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The mucosal adjuvant effect of plant polysaccharides for induction of protective immunity against *Helicobacter pylori* infection

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ABSTRACT

Some plant polysaccharides (PPSs) had been used as the adjuvants for systemic vaccination. In this study, we investigated whether PPSs could exhibit adjuvant effect at the mucosa. Groups of mice were intranasally immunized with Epimedium Polysaccharide (EPS), Trollius chinensis polysaccharide (TCPS), Siberian solomonseal rhizome polysaccharide (SSRPS) and Astragalus polysaccharides (APS) together with ovalbumin (OVA). Significantly higher levels of OVA-specific IgG in serum and secretory IgA in saliva, vaginal wash and intestinal lavage fluid were induced after immunization with OVA plus one of the four PPSs compared to OVA alone. Antigen absorption and TLR2 (Toll-like receptor 2) activation may be related to their mucosal adjuvant effect. Of note, when APS used as an adjuvant, intranasally vaccination with recombination UreB (rUreB, Urease subunit B) conferred more robust protection against *Helicobacter pylori* (*H. pylori*). Immunized with rUreB in combination APS resulted in mixed specific Th1 and Th17 immune response, which may contribute to the inhibition of *H. pylori* colonization. Though specific Th2-dominant responses were elicited when the other three PPS intranasally immunized with rUreB, no significant difference in the protective effect were found between those groups and rUreb alone group. Taken together, the four PPSs may be promising candidates for mucosal adjuvant, and APS could enhance rUreB-specific protective immunity against *H. pylori* infection.

1. Introduction

Vaccine is one of the most important ways for preventing the spread of infectious diseases. Though with excellent immunogenicity, traditional vaccine types, such as live-attenuated vaccines and inactivated vaccines, have been limited applied because they might revert to virulence [1]. Various new types of vaccines with high purity and solubility thus have been raised in recent years, such as recombinant subunit vaccines, synthetic peptide vaccines. However, these vaccines alone failed to show a satisfactory immunogenicity [2], and the inclusion of an adjuvant may enhance the immune response [3].

Aluminum-based adjuvants have been widely used in human vaccination, which could induce antibodies response, but do not induce cytotoxic T cell and cell-mediated immunity [4]. Although many new proposed adjuvants for human beings including AS03, AS04 and MF59 can enhance both humoral and cellular immune response, they are unable to be administered directly at the mucosa [5]. Mucosal response is of great importance for defense against infections since many pathogenic infections occur at the mucosal surface [6]. Over the past few decades, endeavors have been done to explore new potential mucosal adjuvants. Cholera toxin (CT) and the closely related Escherichia coli heat-labile enterotoxin (LT) had been proved to be potent mucosal adjuvants [7], but clinical trials had found that oral delivery of LT and CT can result in diarrhea [8], whereas intranasal administration of LT can lead to Bell palsy [9]. Interleukin-1 (IL-1), interleukin-12 (IL-12) and interleukin-33 (IL-33) were effective mucosal adjuvants [10], but using cytokine as mucosal adjuvant should not be realistic because of high production costs and potential safety risk [11]. Liposomes can be endocytosed by antigen-presenting cells (APCs) and serve effectively as mucosal vaccine delivery system. But due to their limitations such as instability in low pH and difficult manufacturing process, liposomes have not been widely used

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[12]. Therefore, there are many challenges in successful development of mucosal adjuvant.

Many studies indicated that PPSs, with the advantage of special features and low toxicity [13], had been employed as a potent vaccine adjuvant. Taiwan Ganoderma lucidum polysaccharide PS-F2 can stimulate DC maturation, antibody production, Th1 polarized immune response and antitumor cytotoxic T cell activation [14]. When injected with rabbit hemorrhagic disease vaccine, epimedium polysaccharide (EPS) and astragalus polysaccharides (APS) can significantly enhance serum antibody titer and promote lymphocyte proliferation, which showed better adjuvant effect than aluminum adjuvant [15]. Low doses of APS and isatis root polysaccharide, high doses of Achyranthes bidentata polysaccharides and Chinese yam polysaccharide can enhance the antibody titer, peripheral blood T lymphocyte proliferation and CD4+/CD8+ratio [16]. A polysaccharide-protein complex isolated from Lycium chinense induced DC maturation, and up-regulated the expression of CD40, CD80, CD86 and MHC class II molecules [17]. However, these PPSs were usually used as the adjuvants for systemic vaccination. It is unknown whether PPSs could exhibit adjuvant effect at the mucosa.

In the preliminary experiments, over 10 PPSs were screened in mice to identify those capable of exerting potent mucosal adjuvant activity. EPS, Trollius chinensis polysaccharide (TCPS), Siberian solomonseal rhizome polysaccharide (SSRPS) and APS showed mucosal adjuvant effect (data not shown). In this study, we systematically evaluated the mucosal adjuvant effect of the above four PPSs by intranasal immunization with ovalbumin (OVA). Then, the four PPSs were combined with recombination urease subunit B (rUreB), a candidate vaccine against *H. pylori*, and the protective efficacy was determined by challenging mice orally with *H. pylori* B0, which expresses significant urease activity [18]. Systemic and mucosal immune responses were also evaluated.

2. Materials and methods

2.1. Animals and cell lines

SPF female BALB/c mice of 6–8 weeks were purchased from the Experimental Animal Center of Third Military Medical University. All animal experiments were approved by the Animal Ethical and Experimental Committee of the Third Military Medical University. Human normal kidney epithelial cells, HK-2 was purchased from ATCC (American Type Culture Collection). HEK-Blue™-mTLR2 cells were purchased from InvivoGen (San Diego, CA, USA). Cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL Normocin, 50 U/mL penicillin, 50 g/mL streptomycin and passaged when 70% confluence was reached according to the manufacturer's instructions. Cells were scraped and resuspended in HEK-Blue™ Detection medium (InvivoGen, San Diego, CA, USA), and then SEAP was observed by using a spectrophotometer at 620 nm [19].

