responses. The rabies-specific VNA titers, the most

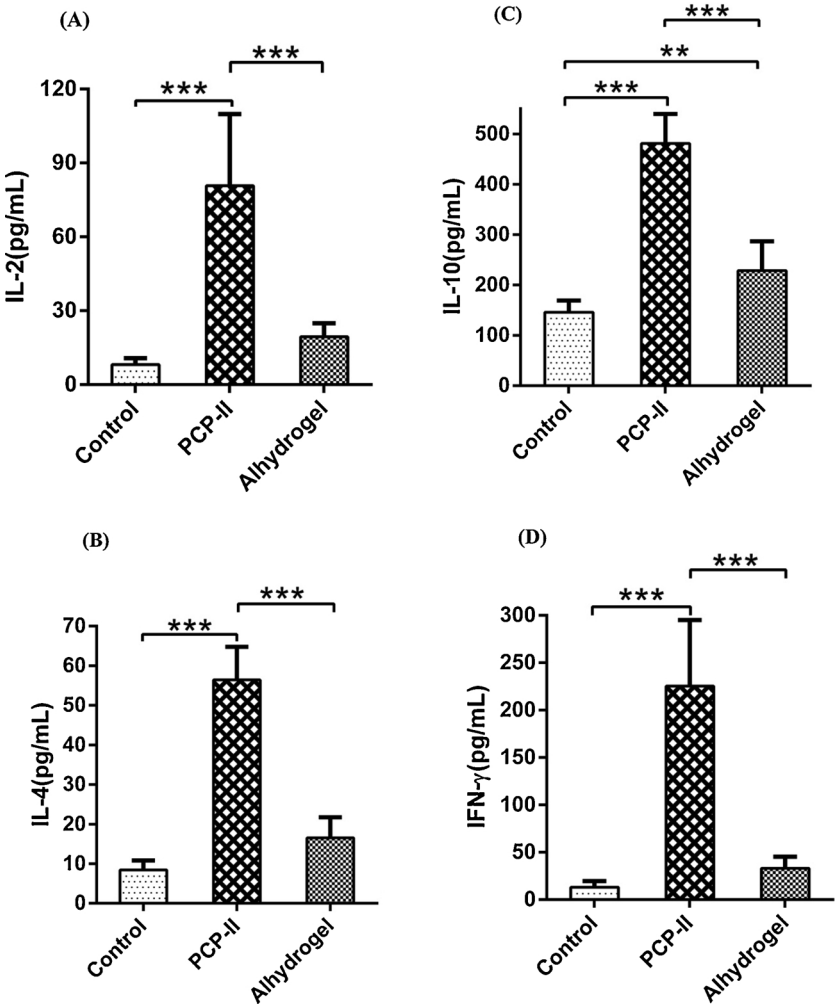

Fig. 5. ELISAs of the levels of IL-2, IL-4, IL-10 and IFN-γ secreted by splenocytes. Spleens were isolated from 6 mice of each group at 2 weeks after the last vaccination, and splenocytes were cultured and stained as described in the Materials and Methods. The levels of IL-2 **(A)**, IL-4 **(B)**, IL-10 **(C)** and IFN-γ **(D)** were measured using commercial ELISA kits. Representative data are presented as the means ± SD of 6 mice from each group and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

