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Adjuvant effect of polysaccharide from fruits of *Physalis alkekengi* L. in DNA vaccine against systemic candidiasis

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ABSTRACT

Adjuvant effect mediated by polysaccharide (PPSB) isolated from the fruits of *Physalis alkekengi* L. in DNA vaccine was evaluated in mice. Recombinant plasmid containing epitope C (LKVIRK) from heat shock protein 90 (HSP90) of *Candida albicans* (*C. albican*) was used as DNA vaccine (pD-HSP90C). The results indicated that PPSB significantly enhanced specific antibody titers IgG, IgG1, IgG2b, and concentration of IL-2 and IL-4 in sera of mice immunized with pD-HSP90C ($p < 0.05$). More importantly, it was found that the mice immunized with pD-HSP90C/PPSB not only had fewer CFU (colony forming unites) in the kidneys than mice immunized with pD-HSP90C, but also a statistically significant higher survival rate over PBS-injected group ($p < 0.05$) when the immunized mice were challenged with living *C. albican* cells. However, no statistically significant difference in survival rate was observed between pD-HSP90C-immunized group and PBS-injected group. Therefore, PPSB can be considered as a promising adjuvant eliciting both Th1 and Th2 responses to enhance the efficacy of DNA vaccines.

1. Introduction

Candidiasis is a disease caused by members of the genus, Candida. The most common species that causes candidal infections is *Candida albicans* (*C. albicans*). Systemic candidiasis arises as a result of a normal commensal fungus when the normal microflora balance is disrupted in the host (LeibundGut-Landmann, Wüthrich, & Hohl, 2012; Magliani et al., 2012; Vecchiarelli, Pericolini, Gabrielli, & Pietrella, 2012). Over the past two decades, morbidity and mortality of systemic candidal infection has increased significantly even in the presence of antifungal therapy (Gudlaugsson et al., 2003; Hajjeh et al., 2004; Lin, Schranz, & Teutsch, 2001; Pfaller et al., 2004). Consequently, antifungal vaccines is currently considered one of the most attractive strategies (Brown, Denning, & Levitz, 2012; Netea & Brown, 2012).

Previous findings revealed that antibodies of 47-kDa antigen, which is a breakdown product of heat shock protein 90 (HSP90), are protective in systemic candidiasis. Antibodies of 47-kDa antigen are against epitope C (LKVIRK) which is shared by *C. albicans* and human HSP90 in all patients infected with systemic candidiasis (Matthews & Burnie, 1992; Matthews, Burnie, Howat, Rowland, & Walton, 1991; Matthews, Burnie, & Lee, 1991; Matthews, Burnie, & Tabaqchali, 1984). Matthews et al. (Matthews, Hodgetts, & Burnie, 1995) have shown recombinant antibodies to epitope LKVIRK can mediate protection by neutralizing the circulating candida HSP90; Wang and co-workers (Yang et al., 2005) further indicated that hybrid-phage expressing epitope C (LKVIRK) can confer protective immune responses against systemic candidiasis. Although DNA vaccines represent an attractive strategy for developing new vaccines as new technologies become available, only one study was found investigating the protective effect of DNA vaccine encoding HSP90 in murine model of systemic candidiasis. The results showed that the intramuscular application of DNA vaccine HSP90 was poorly immunogenic and did not provide any significant protection (Raska et al., 2005). Similarly, we focused on the development of DNA vaccine (pD-HSP90C) containing epitope C (LKVIRK) of *C. albican* HSP90 against systemic candidiasis in this study.

Compared to traditional vaccines, DNA vaccines are relatively inexpensive, homogeneous, heat stable, and safe (Best et al., 2009; Hirao et al., 2008). However, DNA vaccines are poorly immunogenic, despite the fact that they can induce humoral and cellular immune responses (Ferraro et al., 2011). An attractive approach to improve immunogenicity of DNA vaccines is the co-administration

Abbreviations: CFU, colony forming unites; HSP90, heat shock protein 90; pD-HSP90C, a recombinant plasmid containing epitope C (LKVIRK) from HSP90 of *C. albicans*; PPSB, polysaccharide isolated from fruits of *P. alkekengi*; rP-HSP90C, a recombinant protein containing epitope C (LKVIRK) from HSP90 of *C. albicans*.