2.2. Reagents

EPS and TCPS were prepared by the method as described previously [20]. SSRPS (S26701) and APS (SA9790) were obtained from Solarbio (Beijing, China) and Yuanye (Shanghai, China). Albumin from chicken egg white (A5503) was purchased from Bellancom Chemistry (Beijing, China). rUreB was expressed in *Escherichia coli* as previously described [21]. Mouse IL-17A precoated ELASA kit (DKW12-2170-096), Mouse IL-4 precoated ELASA kit (DKW12-2040-096) and Mouse IFN-γ precoated ELASA kit (DKW12-2000-096) were purchased from Dakewe (Shenzhen, Guangdong, China). *Helicobacter Pylori* Medium (HB8674) was the product of Hopebio (Qingdao, Shandong, China). Anti-Rabbit IgG Fc (HRP) (ab97200), Anti-Mouse IgA alpha chain (HRP) (ab97235), Goat Anti-Mouse IgG1 heavy chain (HRP) (ab97240) and Goat Anti-Mouse IgG2a heavy chain (HRP) (ab97245) were the product of abcam (Cambridge, MA, USA).

2.3. Hemolysis assay

Mice blood was washed three times with saline by centrifugation at 2500 rpm for 5 mins. The cell suspension was prepared by finally diluting the pellet to 2% in saline. A volume of 0.5 mL of the cell suspension was mixed with 0.5 mL of diluent containing 10, 5, 2.5 and 1.25 mg/mL of PPS in saline. The mixtures were incubated for 3 h at 37 , and centrifuged at 2500 rpm for 10 mins. The free hemoglobin in the supernatants was measured spectrophotometrically at 570 nm with a microplate reader (BIO-RAD, Berkeley, CA, USA.). Water was used as a positive control and saline was used as a negative control. Each experiment included triplicates at each concentration. Hemolysis rate (%) = (sample absorption - negative control absorption)/(positive control absorption - negative control absorption) × 100% [22].

2.4. Cell viability

The logarithmic growth phase HK-2 cells were digested by 0.25% trypsin, then the cells were centrifuged, and diluted with DMEM medium to a 5×10^4 cells/mL. Cell suspension (180 μL) was added to each well of a 96-well plate and stabilized for 12 h. PPSs in gradient concentration (20 μL) were then added to the experimental group, and the same amount of PBS was added as the control group, and each group has four duplicate wells. The plate was incubated for 48 h at 37 °C in an incubator shaker, and then 20 μL of a 10% 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT) solution (5 mg/mL) diluted in PBS was added to each well [23]. After 4 h incubation, the mixture was centrifuged and 200 μL of dimethylsulfoxide (DMSO) was added into the sediment, shaking for 1 min on a shaker. Cell viability was calculated using the absorbance (A) measured with a microplate reader (BIO-RAD, Berkeley, CA, USA.) at a detection wavelength of 570 nm, Cell viability = A (experiment group)/A (control group) × 100%.

2.5. Microscale thermophoresis (MST)

The OVA protein was fluorescently labeled according to the NanoTemper Monolith NT Protein Labeling Kit RED-NHS kit procedure, and excess dye was discarded within 45 mins after marking. Four PPSs were gradient dissolved in a buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM, $MgCl_2$, 0.05% Tween-20) from the highest concentration of 1250 μg/mL to the lowest of 1.2 μg/mL. A mixture of 10 μL non-labeled molecules with gradient dilution and 10 μL labeled molecules with fixed concentration, was set for reaction, and then was loaded into glass capillaries and analyzed by Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) [24].

2.6. TLR2 signaling assay

HEK-Blue™ Detection was prepared according to manufacturer's recommendation. In a flat bottom 96-well plate, the test sample and negative control were 20 μL PPS, and 20 μL endotoxin-free water, respectively. HEK-Blue™ mTLR2 cells passaged to 70% confluence was scraped, centrifuged and resuspended in the prewarm detection with a concentration of 2.8×10^5 cells/mL. Cell suspension (180 μL, approximately 5×10^4 cells) was then immediately added to each well, and incubated the plate at 37 °C in 5%

CO_2 for 14 h. Secreted Embryonic Alkaline Phosphatase (SEAP) activity can be determined using a spectrophotometer at 620 nm [25].

2.7. Animal immunization

BALB/c mice were immunized intranasally three times at two-week intervals with PBS, the antigen (10 μg OVA or 5 μg rUreB), or the antigen plus one of the four PPSs (50 ug/mice). As a positive control, one group of mice was adjuvanted with 1.2 μg LTK63. The final volume for each immunization was 20 μL. On day 33, saliva and vaginal wash were collected and mice were sacrificed, sera and for further analysis [26].

2.8. Antibody production assay

Serum specific IgG levels were determined by standard indirect ELISA. First, 96-well microplates were coated overnight with the antigen (OVA/rUreB). After blocking with 5% bovine serum albumin (BSA), levels of antigen-specific IgG antibodies were measured by testing serial dilutions of 1:1000 pre-diluted sera followed by detection with an HRP-coupled goat anti-mouse IgG as secondary antibody. Color was developed by addition of o-phenylenediamine dihydrochloride (OPD) together with H_2O_2. The absorbance at 450 nm was measured in a microplate reader (Bio-Rad). The ELISA titer is defined as the highest dilution yielding an absorbance of two-fold that of normal control mouse sera. The level of specific sIgA level in saliva, vaginal wash or intestinal lavage fluid was measured by ELISA as described above.

2.9. H. Pylori challenge and quantification

Two weeks after the last immunization with rUreB or rUreB adjuvanted with one of the four PPSs, mice were challenged orally four times with 10^9 BALB/c mouse-adapted *H. pylori*. Four weeks post challenge, the colonization by *H. pylori* was quantified by real-time PCR using the TaqMan method, amplifying 16S rDNA of *H. pylori* as previously described [24]. The following primers were used: forward, 5′-TTTGTTAGAGAAGATAATGACGGTATCTAAC-3′, reverse, 5′-CATAGGATTTCACACCTGACTGACTATC-3′, probe, 5′-FAM-CGTGCCAGCAGCCGCGGT-TAMRA-3′ [27]. The assay was carried out on a Bio-Rad iQ5 multicolor Real-time PCR Detection System using the absolute quantification option.

