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Isatis indigotica root polysaccharides as adjuvants for an inactivated rabies virus vaccine

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ABSTRACT

Adjuvants can enhance vaccine immunogenicity and induce long-term enhancement of immune responses. Thus, adjuvants are important for vaccine research. Polysaccharides isolated from select Chinese herbs have been demonstrated to possess various beneficial functions and excellent adjuvant abilities. In the present study, the polysaccharides IIP-A-1 and IIP-2 were isolated from *Isatis indigotica* root and compared with the common vaccine adjuvant aluminum hydroxide via intramuscular co-administration of inactivated rabies virus rCVS-11-G into mice. Blood was collected to determine virus neutralizing antibody (VNA) titers and B and T lymphocyte activation status. Inguinal lymph node samples were collected and used to measure B lymphocyte proliferation. Splenocytes were isolated, from which antigen-specific cellular immune responses were detected via ELISpot, ELISA and intracellular cytokine staining. The results revealed that both types of polysaccharides induce more rapid changes and higher VNA titers than aluminum hydroxide. Flow cytometry assays revealed that the polysaccharides activated more B lymphocytes in the lymph nodes and more B and T lymphocytes in the blood than aluminum hydroxide. Antigen-specific cellular immune responses showed that IIP-2 strongly induced T lymphocyte proliferation in the spleen and high levels of cytokine secretion from splenocytes, whereas aluminum hydroxide induced proliferation in only a small number of lymphocytes and the secretion of only small quantities of cytokines. Collectively, these data suggest that the polysaccharide IIP-2 exhibits excellent adjuvant activity and can enhance both cellular and humoral immunity.

1. Introduction

Rabies is a fatal form of encephalomyelitis caused by the rabies virus, to which all warm-blooded animals, including humans, are susceptible [1,2]. According to the World Health Organization, approximately 55,000 people die from rabies each year worldwide [3,4]; this total includes more than 3000 people in China [5]. China is second worldwide in terms of the number of rabies-related deaths. Because the mortality rate for rabies is nearly 100% once clinical symptoms appear, the most effective way to prevent the spread of rabies is through inoculation with a vaccine [2]. Previous studies have shown that domestic dogs are responsible for over 95% of human rabies cases [6] and that the immunization of >70% of all domesticated dogs may be sufficient to prevent rabies transmission to humans and avoid a rabies epidemic [7,8].

Most commercial rabies vaccines for human and veterinary use in China are inactivated cell culture vaccines. Inactivated cell culture vaccines are safe and easy to use and store. However, at least 3 injections are required to provide a sufficient virus neutralizing antibody (VNA) titer (at least 0.5 IU/ml), which is a reliable indicator that immune protection against rabies virus infection has been achieved [9,10]. In fact, the rabies neutralizing antibody titer is the most reliable indicator of immune protection [11]. Previous studies have shown that nearly one-third of dogs that received only one injection of a commercial rabies vaccine failed to produce a VNA titer of 0.5 IU/ml [12–15]. The use of multiple injections translates into higher costs and therefore becomes unaffordable for developing countries. Similar circumstances exist for humans. Most human rabies vaccinations are given post-exposure [9], so

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the rapid generation of an antibody response following rabies vaccination is very important. The current post-exposure prophylaxis schedule requires at least 4 injections to be effective, and rabies immune globulin is required in serious cases [10]. Vaccine adjuvants are widely used to accelerate and boost immune responses. Aluminum hydroxide is a commonly used vaccine adjuvant; however, its contribution to early antibody responses is limited, and it may cause side effects in some cases [16–19]. In addition, some studies have shown that aluminum hydroxide may even delay early antibody production [20]. Interestingly, previous records in the Chinese Pharmacopoeia describing the use of aluminum hydroxide in human rabies vaccines were deleted from its fifth edition. Therefore, a novel adjuvant must be developed to increase the effectiveness of the inactivated rabies veterinary vaccine to provide better immune coverage for domestic dogs. This in turn should help block rabies transmission to humans and if approved for human use, could reduce the dosing schedule required for post-exposure prophylaxis.

Recent research has shown that many plant polysaccharides, specifically those derived from Chinese herbs, can enhance immunogenicity and be used to promote both humoral and cellular immunity. These polysaccharides are natural, safe and non-residual [21,22].

The use of *Isatis indigotica* root as a medicine in China can be traced back to the beginning of the Common Era. It is believed that this root can stimulate the body's resistance to influenza and severe acute respiratory syndrome (SARS) and may even prevent these conditions [23–26]. The polysaccharide IIP-A-1 is an alpha-glucan isolated from the roots of *I. indigotica*; its chemical structure and adjuvant activities in influenza H1N1 and hepatitis B surface antigen (HBsAg) vaccines were described in a previous study [27]. IIP-2, another polysaccharide isolated from these same roots, is an arabinogalactan with a molecular weight of 66,400 Da. IIP-2 is composed of arabinose and galactose at a ratio of 1.0:1.5. Its structure and activity as an adjuvant were previously described [28]. Previous studies have also shown that arabinogalactan can be used either to potentiate immune responses or as an adjuvant in human or animal vaccines [29–31].

The present study evaluated the use of the polysaccharides IIP-A-1 and IIP-2 as adjuvants for an inactivated rabies virus vaccine in mice. The effects of antigen-specific humoral and cellular immune responses and their protective capacity when challenged with virulent rabies virus were also analyzed.