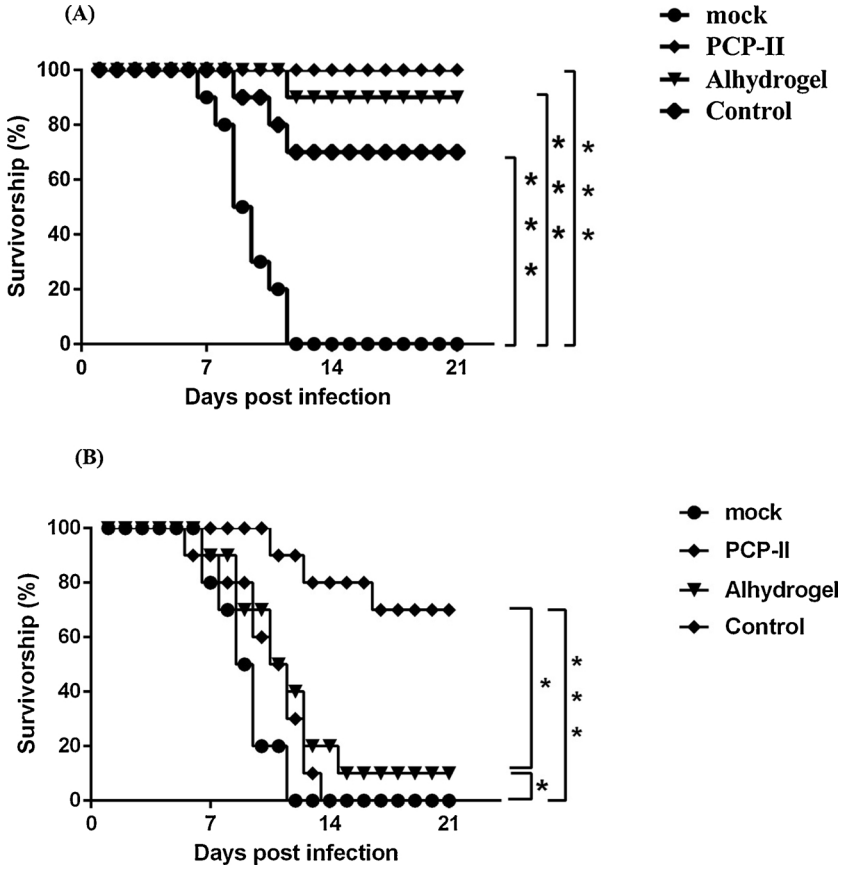

Fig. 6. Challenge test in mice. All mice in each group ($n = 10$) were challenged via intramuscular injection with 100 × IMLD50 of RABV street stain HuNPB3 at 4 weeks after the second vaccination, and the mice were observed for 21 days. The numbers of surviving mice in each group at different time points after the challenge test were recorded.
Post-exposure immune test in mice. Mice were challenged with 10 × IMLD50 HuNPB3 24 h before immunization and were observed for 21 days. The results are shown in (B). The numbers of surviving mice in each group at different time points after challenge were recorded.
Significant difference of survival group were assessed by Kaplan-Meier (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

important index of the effectiveness of a rabies vaccine, were measured in mice. The VNA titer of mice in the PCP-II group was significantly higher than that of mice in the control and aluminum hydroxide groups ($p < 0.05$). Cellular immune responses are also very important in resisting rabies infections and clearing rabies virus from the central nervous system (Johnson and Cunningham et al., 2010). $CD4^+$ T cells that differentiate into Th1 cells and secrete type 1 cytokines, such as IL-2 and IFN-γ, can activate $CD8^+$ T cells and perform anti-viral functions (Sant and McMichael, 2012; Swain and McKinstry et al., 2012). Additionally, Th2 cells, which are also derived from $CD4^+$ T cells, secrete type 2 cytokines, such as IL-4 and IL-10, which induce B cell-helper activity to stimulate the humoral immune response and increase antibody production (Nair and Bayer et al., 2011). In our study, we evaluated rabies-specific cellular immune responses by ELISpot and ICS assays. The frequency of IL-4- or IFN-γ-secreting SFCs was significantly higher in mice receiving PCP-II than that in the control and alhydrogel-receiving mice ($p < 0.05$). The frequency of IL-4- and IFN-γ-secreting $CD4^+$ and $CD8^+$ cells among splenocytes were measured, and similar results were obtained. Then, splenocytes were stimulated, and the secretion of IL-2, IL-4, IL-10 and IFN-γ was measured. All of these results indicate that PCP-II can activate splenocytes and stimulate these cells to secrete IL-2, IL-4, IL-10 and IFN-γ. These findings suggest that PCP-II can mediate immune responses via both the Th1 and Th2 pathways. Flow cytometry analysis showed that the mice in the PCP-II group contained more activated T and B lymphocytes in their blood and lymph nodes than the mice in the other groups. The results of the challenge and post-exposure tests showed that PCP-II mixed with a whole killed rabies vaccine can fully or partially protect mice from lethal challenge with a street rabies virus. Induction of B and T cell proliferation and enhancement of the secretion of cytokines may be the main mechanisms underlying the immunostimulatory effects of PCP-II. Furthermore, we evaluated the immune-enhancing effects of PCP-II in