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of immunologic adjuvants with DNA vaccines. In fact, a number of adjuvants have been explored to improve plasmid DNA immunogenicity by directly stimulating the immune system or enhancing plasmid DNA expression (Greenland & Letvin, 2007). Unfortunately, alum adjuvant is not optimally effective for diseases where cell-mediated immunity is required for protection (Aguilar & Rodríguez, 2007; Guy, 2007; Reed, Bertholet, Coler, & Friede, 2009; Schijns & Lavelle, 2001). Although QuilA can stimulate Th1 and Th2 immune responses, high toxicity and the undesirable haemolytic effect limit its use in human vaccines (Sun, Xie, & Ye, 2009; Vitoriano-Souza et al., 2012). Therefore, novel adjuvant capable of strongly eliciting both humoral and cellular immune responses (both Th1 and Th2 responses) is necessary to maximize the efficacy of new or existing vaccines (Petrovsky, 2006; Silva, Cooper, & Petrovsky, 2004).

It has been reported that polysaccharides from herbal plants play a critical role in immune system functions, and they are less immunogenic, non-toxic, and biodegradable. Thus, polysaccharides can be one of the ideal adjuvant candidates (Gao, Wang, Wang, & Wang, 2013; Petrovsky & Cooper, 2011; Pi et al., 2013; Sun, 2011; Sun, Liu, Yu, & Gong, 2010). *Physalis alkekengi* L. var. *francheti* (*Mast.*) *Makino* (*P. alkekengi*) is widely distributed in Europe and Asia countries including Russia, China, Japan, *etc.* Our group previously reported the purification, structure characterization, and the preliminary activity assessment of polysaccharide PPSB (designated PPSB below) isolated from fruits of *P. alkekengi* (Tong, Liang, & Wang, 2008; Li et al., 2011). The present study further investigates adjuvant efficacy of polysaccharide in DNA vaccine.

In this study, we constructed DNA vaccine pD-HSP90C containing epitope C (LKVIRK) of *C. albican* HSP90, and detected the immunologic enhancement of PPSB in mice immunized with DNA vaccine pD-HSP90C.

2. Materials and methods

2.1. Preparation of polysaccharide PPSB isolated from fruits of P. alkekengi

We have previously reported the purification, structure characterization, and the hypoglycemic activity assessment of polysaccharide PPSB isolated from fruits of *P. alkekengi* (Tong, Liang, & Wang, 2008). PPSB (Mw = 27 kDa) is an acid heteropolysaccharide consisting of Ara, Gal, Glc and GalA in ratio of 2.6:3.6:2:1. Briefly, fresh fruits (1 kg) of *P. alkekengi* were decocted with distilled water at 100 °C for 3 h. The crude polysaccharide (CP) was extracted from the decoction by precipitating with 85% ethanol. CP dissolved in distilled water was frozen at −20 °C, thawed repeatedly to remove insoluble materials by centrifugation. CP was further precipitated with 50% ethanol to discard the residue, and the supernatant was further precipitated with 70% ethanol to obtain precipitate (PPSA). After being deproteinated by a combination of proteinase and Sevag method, PPSA was further purified on a Sepharose CL-6B column eluted with 0.15 mol/L NaCl, and the main polysaccharide fraction (PPSB) was collected, dialyzed and lyophilized. PPSB was used as an adjuvant in DNA vaccine. The endotoxin level in PPSB solution was less than 0.5EU (endotoxin unit)/mL. The solution of PPSB was sterilized by 0.22 μm millipore filter for all animal experiments.

2.2. Experimental animals

Six- to eight-week-old male ICR mice (body weight 20 ± 2 g) were obtained from Beijing HFK Bio-Technology Co., LTD, Beijing, China. Animals were maintained in the animal facility of Northeast Normal University following the guidelines of LARC (Laboratory Animal Resource Center) of NENU (Northeast Normal University). All surgeries were performed under diethyl ether anesthesia, and all efforts were made to minimize suffering.

2.3. Candida albicans and sera

C. albicans strain ATCC 10231, which is a laboratory-adapted strain with less virulence in animal models, was obtained from Institute of Microbiology, Chinese Academy of Science, Beijing, China. *C. albicans* was grown on Sabouraud's medium for 12 h at 30 °C. The yeast cells were collected to infect ICR mice. The human sera were from the First Clinical Hospital of Jilin University, Changhun, China. Permissions of using sera were obtained from The Regional Ethical Review Board of Jilin University and Northeast Normal University. Written consents were obtained from patients infected with systemitic candidiasis and healthy people.