2. Materials and methods

2.1. Viruses, cells, polysaccharides and mice

The rabies virus wtCVS-11 was provided by the Chinese Center for Disease Control and Prevention. Recombinant virus rCVS-11-G was recovered and stored in our laboratory, as previously described [32]. A street rabies virus strain, HuNPB3, was isolated from a pig in Hunan Province in 2006 and has been stored in our laboratory. The rCVS-11-G strain was propagated in BSR cells, which 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 grown in DMEM supplemented with 10% FBS. The polysaccharides IIP-A-1 and IIP-2 were kindly provided by Professor Shan Junjie of the Beijing Institute of Pharmacology and Toxicology.

BALB/C mice (6- to 8-week-old females) were purchased from the Changchun Institute of Biological Products (Changchun, China). 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.

2.2. Immunogen preparation

BSR cells were infected with rCVS-11-G at an MOI of 0.1. The titer of virus collected was 10^8 50% tissue culture infectious dose units ($TCID_{50}$)/ml. rCVS-11-G was inactivated by mixing with 0.03% β-propiolactone; the mixture was incubated overnight at 4 °C and then for 2 h at 37 °C. Inactivated rCVS-11-G was mixed with IIP-A-1 or IIP-2 and incubated overnight at 4 °C. Aluminum hydroxide was mixed completely with inactivated rCVS-11-G at a volumetric ratio of 1:4.

2.3. Mouse immunization and challenge

Mice were randomly divided into 5 groups with 20 mice per group and were inoculated twice with 50 μl of inactivated rCVS-11 (5×10^6 $TCID_{50}$) mixed with different adjuvants at 2-week intervals. A control group was injected twice with 50 μl of inactivated rCVS-11 (5×10^6 $TCID_{50}$) only, and a mock group was injected twice with PBS at the same time points. For each dose, 200 μg of either IIP-A-1 or IIP-2 was included. The mice were challenged with 100× of the 50% intramuscular mouse lethal dose ($IMLD_{50}$) of street rabies virus strain HuNPB3, which was injected into the forelimb muscle 42 days after the first immunization. Following this, the mice were observed for an additional 21 days. During the observation period, all of the mice that developed clinical signs of rabies were humanely euthanized by cervical dislocation under isoflurane anesthesia.

2.4. Antibody response assay

Blood was collected from mice 3, 7, 14, 21, 28 and 42 days after the first immunization by retro-orbital plexus puncture. 6 mice from each group was randomly selected at each time point to represent the mean VNA titers. VNA titers were determined using a fluorescent antibody virus neutralization (FAVN) test [33].

2.5. Interferon-γ and interleukin-4 enzyme-linked immunospot assays

Spleens were collected from 3 mice from each group on day 14 after the second vaccination, and splenocytes were isolated and suspended at a concentration of 1×10^6/ml in RPMI 1640 medium supplemented with 10% FBS. Splenocyte suspensions were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml. The cell suspensions were then incubated at 37 °C for 24 h. The number of cells which produced interferon (IFN)-γ and interleukin (IL)-4 in the splenocytes was measured using an enzyme-linked immunospot (ELISpot) assay (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 determined using an automated ELISpot reader (AID GmbH, Strasberg, Germany).

2.6. Flow cytometry assays to assess intracellular cytokine staining

Splenocytes were isolated from 3 mice from each group at 14 days after the second immunization, and splenocyte suspensions (1×10^6 cells/ml) were prepared in RPMI 1640 medium containing 10% FBS. The splenocyte suspensions were stimulated with inactivated HuNPB3 at a concentration of 10 μg/ml and were cultured with a protein transport inhibitor (containing monensin) (BD Biosciences, Franklin, TN, USA) at 37 °C. The cell suspensions were

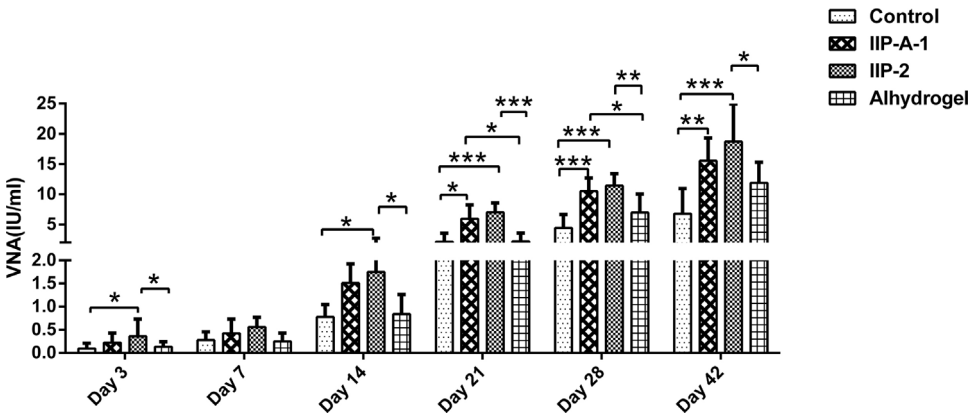

Fig. 1. Production of rabies-specific antibodies in mice following the administration of rCVS-11-G mixed with different adjuvants or no adjuvant (control). Mice were immunized twice by intramuscular injection in the hind leg at 2-week intervals. Blood was collected from 6 randomly selected mice of each group at 3, 7, 14, 21, 28 and 42 days after the first immunization. Rabies-specific antibody titers were measured using an FAVN test. Representative data are shown as the means ± SDs of 6 mice from each group and were analyzed using one-way ANOVA (*$p < 0.05$, **$p < 0.01$).

collected after 6 h, and the cells were surface-stained with anti-mouse CD4 and anti-mouse CD8 antibodies (BD Biosciences) for 30 min at 4 °C. The cells were then permeabilized for 30 min at 4 °C with Cytofix/Cytoperm (BD Biosciences) and stained with anti-mouse IFN-γ and anti-mouse IL-4 antibodies (BD Biosciences) for 30 min at 4 °C. The stained cells were analyzed using a flow cytometer.