2.10. ELISA for cytokines

Splenic lymphocytes from the immunized mice were re-stimulated with rUreB for 48 h. The coculture supernatants were collected and kept at −80 °C until used. Cytokines from Th1/Th2/Th17 cells, such as IFN-γ, IL-4, and IL-17A, were measured by ELISA kit (Dakewe, China) according to the manufacturer's instructions. Absorbance was detected using a microplate reader (Thermo Fisher, USA) at 450 nm.

2.11. Statistical analysis

All data were analyzed using the SPSS 21.0 statistical package. Continuous variables were expressed as means ± standard deviation (SD). Inter-group differences were analyzed by using one-way ANOVA, and differences with a p value ≤ 0.05 were considered statistically significant. Pairwise comparisons were further performed if Inter-group differences were statistically significant, and for Bonferroni test, significant level $\alpha' = \alpha/k$ (k is the number of comparison times).

3. Results

3.1. In vitro safety of the four PPSs

To evaluate the possibility of in vivo application of the four PPSs, the cytotoxicity to the HK-2 cells was determined by MTT assay. Incubation of ECPS, TCPS, SSRPS and APS with the concentration of 2.5–10 mg/mL showed that those PPSs had almost no cytotoxicity to the HK-2 cells (Fig. 1A). The degree of hemolysis caused by the four PPSs exposed to mice blood cells is shown in Fig. 1B and C. The percent hemolysis was concentration dependent. At 3 h, the

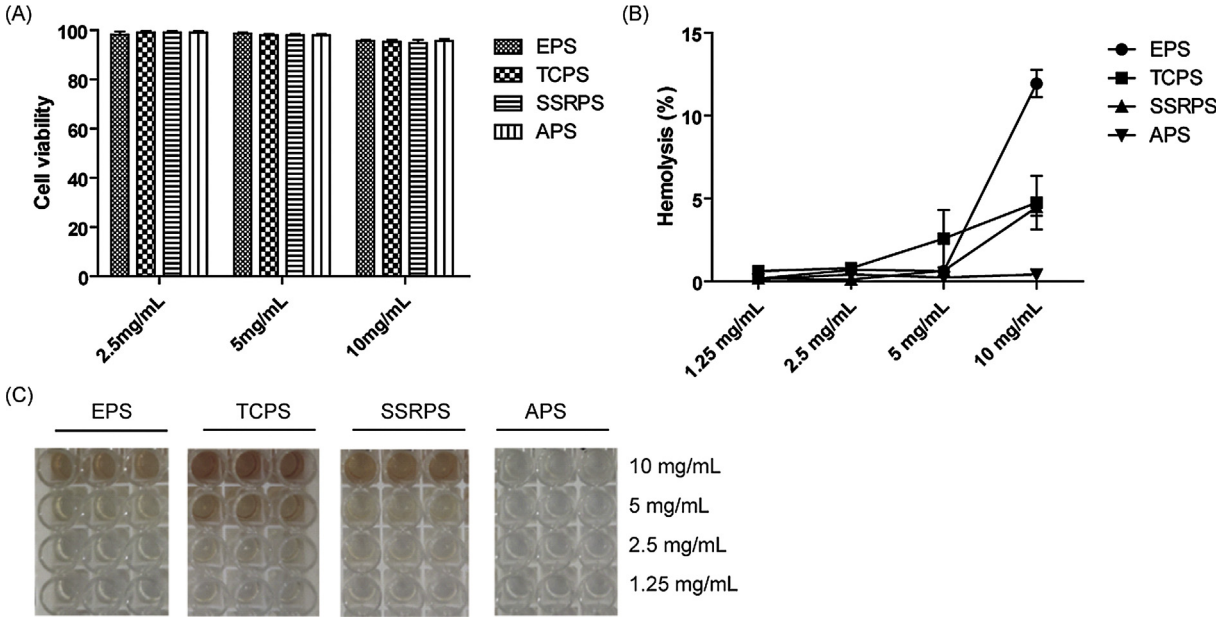

Fig. 1. Cell viability and Hemolysis assay. (A) HK-2 cells were incubated with different PPSs for 48 h, and then cell viability was quantified with an MTT assay (data are expressed as mean ± S.D., n = 3). (B) Hemolytic effects were investigated by incubating erythrocytes with the diluent containing 10, 5, 2.5 or 1.25 mg/mL of different PPSs in saline (data are expressed as mean ± S.D., n = 3). (C) Hemolysis was observed in a flat-bottom 96-well microtiter plate.

four PPSs caused <2% hemolysis even at the concentration of 5 mg/mL. Those results indicated that those PPSs were non-toxic at the concentration below 5 mg/mL and were suitable as mucosal adjuvants.

3.2. Intranasal immunization with OVA plus the PPS elicits a strong specific serum IgG response

Mice were intranasally immunized with OVA plus one of the four PPSs, and OVA-specific serum IgG titers were measured by