2.4. Construction and preparation of recombinant protein rP-HSP90C

rP-HSP90C was a recombinant protein containing epitope C (LKVIRK) from HSP90 of *C. albicans*. The recombinant protein rP-HSP90C was prepared for analysis of specific antibody against rP-HSP90C by ELISA and Western blot. The recombinant plasmid (pET28a-HSP90C) containing epitope C (LKVIRK) from HSP90 of *C. albicans* was constructed by our lab. The complete genome was isolated from *C. albicans* strain ATCC 10231 by phenol–chloroform extraction method, and used as the template for PCR (Santhanam & Burnie, 2000). Briefly, genes (700 bp) containing epitope C (LKVIRK) was amplified by PCR; it was then cloned into the prokaryotic expression plasmid pET28a by the EcoRI and XhoI restriction enzyme sites. pET28a-HSP90C was confirmed by DNA sequencing. rP-HSP90C was expressed in *E. coli* BL21(DE3) by pET28a-HSP90C under the control of the T7 promoter and *lac* operator. rP-HSP90C was expressed as a fusion protein containing T7 and His tag peptides, and purified by NiNTA agarose column (Qiagen). The antigenicity of rP-HSP90C was analyzed with sera of patient infected with systemic *C. albicans* in addition to sera of mice immunized with rP-HSP90C by Western blot. The following is the inserted sequences:

GAATTC(EcoRI)ATCACTGATGATGCTGAAGAGTTGATTCCAGAA-TGGTTAAGTTTCATCAAGGGGGTTGTCGATTCCGAAGACTTGCCATT-GAACTTGTCCAGAGAAATGTTGCAACAAAACAAGATTTTGAAAGTT-ATCAGAAAG(epitope C)AACATTGTCAAAAAGATGATTGAAACTTTC-AATGAAATCTCTGAAGACCAGGAACAATTCAACCAATTCTACACTGC-TTTCTCCAAGAACATCAAGTTGGGTATTCATGAAGATGCTCAAAACA-GACAATCTTTGGCTAAATTGTTGAGATTCTACTCTACCAAATCTTCTG-AAGAAATGACTTCCTTGTCTGACTACGTTACTAGAATGCCAGAACAC-CAAAAGAATATCTACTACATCACTGGTGAATCCATCAAAGCCGTTGA-AAAATCACCATTCTTGGATGCCTTGAAAGCTAAGAACTTTGAAGTCT-TGTTCATGGTGGATCCAATCGATGAATATGCCATGACTCAATTGAAG-GAATTTGAAGACAAGAAATTGGTTGATATTACCAAAGACTTTGAATT-GGAAGAAAGTGACGAAGAAAAAGCTGCTAGAGAAAAGGAAATCAA-AGAATACGAACCATTGACCAAAGCTTTGAAAGATATTCTTGGTGATC-AAGTTGAAAAAGTTGTTGTTTCCTACAAACTTGTTGATGCTCCAGCTG-CCATTAGAACTGGTCAATTTTAA(stop code)CTCGAG(XhoI)

2.5. Recombinant protein rP-HSP90C immunization

In order to detect antigenicity of rP-HSP90C, ICR mice (5 mice/group) were immunized with 50 μg rP-HSP90C intraperitoneally three times at a 10-day interval (PBS used as the negative control). The mice were sacrificed one week after the last immunization. The immunized mice sera were collected to detect the specific antibody against rP-HSP90C by Western blot.

2.6. SDS-PAGE analysis for C. albicans total proteins and recombinant protein rP-HSP90C

C. albicans were grown overnight on Sabouraud's medium at 37 °C and harvested by centrifugation at 4000 rpm for 15 min. It was washed with buffer (50 mmol/Tris–HCl, pH6.8) prior to ultrasonic fragmentation (Matthews, Burnie, & Tabaqchali, 1987). The fragmented *C. albicans* were centrifuged at 4000 rpm for 15 min and proteins from the supernatant were detected by SDS-PAGE on 13% polyacrylamide gel (Matthews et al., 1991a,b). Recombinant protein rP-HSP90C was analyzed by SDS-PAGE with 12% polyacrylamide gel. The protein bands were stained by coomassie brilliant blue.

2.7. Western blot

The proteins (either recombinant protein rP-HSP90C or *C. albicans* total proteins) were transferred onto nitrocellulose membrane in buffer (25 mM Tris, 192 mM glycin, 20% methanol, pH 8.3) after electrophoresis, and were later blocked at 4 °C overnight with buffer (150 mM NaCl, 10 mM Tris, 3 (w/v) % skim milk, 0.05% Tween 20, pH7.5). The nitrocellulose membrane with transferred proteins was incubated with sera from immunized mice, patient sera, and healthy sera at 37 °C for 2 h after being washed three times in buffer (150 mM NaCl, 10 mM Tris–HCl, 0.05% Tween 20, pH 7.5). All sera were diluted to 1/40 when used. After being washed, the nitrocellulose membrane was incubated with peroxidase-conjugted goat anti-mice or goat anti-human HRP-IgG (Vector Laboratories Inc., USA) and stained with 3-amino-9-ethylcarbozole (AEC) to detect conjugated protein bands (Wang et al., 2006).