2.7. Flow cytometry assays to analyze B and T cell populations in lymph nodes and blood collected from mice

Inguinal lymph node samples were harvested from 3 mice from each group at 3 and 6 days after the first immunization. Cells were isolated, and single-cell suspensions were prepared in phosphate-buffered saline (PBS) and stained for 30 min at 4 °C with CD19 and CD40 antibodies (BD Bioscience) to label B cells. The stained cells were washed twice with PBS and analyzed using a flow cytometer.

Whole-blood samples were collected from 3 mice from each group at 6 days after primary immunization by retro-orbital plexus puncture. Peripheral blood mononuclear cells (PBMCs) were isolated, and single-cell suspensions (1×10^6 cells/mL) were prepared in PBS. The cells were stained for 30 min at 4 °C with anti-mouse CD19 and CD40 (BD Biosciences) to label B cells and CD3, CD4 and CD8 monoclonal antibodies (BD Biosciences) to label T cells. The labeled cells were washed twice with PBS and analyzed using a flow cytometer.

2.8. IL-2, IL-4, IL-10 and IFN-γ enzyme-linked immunosorbent assays

Splenocytes were isolated from 6 mice from each group at 28 days after the first vaccination and then suspended in RPMI 1640 medium containing 10% FBS at a concentration of 2×10^6 cells/ml. The splenocyte suspensions were stimulated with inactivated HuNPB3 at a final concentration of 10 μg/ml and then incubated at 37 °C. The supernatants from the suspensions were collected at 48 h post-stimulation and measured using an enzyme-linked immunosorbent assay (ELISA) (Mouse IL-2, IL-4, IL-10 and IFN-γ ELISA kit, Mabtech AB) according to the manufacturer's instructions.

2.9. Post-exposure immune test in mice

Mice were divided into 4 groups with 10 mice per group and were challenged with 10× $IMLD_{50}$ of street rabies virus strain HuNPB3 in the muscle of the forelimb. Twenty-four hours after the challenge, the mice were immunized with 10^7 $TCID_{50}$ of rCVS-11-G mixed with different adjuvants (alhydrogel, IIP-A-1 or IIP-2); the mock group was injected with PBS. Each group was immunized twice at 24 and 96 h after the challenge. The mice were observed for 21 days, and all mice that developed clinical signs of rabies during the observation period were humanely euthanized by cervical dislocation under isoflurane anesthesia.

2.10. Statistical analysis

Data are expressed as the means ± standard deviations (SDs). Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) to determine statistically significant differences in the generated data by one-way analysis of variance (ANOVA). Statistically significant differences in survivor ratios were determined by Kaplan–Meier analysis. The results were considered significant if $p < 0.05$ and very significant if $p < 0.01$.

3. Results

3.1. Enhancing effects of polysaccharides on rabies neutralizing antibody titers in mice

Blood was collected to determine antibody titers at predetermined times after immunization. Fig. 1 shows the mean VNA titers against rabies virus in mice immunized with rCVS-11-G mixed with different adjuvants. None of the antibody titers rose above 0.5 IU/ml by 3 days after the first immunization, but the titers in the mice immunized with the vaccine in addition to IIP-2 rose to 0.56 IU/ml by 7 days after the first immunization. The mean VNA titer of all four groups rose above 0.5 IU/ml by 14 days after the first immunization; the second immunization was done after we collected the blood at the fourteenth day after the primary immunization. The antibody titers in the mice in the two polysaccharide test groups were higher than those in the alhydrogel group. The VNA titers in the mice from the mock group did not rise above 0.02 IU/ml (data not shown and Fig. 1).

3.2. Antigen-specific cellular immune responses induced by the addition of polysaccharides

After confirming that the studied polysaccharides could enhance VNA responses in mice, we used an ELISpot assay to detect antigen-specific IFN-γ and IL-4 activities in splenocytes. As shown