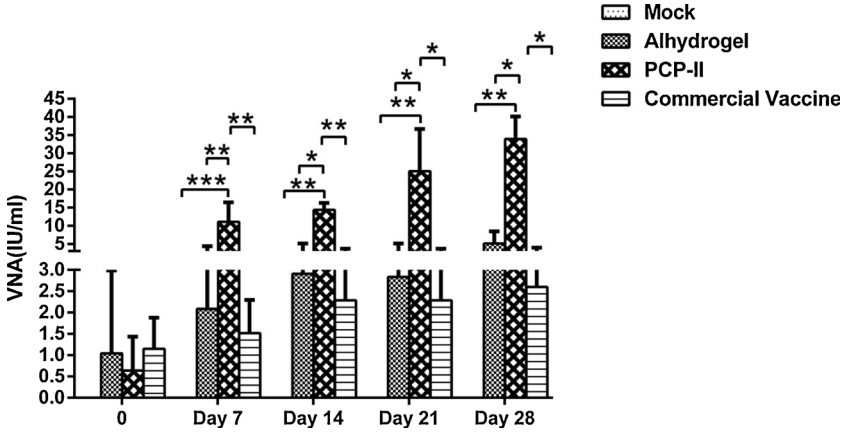

Fig. 7. Booster immunization test in dogs. Blood samples were collected from 5 dogs in each group, and serum samples were isolated as described in the Materials and Methods. The VNA titers were determined via FAVN tests. Representative data are presented as the means ± SD of 5 dogs from each group and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

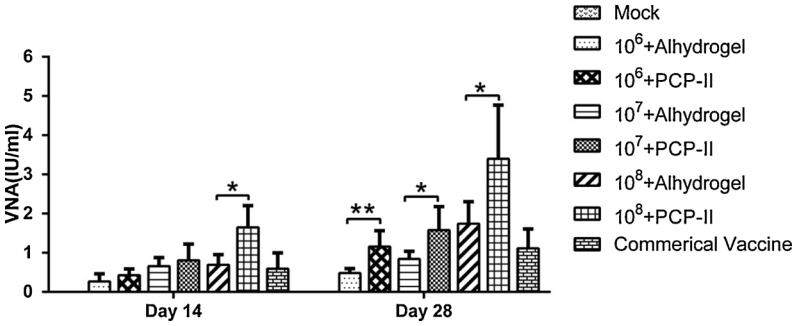

Fig. 8. Single-dose immunization test in dogs. Blood samples were collected from 5 dogs in each group, and serum samples were isolated as described in the Materials and Methods. The VNA titers were determined via FAVN tests. Representative data are presented as the means ± SD of 5 dogs from each group and were analyzed via one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

dogs. The rabies-specific VNA titers of the dogs in the PCP-II group were significantly higher than those of the dogs in the aluminum hydroxide and commercial vaccine groups.

In the present study, the polysaccharide PCP-II was found to enhance antigen-specific cellular immune responses in splenocytes by activating T cells and promoting cytokine secretion. In contrast, aluminum hydroxide failed to exert a significant effect on either splenocytes or lymphocytes. Together, these results suggest that this polysaccharide can significantly enhance cellular immune responses and accelerate antibody responses to an inactivated rabies vaccine, and these effects might reduce the PEP dose and schedule. These results highlight the potential utility of PCP-II in developing a more effective and affordable post-exposure rabies vaccine for use in humans.

Compared with the common veterinary rabies vaccine adjuvant, aluminum hydroxide, PCP-II significantly accelerated and enhanced antibody production in dogs injected with an inactivated rabies vaccine and only one injection can be effective which makes it highly valuable to be used as adjuvant for veterinary rabies vaccine. In conclusion, our findings suggest that PCP-II is an excellent candidate rabies vaccine adjuvant for veterinary or even human use.

Conflict of interest

The authors declare no conflicts of interest.

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