2.8. Construction and preparation of recombinant DNA vaccine

DNA vaccine pD-HSP90C was a recombinant plasmid containing epitope C (LKVIRK) from HSP90 of *C. albicans*. It was prepared by our lab. The complete genome, which was isolated from *C. albicans* strain ATCC 10231 by phenol–chloroform extraction method, was used as the template for PCR (Santhanam & Burnie, 2000). Genes (600 bp) containing epitope C (LKVIRK) from HSP90 of *C. albicans* was amplified by PCR, and cloned into mammalian expression vector pcDNA3.1 by the EcoRI and XhoI restriction enzyme sites. The recombinant plasmids pD-HSP90C was confirmed by DNA sequencing. Plasmids pD-HSP90C and pcDNA3.1 were transformed to *E. coli.* DH5α. pD-HSP90C and pcDNA3.1 were purified for vaccination with the aid of EndoFree Plasmid Mega kit (Tiangen, China). The following is the inserted sequences:
CTCGAG(XhoI)TTCATCAAGGGGGTTGTCGATTCCGAAGACTTGCCA-TTGAACTTGTCCAGAGAAATGTTGCAACAAAACAAGATTTTGAAAGT-TATCAGAAAG(epitopeC)AACATTGTCAAAAAGATGATTGAAACTTT-CAATGAAATCTCTGAAGACCAGGAAAATTCAACCAATTCTACACTGC-TTTCTCCAAGAACATCAAGTGGGTATTCATGAAGATGCTCAAAACAG-ACAATCTTTGGCTAAATTGTTGAGATTCTACTCTACCAAATCTTCTGA-AGAAATGACTTCCTTGTCTGACTACGTTACTAGAATGCCAGAACACC-AAAAGAATATCTACTACATCACTGGTGAATCCATCAAAGCCGTTGAA-AAATCACCATTCTTGGATGCCTTGAAAGCTAAGAACTTTGAAGTCTT-GTTCATGGTGGATCCAATCGATGAATATGCCATGACTCAATTGAAG-GAATTTGAAGACAAGAAATTGGTTGATATTACCAAAGACTTTGAATT-GGAAGAAAGTGACGAAGAAAAAGCTGCTAGAGAAAAGGAAATCAA-AGAATACGAACCATTGACCAAAGCTTTGAAAGATATTCTTGGTTAA-(stop code)GAATTC(EcoRI)

2.9. DNA vaccine immunization and electroporation in vivo

Preliminary experiments showed that mice immunized with pD-HSP90C containing PPSB (40 μg/mice) had a higher level of antibody titer against rP-HSP90C as compared to other groups immunized with different PPSB doses and that the peak of antibody titer occurred at one week after the last immunization (data not shown). Thus, PPSB (40 μg/mice) was used for all experiments.

The male ICR mice (6 mice per group) were immunized with either pD-HSP90C (10 μg/mice) alone or in presence of PPSB (40 μg/mice) twice at a 14-day interval. PBS and pcDNA3.1 (10 μg/mice) were used as controls. The mice were immunized intramuscularly in tibialis anterior muscle followed by electroporation with two-needle array electrodes (BTX, San Diego, CA) (Widera et al., 2000). Electroporation parameters *in vivo* were 20 V/mm distance between the electrodes; 50-ms pulse length; 6 pulses with reversal of polarity after 3 pulses, at 1, given by a BTX 820 square were genetor. The mice were sacrificed two weeks after the second immunization, and the immunized mice sera were collected to detect the titer of specific antibody against rP-HSP90C by ELISA and Western blot.