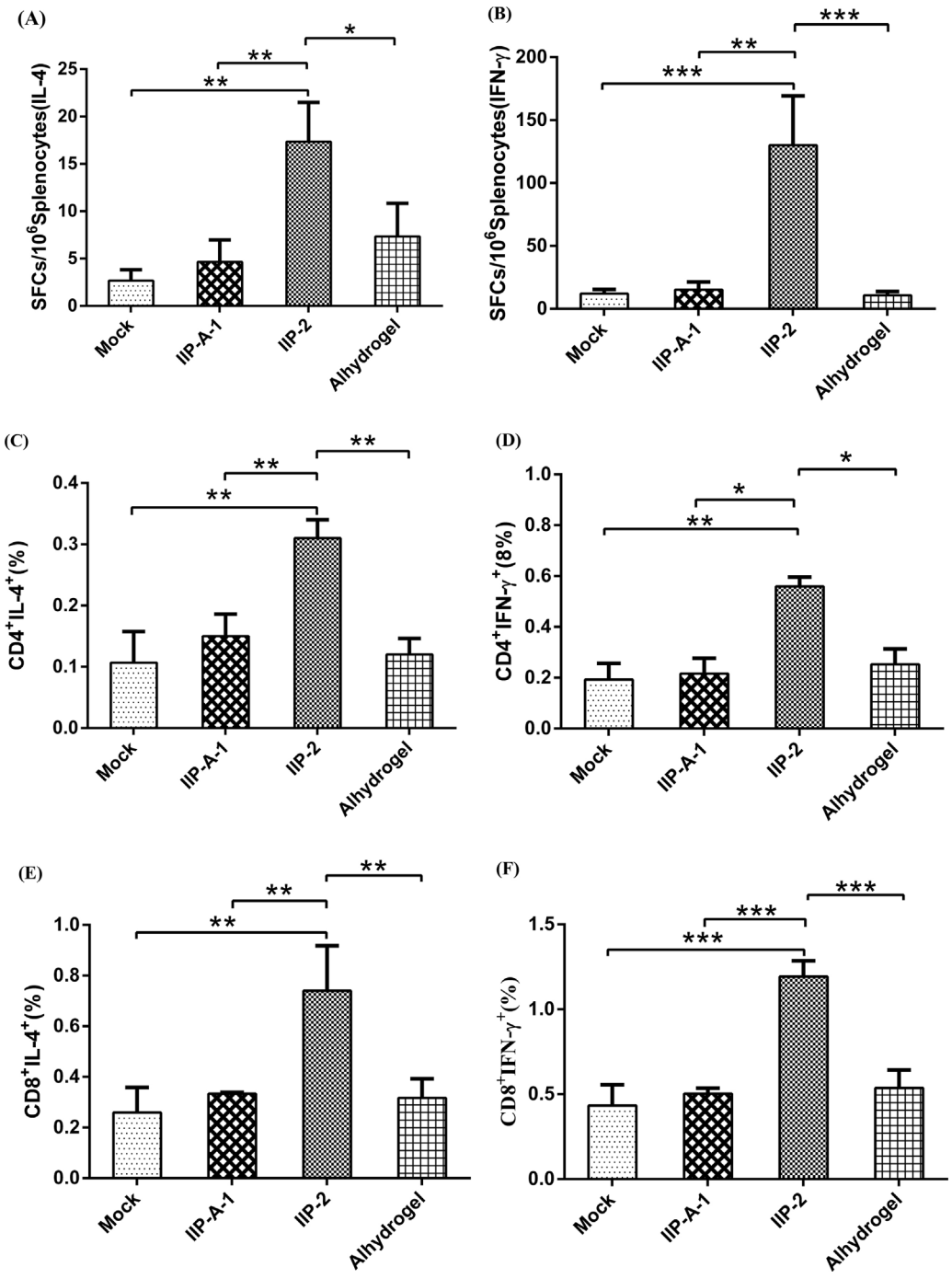

Fig. 2. ELISpot analysis of IFN-γ and IL-4 secretion and ICS assays for antigen-specific $CD4^{++}$ and $CD8^{++}$ T cell secretion of IFN-γ and IL-4 in mouse splenocytes. Spleens were collected from 3 mice per group 14 days after the second vaccination, and splenocytes were assayed by ELISpot and ICS assays. 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 collected from 3 mice per group 14 days after the second vaccination and were stained with mouse anti-CD4, anti-CD8, anti-IFN-γ and anti-IL-4 monoclonal antibodies. $CD4^+$ cells secreting IL-4 (C) or IFN-γ (D) and $CD8^+$ cells secreting IL-4 (E) or IFN-γ (F) are shown in Fig. 3. The data represent the subtraction value means and SDs of 3 mice and were analyzed using one-way ANOVA ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

in Fig. 2A and B, there were greater numbers of SFCs in the mice injected with IIP-2 than in the mice from the other groups.

We also analyzed the capacities of these adjuvants to induce IFN-γ and IL-4 secretion from $CD4^+$ and $CD8^+$ T cells by intracellular cytokine staining (ICS). As shown in Fig. 2D and F, IIP-2 induced greater numbers of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells than the other adjuvants tested; similar results were also observed for IL-4-secreting $CD4^+$ and $CD8^+$ T cells (Fig. 2C and E). The results from the other groups showed no significant differences.

3.3. Enhancing effects of polysaccharides on B cell activation in lymph nodes

To investigate whether polysaccharides can function as adjuvants to further induce B cell activation, lymph nodes were collected from the mice. Cells were isolated from the collected lymph nodes and analyzed by flow cytometry. As shown in Fig. 3A and B, the numbers of activated B cells ($CD19^+CD40^+$) in the lymph nodes were significantly higher in the IIP-A-1 and IIP-2 groups than