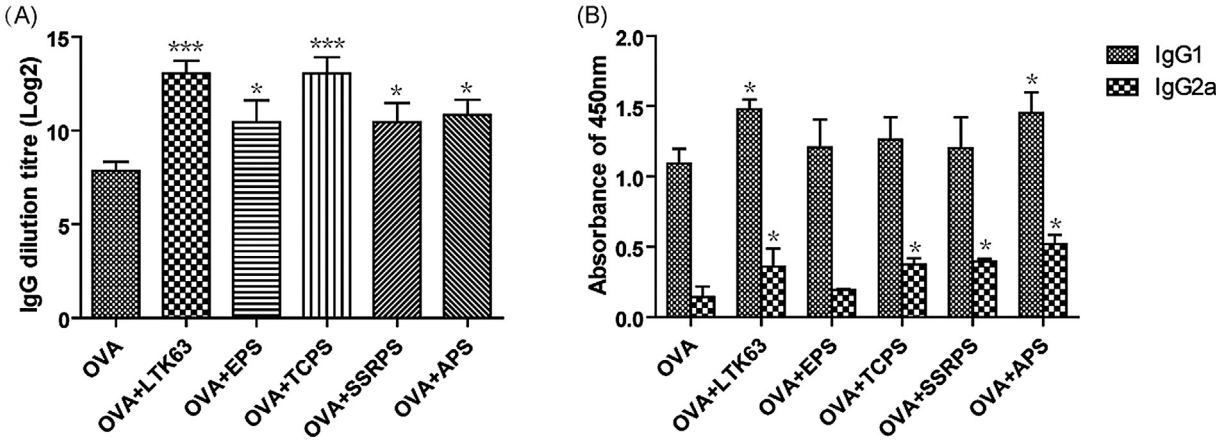

Fig. 2. Serum OVA-specific IgG antibody profile of mice immunized with OVA plus different PPSs. BALB/c mice were immunized intranasally three times at two-week intervals with OVA or OVA plus one of the four PPSs. As a positive control, one group of mice was adjuvanted with LTK63. The serum was collected at two weeks after the last immunization. (A) OVA-specific serum IgG antibody titers were measured by ELISA. (B) The level of specific IgG1 and IgG2a against OVA in serum samples were tested by ELISA. Data are expressed as mean ± S.D., n = 5. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, compared with OVA alone group.

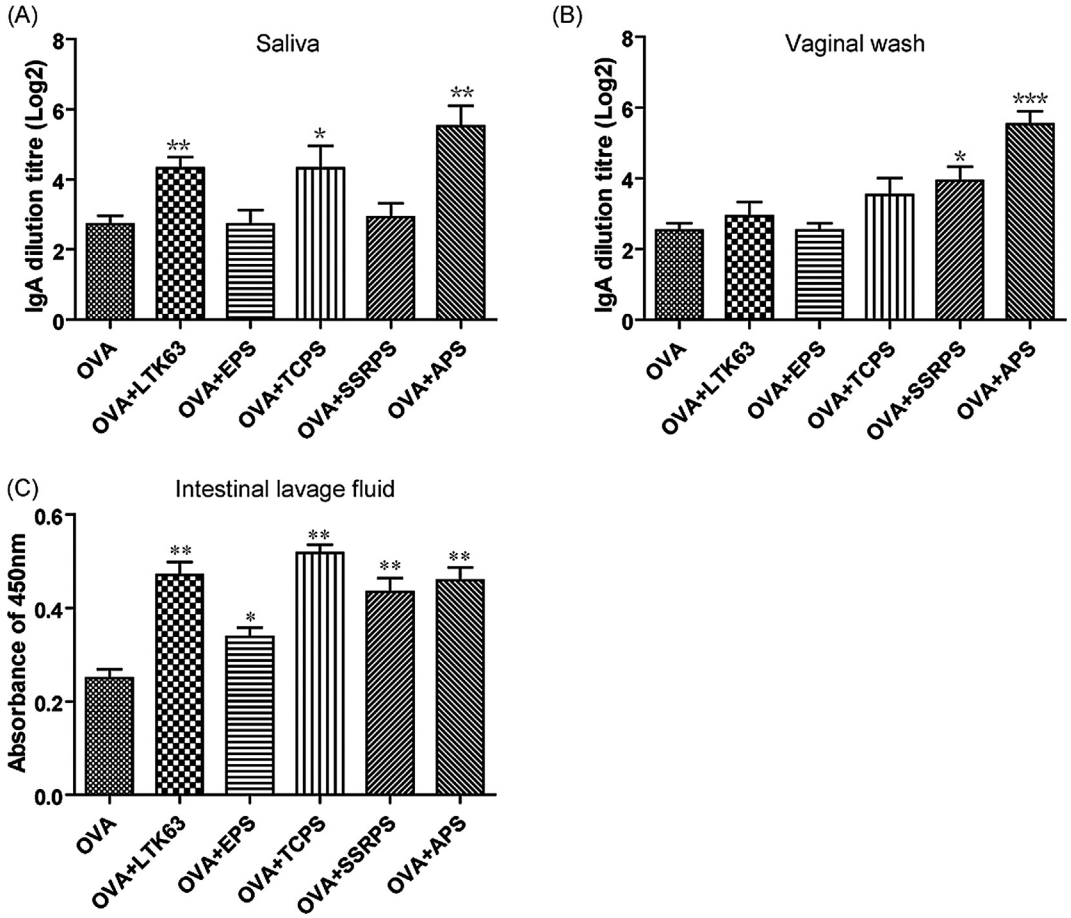

Fig. 3. Secretory IgA level in saliva (A), vaginal wash (B) or intestinal lavage fluid (C). BALB/c mice were immunized intranasally three times at two-week intervals with OVA or OVA plus one of the four PPSs, LTK63 as a positive control. The mucosal secretions was collected at two weeks after the last immunization. Data are expressed as mean ± S.D., n = 5. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, compared with OVA alone group.