2.10. Measurement of specific antibody against rP-HSP90C in sera of immunized mice

rP-HSP90C specific antibodies (IgG, IgG1, and IgG2b) in sera of the immunized mice were evaluated by an indirect ELISA based on the methods of Yang (Yang et al., 2005). Briefly, microtiter plates (Nunc) were coated with 100 μL rP-HSP90C (50 mg/L) in 0.05 mol/L carbonate-bicarbonate buffer pH 9.6 for 24 h at 4 °C. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20, and blocked with PBS containing 5% skim milk powder at 37 °C for 1 h. After washing the wells with PBS containing 0.05% (v/v) Tween 20 three more times, 100 μL of a series of diluted sera from immunized mice or PBS containing 5% skim milk powder were added to the triplicate wells as control. The plates were then incubated for 1 h at 37 °C, followed by being washed with PBS containing 0.05% (v/v) Tween 20. Aliquots of 100 μL of goat anti-mouse IgG-HRP, IgG1-HRP, and IgG2b-HRP (diluted 1:5000 with PBS containing 5% skim milk powder respectively) were added to each plate. The plates were further incubated for 1 h at 37 °C. Substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to each well after being washed with PBST, and the plate was incubated for 15 min at room temperature. Reaction was terminated by adding 50 μL of 2 mol/L H_2SO_4 to each well, and optical density (OD) was detected at 450/630 nm with ELISA reader (Molecular devices, spectra MAX190, USA). The end point titer was used in this study. Antibody titer was expressed by Log_2 value of the highest dilution of serum that gave an absorbance value which exceeded an optical density of 0.05 and that was twofold greater than that of a matched dilution of sera from PBS-injected mice (Derrick, Yang, & Morris, 2005; Sun, 2006).

2.11. Detection of cytokines in sera of immunized mice

The quantities of IL-2, IL-4 in sera of immunized mice were determined by commercial ELISA kits following to the manufacturer's instruction. Briefly, sera of immunized mice or cytokine standards were added to 96-well flat-bottom microtiter plates coated with coating antibody, and plates were then incubated at 37 °C for 0.5 h. A HRP-Conjugate reagent was added to each well after the plates were washed. Plates were then incubated at 37 °C for 0.5 h, and they were washed and developed with tetramethyl benzidine (TMB) at 37 °C for 15 min. The reaction was stopped by addition of stop solution (50 μL). The absorbance was measured in an ELISA reader at 450 nm.

2.12. DNA vaccine pD-HSP90C vaccination and challenge

The male ICR mice (10 mice per group) were immunized with either pD-HSP90C (10 μg/mice) alone or in presence of PPSB

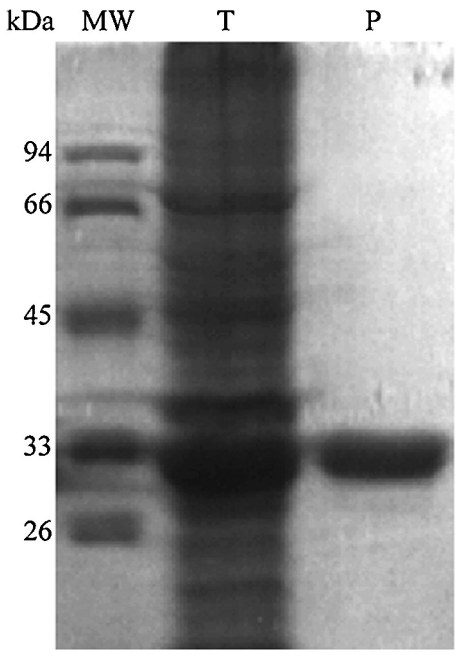

Fig. 1. SDS-PAGE of recombinant protein rP-HSP90C.
Purified rP-HSP90C was analyzed by SDS-PAGE (12%). Total expressed proteins (T). Purified protein rP-HSP90C (P).