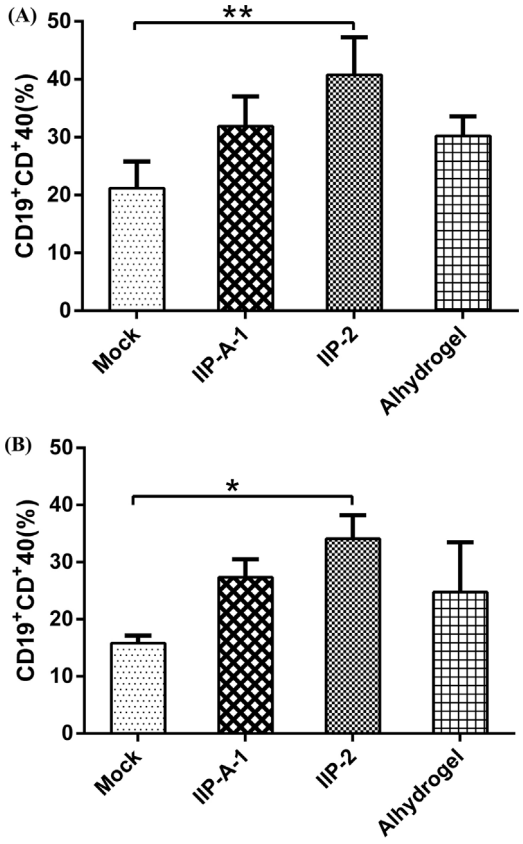

Fig. 3. Flow cytometry analysis of B cells isolated from lymph nodes. Lymph nodes were collected from 3 mice per group and cultured as described in Section 2. Different adjuvants activated $CD19^+CD40^+$ B cells at 3 (A) and 6 (B) days after the first immunization. Representative data are shown as the means ± SDs of 3 mice per group and were analyzed using one-way ANOVA ($^*p < 0.05$, $^{**}p < 0.01$).

in the alhydrogel group at days 3 and 6 after the first immunization. The representative scatter plots of B cells ($CD19^+CD40^+$) in the lymph nodes 3 and 6 days post the first immunization are shown in Fig. S1(A and B).

3.4. Enhancing effect of polysaccharides on B and T cell recruitment in the blood

To further investigate the use of polysaccharides as adjuvants, blood was collected, and PBMCs were isolated, cultured and stained as described above. The resultant cell suspensions were analyzed by flow cytometry. Fig. 4A and B shows T cell recruitment ($CD3^+CD4^+$, $CD3^+CD8^+$), and Fig. 4C shows B cell activation ($CD19^+CD40^+$) in blood. IIP-2 activated the greatest numbers of B and T cells among four tested adjuvant conditions. Fig. S2(A and B) shows the representative scatter plots of T cell ($CD3^+CD4^+$, $CD3^+CD8^+$) and B cell ($CD19^+CD40^+$) in blood.

3.5. Enhancing effects of polysaccharides on splenocyte cytokine secretion

Levels of the cytokines IL-2, IL-4, IL-10 and IFN-γ, all of which are secreted by splenocytes, were measured using commercial ELISA kits. As shown in Fig. 5A and D, the levels of IL-2 and IFN-γ secreted from splenocytes isolated from mice in the IIP-A-1 and IIP-2 groups were significantly higher than those detected in the other experimental groups. In Fig. 5B and C, the levels of IL-4 and IL-10 in all polysaccharide groups were higher than those in the mock and alhydrogel groups.

3.6. Immunization with polysaccharide adjuvants protects mice against lethal challenge with a street rabies virus strain

To evaluate whether the immune responses induced by the administration of inactivated rCVS-11-G mixed with polysaccharides could provide protection against a rabies infection, mice were challenged with street rabies virus strain HuNPB3. All mice from the polysaccharide groups survived, whereas one mouse from the aluminum hydroxide group and three mice from the control group did not. None of the mice from the PBS group survived. The results of the challenge test are shown in Fig. 6A. Clinical symptoms and RT-PCR results produced by assessing total RNA from brain tissues collected from the dead mice revealed that the mice died from rabies infection (data not shown). The results of the challenge test suggest that IIP-A-1 and IIP-2 can provide complete protection against street rabies virus infection when mixed with an inactivated rabies vaccine.

To determine the protective effects of polysaccharides as adjuvants in post-exposure prophylaxis, mice were challenged with HunPB3 24 h before immunization. Only one mouse from the aluminum hydroxide group survived the challenge. In contrast, 3 mice from the IIP-A-1 group and 7 mice from the IIP-2 group survived for the duration of the observation period, whereas all mice in the mock group died (Fig. 6B). Clinical symptoms and RT-PCR results produced by assessing total RNA isolated from brain tissues collected from the dead mice indicated that the mice died from rabies infection (data not shown).