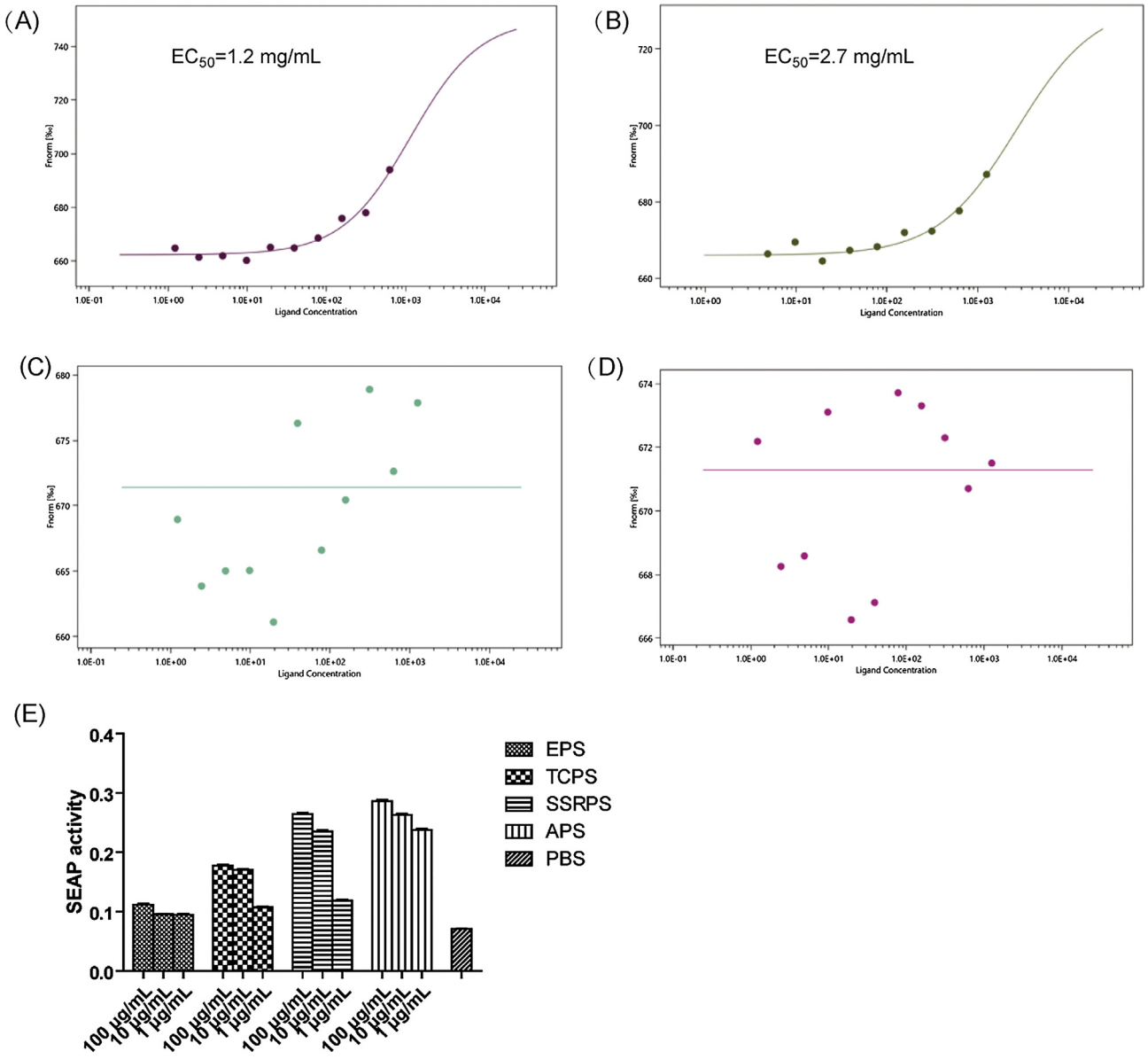

Fig.4. MST analysis and TLR2 signaling assay. (A) The affinity of OVA to EPS, TCPS, SSRPS or APS was measured by MST and the EC_{50} values were calculated respectively. (B) The effect of different PPS treatment on secreted embryonic alkaline phophatase (SEAP) release from HEK-Blue™ mTLR2 cells. Data are expressed as mean ± S.D., n = 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ELISA. As shown in Fig. 2A, combination with the four PPSs enhanced the level of specific IgG, and the titer of IgG antibodies induced by OVA+TCPS was similar to that of LTK63, which has strong mucosal adjuvant properties. IgG2a and IgG1 were markers for Th1 and Th2 responses respectively, and the levels of OVA-specific IgG2a and IgG1 were also determined. Compared with OVA alone group, both OVA-specific Th1 and Th2 response was elevated by APS or LTK63, however, only Th1 response were significantly induced by TCPS and SSRPS (Fig. 2B). Those results suggested that different PPSs may induce distinct types of CD4+T cell responses.

3.3. Intranasal immunization with OVA plus the PPS enhances secretary IgA level at mucosal sites

As for the mucosal immunization, secretions (saliva, vaginal wash and intestinal lavage fluid) were collected to detect the level of secretary IgA (sIgA). As shown in Fig. 3A-C, all four PPSs enhanced sIgA in intestinal lavage fluids, and APS enhanced sIgA in all secretions. There was no significant difference in the humoral response between each PPSs alone and PBS groups, including the titer of specific serum IgG and mucosal sIgA (data not shown).

3.4. Antigen absorption and TLR2 activation may be related to mucosal adjuvant effects of the PPSs

Strong adsorption was considered to be one of the most important mechanisms for vaccine adjuvants to work [28]. In this study, MST was used to quantify the binding affinities of PPSs with OVA. As shown in Fig. 4A–D, both EPS and TCPS, but neither SSRPS nor APS, could absorb to OVA with EC_{50} of 1.2 mg/mL and 2.7 mg/mL. This result indicated that the mucosal adjuvant activity of EPS and TCPS may be associated with their adsorption to antigen.

Studies have shown that the immunomodulatory effects of PPSs may be related to their activation of TLR2 [29,30]. To further study the underlying mechanism of PPSs as mucosal immune adjuvants,

they were experimentally tested in luciferase reporter assays using HEK293-TLR2 cells. As shown in Fig. 4E, all four PPSs significantly augment the NF-kB activation in TLR2 reporter cells in a dose-dependent manner. Among them, APS has the strongest activation effect. In addition to antigen absorption, the role of these four PPSs in mucosal immune adjuvant may be also related to their ability to activate TLR2.

3.5. APS enhanced rUreB-induced protective effect against H. Pylori infection

Since the four PPSs could help to stimulate antigen-specific mucosal sIgA, especially intestinal sIgA, we further investigated whether intranasal immunization with rUreB and those PPSs could confer more robust protection against *H. pylori* infection. BALB/c mice were immunized with rUreB plus one of the four PPSs, and then orally challenged with mouse-adapted *H. pylori* strain B0. Bacteria colonizing in gastric mucosa were measured by real-time PCR. The level of *H. pylori* colonization in the stomach of all immunized groups was significantly lower than that in the PBS group. In the absence of rUreB, no protective effect against *H. pylori* was observed in mice immunized with each PPSs alone (data not shown). The number of copies of *H. pylori* in the four groups adjuvanted with EPS, TCPS and SSRPS had no significant difference in bacterial load, which was comparable to that in the rUreB group. Particularly, adjuvanted with APS provided better protection than rUreB alone, even better than those adjuvanted with LTK63 (Fig. 5).