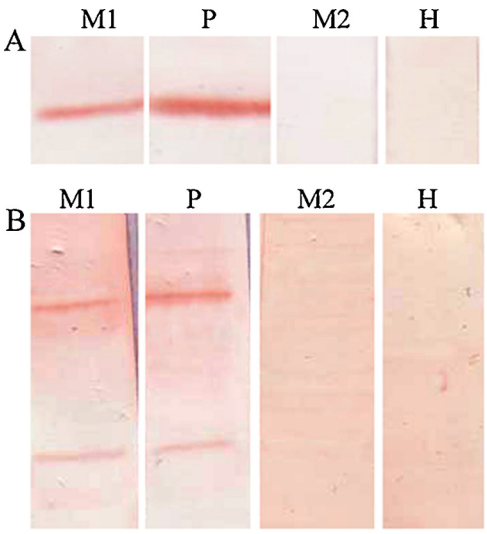

Fig. 2. Antigenicity analysis of recombinant protein rP-HSP90C. In order to detect antigenicity of rP-HSP90C, ICR mice were immunized with 50 μg rP-HSP90C or PBS intraperitoneally three times at a 10-day interval; the immunized mice sera were collected one week after the last immunization for analysis of Western blot. Sera from patient infected with systemic *C. albicans* infection and healthy people were used as positive and negative controls respectively. (A) Western blot analysis of recombinant protein rP-HSP90C using sera from the immunized mice, patient, and healthy people. rP-HSP90C was submitted to SDS-PAGE (12%), then transferred to nitrocellulose membrane and incubated with sera from the immunized mice, patient and healthy people. Sera from the mice immunized with rP-HSP90C (M1) and patient (P) recognized rP-HSP90C; however, sera from mice immunized with PBS (M2) and healthy people (H) did not recognize rP-HSP90C. (B) Western blot analysis of *C. albicans* total proteins using sera from immunized mice, patient, and healthy people. Total proteins extracted from *C. albicans* were submitted to SDS-PAGE (13%), then transferred to nitrocellulose membrane and incubated with sera from immunized mice, patient and healthy people. Sera from the mice immunized with rP-HSP90C (M1) and patient (P) recognized 90 kDa and 47 kDa protein bands of total proteins extracted from *C. albicans*; sera from the PBS-injected mice (M2) and healthy people (H) did not recognize any protein bands of *C. albicans* total proteins.

(40 μg/mice) twice at a 14-day interval, while PBS and pcDNA3.1 (10 μg/mice) were used as controls. The mice were immunized intramuscularly in tibialis anterior muscle followed by electroporation with two-needle array electrodes. Electroporation parameters *in vivo* were the same as mentioned above. The immunized mice were infected with live *C. albicans* cells (5×10^7) intravenously by the lateral tail vein two weeks following the second immunization (Yang et al., 2005). Protection was assessed by both monitoring survival rate for 15 days and Candida colony forming unites (CFU) of tissue from the kidneys of immunized mice. Briefly, kidneys of immunized mice were excised aseptically, weighed, and homogenized in sterile saline using tissue homogenizers. The tissue homogenate was diluted in saline and plated on Sabouraud's agar. Protection against systemic *C. albicans* infection was evaluated in terms of CFU per gram of tissue.

2.13. Statistical analysis

Data were expressed as mean ± SD. Survival rate was examined by Log Rank test. One-way ANOVA (SPSS software) was used in analyzing all the other data. *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Recombinant protein rP-HSP90C can be used as an antigen for analysis of specific antibody against rP-HSP90C due to its antigenicity

The DNA fragment containing epitope C (LKVIRK) from HSP90 of *C. albicans* was cloned into the prokaryotic expression to construct recombinant plasmid pET28a-HSP90C. pET28a-HSP90C was transfected into *E. coli* BL21(DE3) to express recombinant protein rP-HSP90C. rP-HSP90C was analyzed by SDS-PAGE shown in Fig. 1. SDS-PAGE analysis indicated rP-HSP90C was successfully expressed, and its molecular weight was approximately 34 kDa which was the same as expected.

To detect antigenicity of rP-HSP90C, sera of immunized mice were collected one week after the last immunization for analyzing the specificity of antibody against rP-HSP90C by Western blot. Western blot of rP-HSP90C (shown in Fig. 2A) revealed that the sera from mice immunized with rP-HSP90C (M1) and patients (P) with systemic *C. albicans* infection recognized rP-HSP90C; however, sera from PBS-injected mice (M2) and healthy people (H) did not recognize rP-HSP90C. The antigenicity of rP-HSP90C was further confirmed by Western blot analysis of total proteins extracted from *C. albicans*. The results (Fig. 2B) demonstrated that the sera from mice immunized with rP-HSP90C (M1) and patients (P) showed 47 kDa and 90 kDa protein bands of total proteins extracted from *C. albicans*; in contrast the sera of PBS-injected mice (M2) or healthy people (H) did not recognize any protein bands of *C. albicans* total proteins. These results indicated that rP-HSP90C has a high level of antigenicity. Therefore, it can be used as an antigen for analysis of specific antibody against rP-HSP90C evoked by DNA vaccine pD-HSP90C with ELISA and Western blot.

3.2. Construction and preparation of DNA vaccine pD-HSP90C

The DNA fragment containing epitope C (LKVIRK) from HSP90 of *C. albicans* was cloned into pcDNA3.1 by EcoRI and XhoI restriction enzyme sites to construct DNA vaccine pD-HSP90C. DNA sequencing showed that the DNA fragment containing epitope C (LKVIRK) of *C. albicans* HSP90 was correctly inserted into the pcDNA3.1 vector. Purified pD-HSP90C was further confirmed by digesting with EcoRI and XhoI restriction enzyme (Fig. 3) and senquencing.