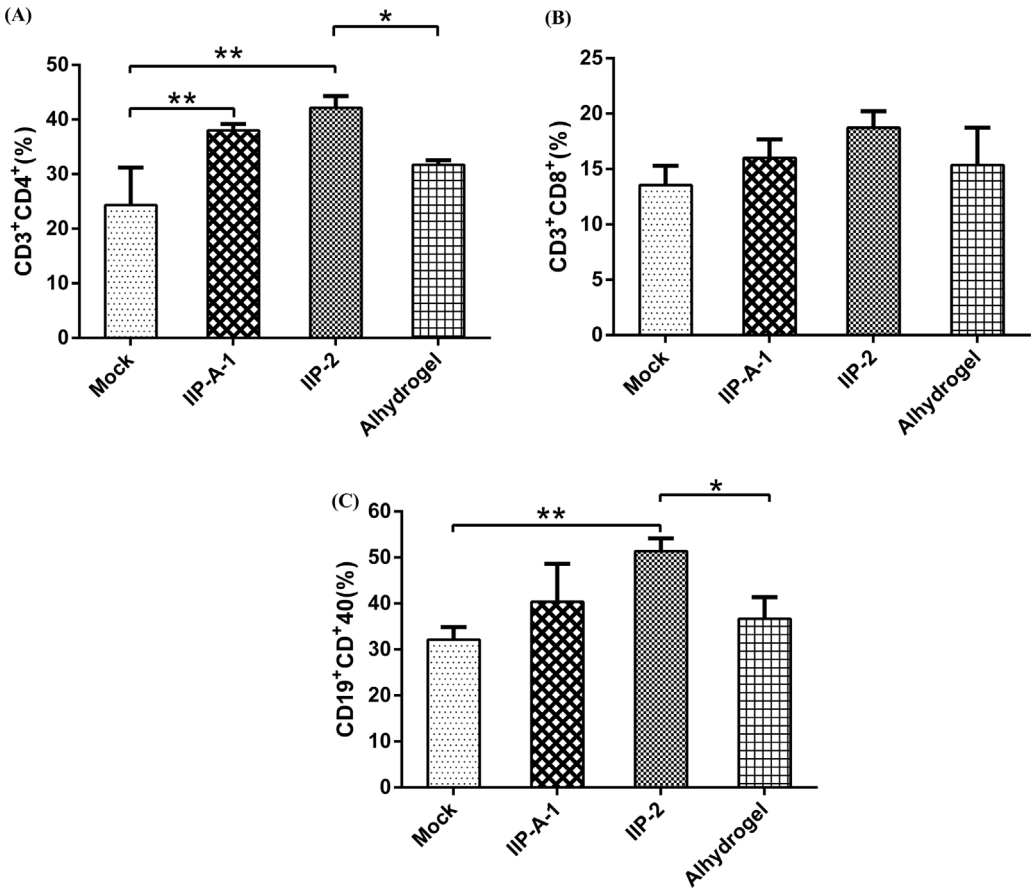

Fig. 4. Blood was collected from mice on day 6 after primary immunization. The proliferation of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells and $CD19^+CD40^+$ B cells following treatments with different adjuvants was measured by flow cytometry. (A) and (B) show $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, and (C) shows B cells following activation with different adjuvants. The cells in (A), (B) and (C) were collected from the blood of 3 mice per group. The data represent the subtraction value means of 3 mice with SDs and were analyzed using one-way ANOVA (*$p < 0.05$, **$p < 0.01$).

4. Discussion

Due to safety concerns, all human rabies vaccines in use today are inactivated virus vaccines. Although these vaccines are safe, they induce only mild immune responses and therefore require multiple injections to generate antibody production, which proceeds at a slow rate [10]. Post-exposure immunization is the main immunization method used in human. The incubation period for rabies can be as short as one week, and rabies immune globulin is needed in severe cases [1]. The recommend dose for equine rabies immune globulin is 40 IU/kg [34], which translates into a need for 2000 IU to 3000 IU of equine immune globulin per individual. In other words, at least 2 vials or doses of rabies immune globulin are required per case. The cost of such a treatment regime is barely affordable for the inhabitants of developing countries. The cost for one dose of equine rabies immune globulin in Cambodia, a developing country in Southeast Asia, ranges from US$20 to US$35. This is far too expensive for the farmers living in the rural areas of Cambodia, whose monthly income is typically less than US$80 [35]. Although the dose recommended for human rabies immune globulin (HRIG) is only 20 IU/kg, but the cost of HRIG is even more expensive. Post-exposure prophylaxis may also fail if insufficient immune globulin is used or if the immune globulin is used improperly [34,36]. Such scenarios occur in developing countries because of their lack of skilled physicians. Thus, because of the high cost of rabies immune globulin, improving the antibody production rate is critical for generating a post-exposure rabies vaccine that is effective for human use, especially in developing countries. An adjuvant that can accelerate the generation of antibody responses and induce cellular immunity would be optimal. Aluminum hydroxide, a traditional, widely used adjuvant, stimulates the immune response by inducing Th2 responses, prolonging the exposure of an antigen to the immune system and delaying antigen clearance from an immunization site [16,17,37]. These mechanisms contribute only slightly to early antibody responses and cellular immunity. Furthermore, side effects, such as severe inflammatory and nervous system concerns, may appear in certain cases. Polysaccharides are emerging as a new type of adjuvant that can be used to stimulate a rapid immune response, thereby serving as a highly valuable vaccine addition.

Although β-glucan is the most well-known immune response stimulant [38], a few studies have reported that α-glucan can also be used as an adjuvant for the same purpose in human and veterinary vaccines. RR1, an (1 → 4)-α-D-glucan with a molecular weight of 550 kDa that was isolated from *Tinospora cordifolia*, has been shown to activate lymphocytes in vitro and stimulate their secretion of IL-1β, IL-6, IL-12, IL-18, IFN-γ and tumor necrosis factor (TNF)-α, but not their secretion of IL-2, IL-4 or IL-10 [39]. IIP-A-1 is a (1 → 4)-α-glucan with a molecular weight of only 3600 Da. IIP-A-1 was evaluated in the current study for its capacity to serve as an immune stimulator. To accomplish this, splenocytes were isolated and cultured in vitro, and the levels of secreted IL-2, IL-4 and IL-10 were assessed; in all three cases, secretion was significantly enhanced (IL-2, $p = 0.006$; IL-4, $p = 0.000$; and IL-10, $p = 0.036$). We also measured rabies-specific VNA titer, the most important gauge of the effectiveness of a rabies vaccine. We found that mice that received IIP-A-1 as a vaccine adjuvant had significantly higher VNA