3.6. rUreB plus APS elicited higher level of gastric and intestinal specific sIgA

In order to investigate the protective mechanism of APS assisted rUreB against *H. pylori*, the levels of rUreB specific IgG antibody in serum of immunized mice were detected. As shown in Fig. 6A, the titer of specific IgG antibodies induced by all PPSs in combination with rUreB were similar to that of rUreB alone group, suggesting that the elevated protective effect against *H. pylori* of APS on rUreB may not be dependent on the induction of serum IgG antibody.

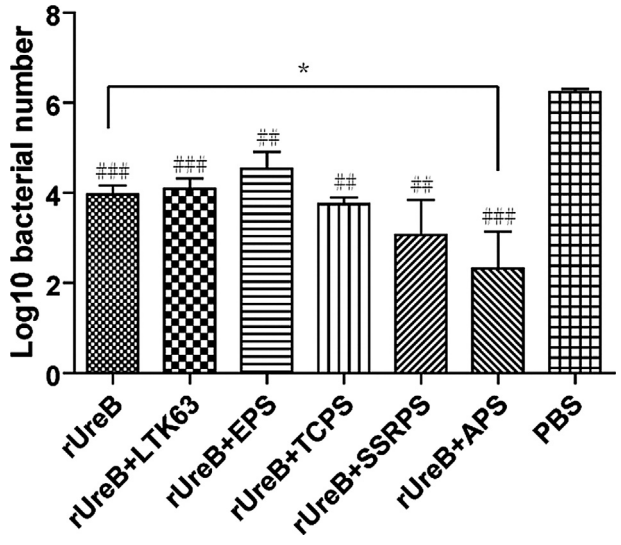

Fig. 5. Colonization of *H. pylori* in stomachs of mice prophylactically immunized with rUreB plus different PPSs. BALB/c mice were immunized intranasally on day 0, 14 and 28 with rUreB or together with different PPS. Controls were treated with PBS. Two weeks after final vaccination, mice were challenged orally four times with *H. pylori* B0. Four weeks post challenge, levels of gastric *H. pylori* colonization were determined by real-time quantitative PCR. Data are expressed as mean ± S.D., n = 10. $^{*}P < 0.05$, $^{\#\#}P < 0.01$ compared with PBS control, $^{\#\#}P < 0.001$ compared with PBS control.

Gastric and intestinal antigen-specific sIgA antibody were believed to play a role in blocking *H. pylori* infection. The levels of local sIgA antibody in the stomach and intestine was also evaluated. As shown in Fig. 6B and C, the titer of specific sIgA antibody in gastric homogenates induced by rUreB plus APS or SSRPS was significantly higher than that in rUreB alone group. Though the gastric sIgA level in the LTK63 group was also increased, but failed to reach significance. In addition, rUreB plus APS or TCPS triggered higher intestinal sIgA production than other treatments. Though immunization with EPS or SSRPS could elevate antigen specific intestinal sIgA, no significant increase was found, possibly due to the large variability in those groups. These results suggested that the ability of APS to enhance both gastric and intestinal rUreB specific sIgA may be related to the significant better protective role against *H. pylori* infection.

3.7. Induction of specific Th1 and Th17 responses by rUreB immunization adjuvanted with APS

To investigate whether vaccine-mediated CD4+T cell response contributed to protective immunity against *H. pylori* infection, splenic lymphocytes collected from immunized mice were restimulated and the CD4+T cell response was measured by the levels of IFN-γ, IL-4 and IL-17 production in supernatants, respectively. As shown in Fig. 7A–C, APS can significantly increase the expression levels of IFN-γ and IL-17, suggesting that APS can effectively enhance rUreB specific Th1 and Th17 response. Many studies have shown that antigen specific Th1 and Th17 immune responses play a crucial role in protecting against *H. pylori* infection [20–22]. The expression level of IL-4 in the supernatants of splenic lymphocytes in rUreB plus EPS or SSRPS was significantly higher than that of rUreB group alone, but no better protective effect was observed, indicating that there may be no significant correlation between vaccine-mediated Th2 response and the protective efficacy in this study.

4. Discussion

Adjuvants are non-specific immunopotentiators which play a critical role in enhancing vaccine-mediated protection. Nevertheless, few adjuvants are licensed for human vaccines, thus there is a critical need to develop safe and effective adjuvants. A variety of active constituents extracted from natural plants have been reported to work as adjuvants, improving humoral and/or cellular immunity, such as polysaccharides, flavonoids and saponins [31,32]. These components always have the advantage of less toxicity, wide availability and easy to produce. In summary, natural plant derived substances are potential vaccine adjuvant candidates.

Natural PPSs are one of the most widely researched plant-derived vaccine adjuvants. Advax^{TM} adjuvant, PS-F2 (ganoderma lucidum polysaccharide), and APS have been respectively used in protection against an actual influenza pandemic, Listeria infection in mice, and systemic candidiasis in humans [33]. However, these PPSs were generally used as the adjuvant for systemic immunization, and their mucosal adjuvant efficacy had not yet been systematically evaluated. The most studied mucosal adjuvants were bacterially-derived adenosine diphosphate (ADP)-ribosylating enterotoxins, including CT, and mutants ofLT. These enterotoxins promoted induction of antigen-specific sIgA antibodies and long-lasting memory when co-administered with antigens orally or intranasally. Safety issues hamper the full realization of the potential of such effective mucosal adjuvants. Intranasal immunization,