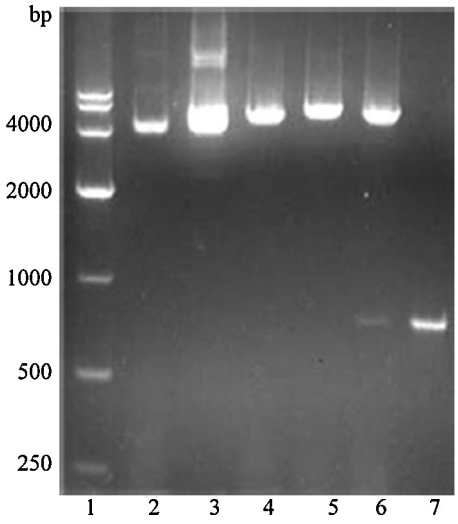

Fig. 3. DNA gel analysis of DNA vaccine pD-HSP90C. 1 Marker. 2 pcDNA3.1. 3 pD-HSP90C. 4 pcDNA3.1 digested with EcoRI. 5 pD-HSP90C digested with EcoRI. 6 pD-HSP90C digested with EcoRI and XhoI. 7 target fragment recovered from PCR.

3.3. DNA vaccine pD-HSP90C can evoke production of specific antibody against rP-HSP90C in mice

To detect the specificity of antibody evoked by DNA vaccine pD-HSP90C, sera of immunized mice were collected two weeks after the last immunization for analyzing the specificity of antibody against rP-HSP90C by Western blot. The sera from patients infected with systemic *C. albicans* and healthy people were used as the positive and negative controls respectively in Western blot.

Western blot analysis of rP-HSP90C (Fig. 4A) demonstrated that sera from the mice immunized with either pD-HSP90C(M4) or pD-HSP90C/PPSB (M5) recognized rP-HSP90C, which patient's sera (P) also recognized. However, the sera of mice immunized with PBS (M1), pcDNA3.1(M2), PPSB (M3)and healthy sera (H) did not recognize rP-HSP90C. More importantly, Western blot analysis of *C. albicans* total proteins (Fig. 4B) revealed that sera from the mice immunized with either pD-HSP90C (M4) or pD-HSP90C/PPSB (M5) recognized 47- and 90-kDa protein bands of *C. albicans* exclusively, which was also recognized by patient sera (P). However, the sera of mice immunized with PBS (M1), pcDNA3.1 (M2), PPSB (M3), and healthy sera (H) did not recognize any protein bands of *C. albicans* total proteins. It was clear that pD-HSP90C was able to evoke the production of specific antibody against rP-HSP90C in mice.

3.4. PPSB can enhance antibody responses against rP-HSP90C in sera of mouse immunized with DNA vaccine pD-HSP90C

Sera of the pD-HSP90C immunized mice were collected two weeks after the last immunization to analyze rP-HSP90C specific antibody titers (IgG, IgG1 and IgG2b) using indirect ELISA. Data shown in Fig. 5 demonstrated that IgG, IgG1, and IgG2b antibody titers in pD-HSP90C-immunized mice were significantly different compared to pcDNA3.1 or PBS group ($p < 0.05$). More importantly, IgG, IgG1, and IgG2b antibody titers in pD-HSP90C-immunized mice were greatly augmented by PPSB ($p < 0.05$). In addition, no specific antibody against rP-HSP90C was found in mice immunized with PBS, pcDNA3.1, or PPSB. The data indicated that PPSB significantly enhanced specific antibody IgG response with higher titers of IgG1 as well as IgG2b in mice immunized with pD-HSP90C.

3.5. PPSB can promote production of IL-2 and IL-4 in sera of mouse immunized with DNA vaccine pD-HSP90C

Analysis of IL-2 and IL-4 (shown in Fig. 6) revealed that there were significant differences comparing groups pD-HSP90C with