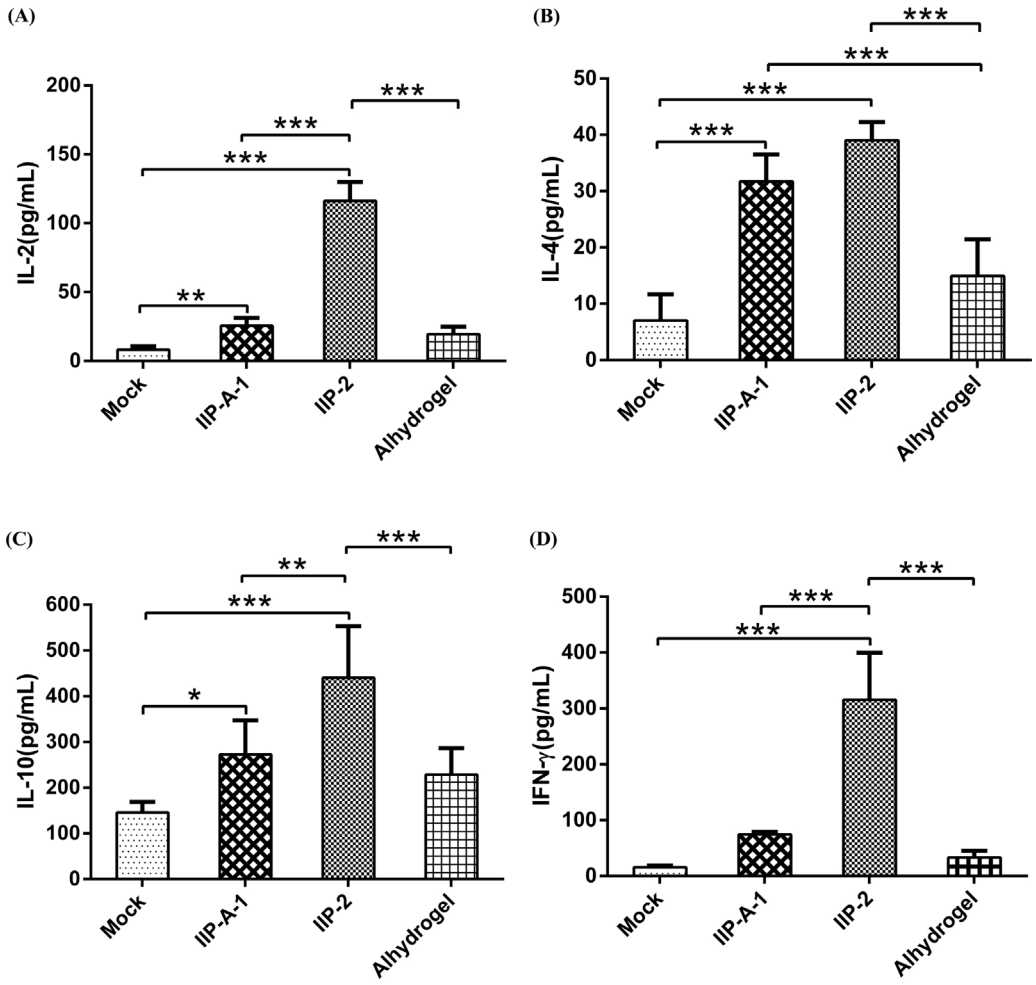

Fig. 5. ELISA results showing the quantities of IL-2, IL-4, IL-10 and IFN-γ secreted by splenocytes. Spleens were isolated from 6 mice per group 14 days after the final vaccination, and isolated splenocytes were cultured and stained as described in Section 2. IL-2 (A), IL-4 (B), IL-10 (C) and IFN-γ (D) levels were measured using a commercial ELISA kit. Representative data are shown as the means ± SDs of 6 mice per group and were analyzed using one-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

titers against the rabies virus than did mice that received aluminum hydroxide on days 21 and 28 post-immunization ($p_{21} = 0.003$ and $p_{28} = 0.045$). Cellular immunity also plays an important role in resistance to rabies infection and may clear the rabies virus from the CNS [40]. Th1 cells, which are derived from CD4 cells, secrete type 1 cytokines, such as IL-2 and IFN-γ, which possess antiviral activities and stimulate $CD8^+$ cell proliferation [41,42]. Additionally, Th2 cells, also derived from CD4 cells, secrete type 2 cytokines, such as IL-4 and IL-10, which induce helper activity in B cells and stimulate the humoral immune response [43]. These cytokine profiles indicate that RR1 mediates immunity via the Th1 pathway, while IIP-A-1 mediates immunity via the Th2 pathway.