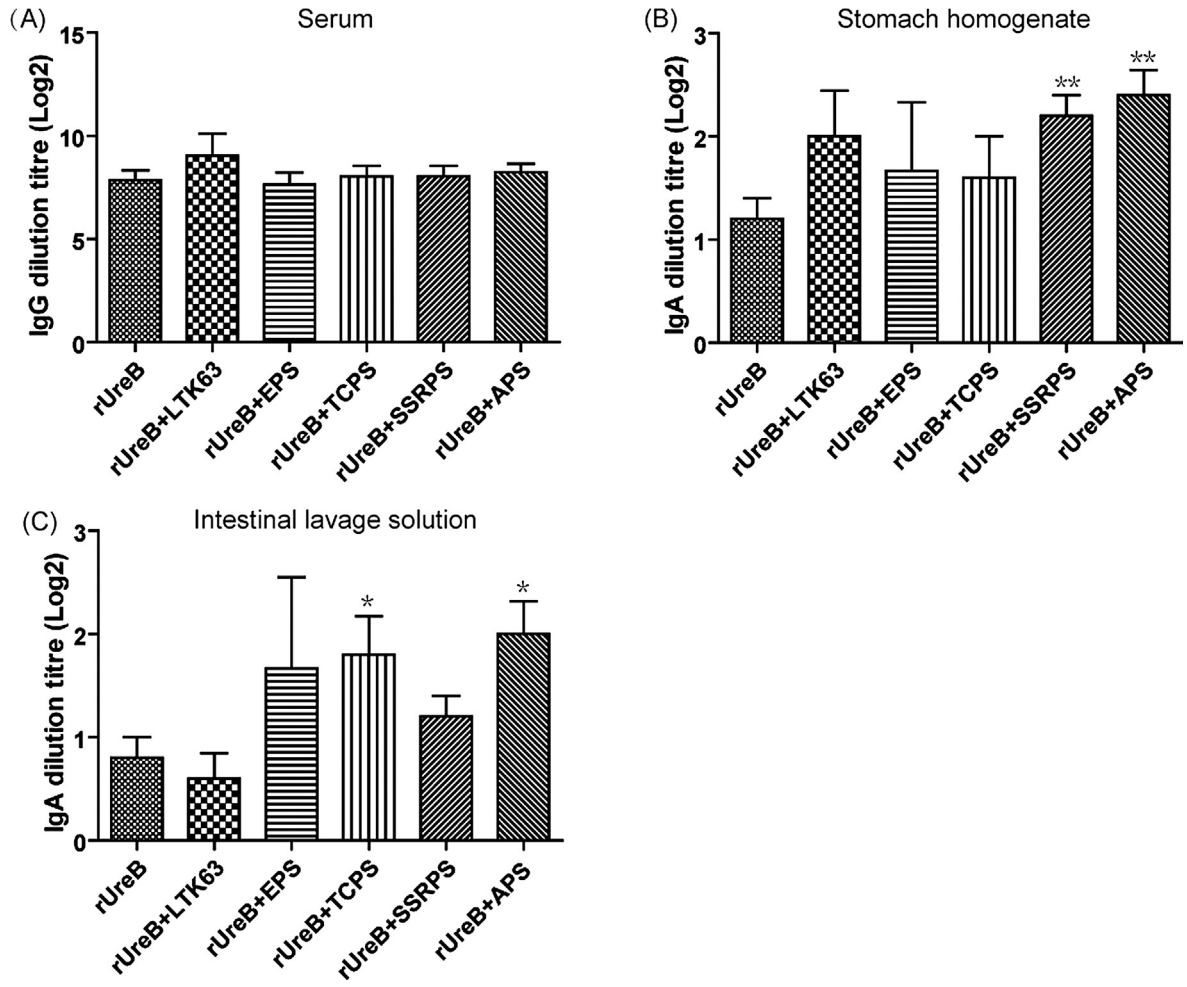

Fig. 6. Anti-rUreB antibody responses elicited by intranasal immunization with rUreB plus different PPS. BALB/c mice were immunized intranasally on day 0, 14 and 28 with rUreB or together with different PPS. The serum, stomach homogenate and intestinal lavage fluid were collected at 2 weeks after final immunization. rUreB-specific serum IgG antibody titers (A), secretory IgA levels in stomach homogenate (B) and intestinal lavage fluid (C) were measured by ELISA. Data are expressed as mean ± S.D., n = 5. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with rUreB alone group.

even low-toxic mutants, could induce Bell's palsy [9]. In this study, EPS, TCPS, SSRPS, and APS had a satisfactory hemolytic tolerance and no significant cytotoxicity when the concentrations were lower than 5 mg/mL. All four PPSs combined with OVA were able to induce specific serum IgG responses, and the titer induced by TCPS was comparable to that of LTK63. Moreover, four PPSs could also significantly enhance specific sIgA production in intestinal lavage fluids, saliva, or vaginal wash, but the ability of different PPSs to stimulate sIgA varied from different mucosal sites. Intranasal immunization in combination with OVA, EPS could elevate the expression of sIgA in intestinal lavage fluids, SSRPS could improve the titer of sIgA in vaginal wash and intestinal lavage fluids, TCPS could increase the expression of sIgA in saliva and intestinal lavage fluids, while APS could significantly increase the sIgA levels in all investigated secretions.

Adjuvants can enhance the immune response to the antigen in different ways, such as antigen absorption, TLR activation and promoting dendritic cells maturation [34,35]. It was realized that strong adsorption of the adjuvant to antigen may ensure a high localized concentration of antigen for a period of time, that may be sufficient to allow antigen uptake and APCs activation [36]. In the current study, EPS and TCPS could significantly adsorb to OVA. Further detection of TLR2 activation indicated that the four PPSs could significantly activate TLR2 with a dose-dependent manner, among which APS showed the strongest activation effect. We hypothesize that PPSs may boost immune response through the two complementary pathways of antigen adsorption and TLR2 activation. However, the mechanisms of the four PPSs were not exactly the same, leading to their different mucosal adjuvant effects when co-administrated with OVA.

A variety of studies had reported the mechanism of vaccine-mediated protection against *H. pylori* infection. Vaccine-induced serum IgG antibody level was considered not to directly be associated with the protection, while specific sIgA antibody in the gastrointestinal tract could contribute to the protective effect [37]. Also studies emphasized that CD4+T cells were essential for vaccine-induced protection against *H. pylori* infection [38], and the importance of urease specific MHC Class II–restricted responses to modulate the protection [39]. Further studies had shown that both Th1 and Th17 response correlate with vaccine-induced protection, and mainly Th17 effector mechanisms are of critical importance to protection against *H. pylori* [40]. In the current study, APS was able to significantly enhance the protective effect of rUreB against *H. pylori*. Enhanced gastric and intestinal rUreB specific sIgA production was observed when mice were intranasally immunized with rUreB plus APS. In addition, APS significantly increased the levels of Th1 and Th17 cytokines in supernatants of splenic lymphocytes from immunized mice. Though specific sIgA and Th2 response were elicited by rUreB adjuvanted with other PPSs, bacterial loads reduction in those groups were