Arabinogalactans are highly branched polysaccharides that can easily dissolve in water; these molecules have high molecular weights and consist of L-arabinose and D-galactose moieties [44]. Several studies have shown that the adjuvant activities of arabinogalactans largely depend on their molecular weights, chemical structures and polysaccharide chain conformations [45,46]. ResistAid™, an arabinogalactan product isolated from the larch plant with a highly branched structure and a 1 → 3,6-β-D-galactan backbone, is composed of arabinose and galactose units in a volumetric ratio of 6:1. Its adjuvant qualities in relation to IgG production were tested orally in a double-blind parallel-group study evaluating the 23-valent pneumococcal vaccine. No signs of toxicity or side effects were observed when ResistAid™ was used at a dose of 4.5 g/day for 10 weeks in healthy volunteers [30]. Larch plant arabinogalactans also appear to have low toxicity, as no side effects or signs of toxicity were apparent in either mice or rats when they were administered at a dose of either 5 g/kg or 500 mg/kg for 3 months [47]. In the current study, another arabinogalactan, IIP-2, which was isolated from the roots of *I. indigotica*, was evaluated. IIP-2 possesses a 1 → 3,6-β-D-galactan backbone similar to that found in larch arabinogalactans. Its adjuvant qualities were assessed using the inactive rabies virus rCVS-11-G. We first measured the rabies-specific antibody response, the most important indicator of vaccine efficacy. The results of an FAVN test showed that mice treated with IIP-2 had significantly higher VNA titers than mice treated with aluminum hydroxide after a second vaccination ($p_{21} = 0.000$, $p_{28} = 0.009$, and $p_{42} = 0.036$). A few studies have reported that arabinogalactans can enhance the effectiveness of cellular immune responses. For example, G1-4a, an arabinogalactan isolated from *T. cordifolia* that has a chemical structure similar to IIP-2, was shown to activate bone marrow-derived dendritic cells (BMDCs) in vitro and spleen dendritic cells in vivo. Secretion of the cytokines IL-12 and TNF-α from BMDCs and T cell stimulation also increased [29]. In the current study, we evaluated antigen-specific cellular immune responses by ELISpot and ICS. Our results indicate that IIP-2 can activate spleen cells and stimulate them to secrete IL-2, IL-4, IL-10 and IFN-γ. These findings suggest that IIP-2 can mediate immune responses though both the Th1 and Th2 pathways. Flow cytometry analysis showed that mice treated with IIP-2 had higher numbers of activated T and B lymphocytes in their blood and lymph nodes than

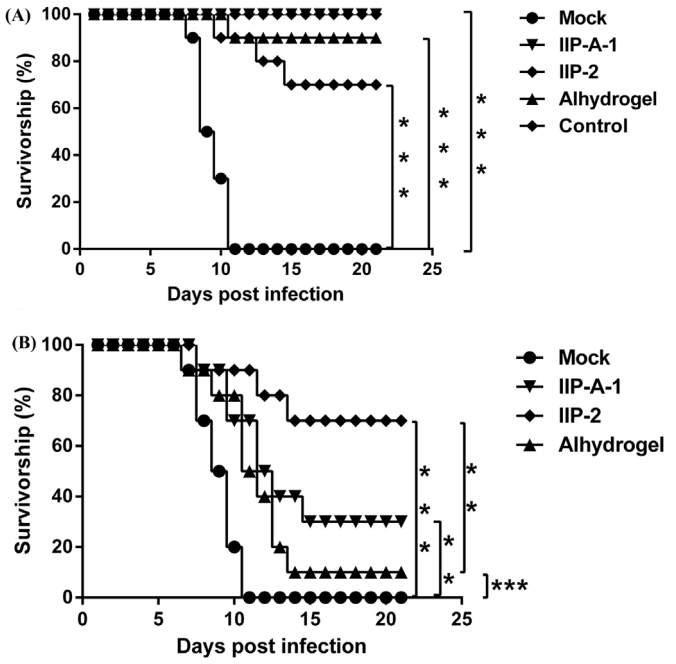

Fig. 6. Challenge test in mice. All mice in each group ($n = 10$) were challenged with 100× $IMLD_{50}$ of the RABV street stain HuNPB3 by intramuscular injection 14 days after the second vaccination and were observed for 21 days. The results are shown in (A). The survival numbers from each group at different times post-challenge are shown. Post-exposure immune test in mice. Mice were challenged with 10× $IMLD_{50}$ HuNPB3 24 h before immunization and were observed for 21 days. The results are shown in (B). The survival numbers from each group at different times post-challenge are shown. Significant differences of survival rates between groups were assessed by Kaplan–Meier (**$p < 0.01$, ***$p < 0.001$).

control mice. The results of challenge and post-exposure immune tests showed that IIP-2 mixed with rCVS-11-G could protect mice from a challenge with a lethal street rabies virus strain.

Both of the polysaccharides analyzed in the current study, particularly IIP-2, enhanced antigen-specific cellular immune responses in spleen cells by activating T cells and promoting cytokine secretion. In contrast, aluminum hydroxide did not significantly affect either spleen cells or lymphocytes. Together, these results suggest that the polysaccharides evaluated here can significantly enhance cellular immune responses and accelerate antibody responses to an inactivated rabies vaccine. Enhanced B and T lymphocyte proliferation and increased cytokine secretion may be the principal mechanisms by which this enhancement is achieved.

Aluminum hydroxide could only induce a slow antibody response that included the secretion of only a few cytokines. Thus, our results suggest that plant root polysaccharides may be more effective for improving antibody responses than traditional adjuvants when used in conjunction with an inactivated rabies vaccine. This increased effectiveness accelerates immune protection and reduces the required vaccine dosage. Overall, our results indicated that both of the polysaccharides tested in this study, in particular IIP-2, can be used as adjuvants in conjunction with an inactivated rabies vaccine to induce robust immune responses in animals and potentially in humans. In conclusion, our work highlights the potential of using polysaccharides to develop a more affordable post-exposure vaccine for use in humans.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.02.023.

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