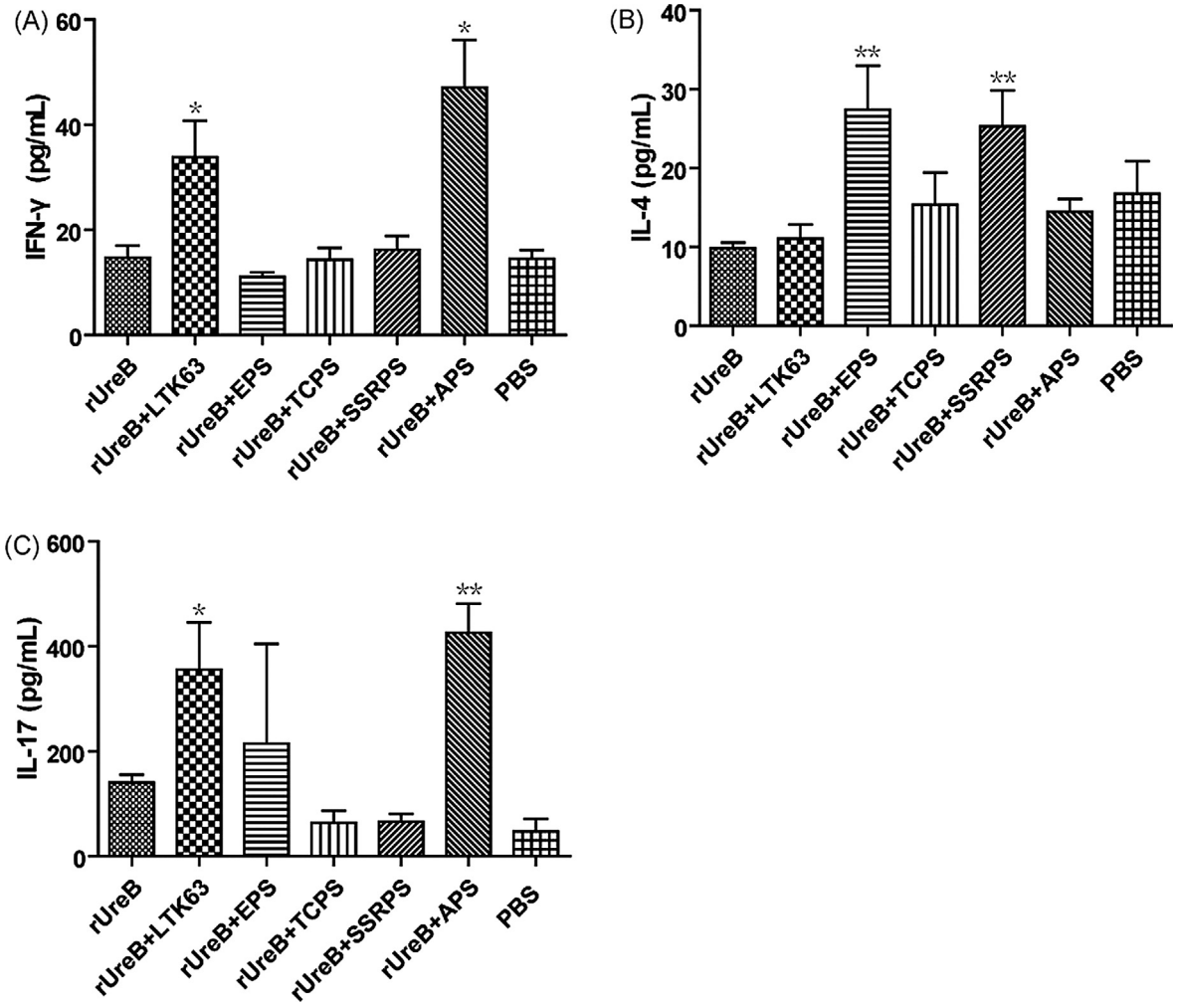

Fig. 7. Cytokines production after stimulation of splenic lymphocytes with rUreB. Mice were immunized intranasally with rUreB or rUreB together with the PPS. Six week after final immunization, splenic lymphocytes from vaccinated group were isolated and stimulated with 250 μg/mL rUreB for 48 h. ELISA assays were used to measure the accumulation of IFN-γ (A), IL-4 (B), and IL-17 (C) in the supernatants of culture. Data are expressed as mean ± S.D., n = 5. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with rUreB alone group.

comparable to that in the rUreB alone group. The collective evidence indicates that the APS-enhanced specific mucosal sIgA production and Th1/Th17 response may contribute to elevated protective efficacy against *H. pylori* infection after intranasal vaccination with rUreB plus APS.

The four PPSs were able to induce elevated specific mucosal sIgA antibodies when OVA or rUreB was used as the antigen. Although together with OVA they augmented serum levels of specific IgG, the level of serum IgG was not significantly improved when combined with rUreB. TCPS and SSRPS significantly increased the level of serum IgG2a antibody in mice immunized with OVA, indicating that they could enhance the OVA-specific Th1 response. However, SSRPS significantly enhanced the expression levels of Th2 cytokine in the supernatant of splenic lymphocytes from rUreB-immunized mice. The antigenic differences between OVA and rUreB may lead to the difference in response when the PPSs were mixed with the two antigens.

In this study, the four PPSs were proved to be candidates for mucosal immune adjuvants. Intranasal immunization with rUreB plus APS provided better protective efficacy against *H. pylori* infection, which may have been mediated by elevated specific sIgA level in gastrointestinal tract and Th1/Th17 CD4+T cell response. In future studies, we will further explore the mechanisms of immune stimulation by the four PPSs. Though systemic cellular response played an important role in the protection against *H. pylori*, the PPSs-induced local cellular response in Peyer's patches will be investigated. In addition, recent studies emphasized the importance of mucosal IgM to modulate the progress of some pathogen infection, especially to prevent infection after mucosal HIV exposure [41], we will try to determine whether the PPSs could enhance the induction of secretary IgM in mucosal sites. For the improvement of their adjuvant activity, chemical structure modification and structure-activity relationship studies would be the object of intensive study.

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