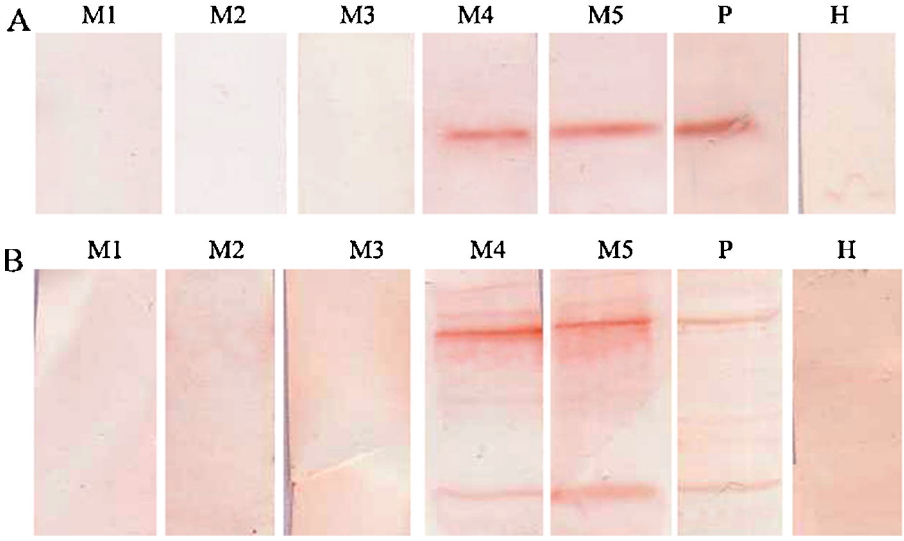

Fig. 4. Western blot analysis for specificity of antibody against rP-HSP90C evoked by DNA vaccine pD-HSP90C. In order to detect specificity of antibody against rP-HSP90C evoked by pD-HSP90C, male ICR mice (6 mice/group) were immunized with either pD-HSP90 alone or in presence of PPSB twice at a 14-day interval. PBS, pcDNA3.1, and PPSB were used as controls. The mice were immunized intramuscularly in tibialis anterior muscle followed by electroporation. The immunized mice sera were collected one week after the last immunization for analysis of Western blot. (A) Western blot analysis of recombinant protein rP-HSP90C using sera of immunized mice. rP-HSP90C was submitted to SDS-PAGE (12%), then transferred to nitrocellulose membrane and incubated with sera from the immunized mice, patient and healthy people. Sera from the mice immunized with pD-HSP90C (M4) or pD-HSP90C/PPSB (M5) recognized rP-HSP90C. However, sera from the mice immunized with PBS (M1), pcDNA3.1 (M2), and PPSB (M3) did not show rP-HSP90C. Patient sera (P) recognized rP-HSP90C while healthy sera (H) did not recognize rP-HSP90C. (B) Western blot analysis of *C. albicans* total proteins using sera of immunized mice. Total proteins extracted from *C. albicans* were submitted to SDS-PAGE (13%), then transferred to nitrocellulose membrane and incubated with sera from immunized mice, patient and healthy people. Sera from the mice immunized with pD-HSP90C (M4) or pD-HSP90C/PPSB (M5) recognized 90 kDa and 47 kDa protein bands of *C. albicans* total proteins. However, sera from the mice immunized with PBS (M1), pcDNA3.1 (M2), and PPSB (M3) did not recognize any protein bands of *C. albicans* total proteins. Patient sera (P) showed 90 kDa and 47 kDa protein bands of *C. albicans* total proteins, while healthy sera (H) did not recognize any protein bands of *C. albicans* total proteins.

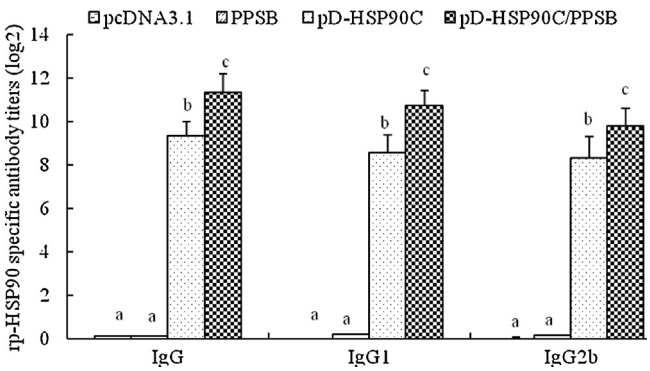

Fig. 5. Effect of PPBS on rP-HSP90C-specific antibody titer IgG, IgG1 and IgG2b in sera of mouse immunized with DNA vaccine pD-HSP90C. ICR mice (6 mice per group) were immunized intramuscularly followed by electroporation with pD-HSP90C in the absence or presence of PPSB (pcDNA3.1, PPSB and PBS used as controls) twice at a 14-day interval. The mice were sacrificed two weeks after the second immunization. The immunized mice sera were collected to detect the titer of specific antibody against rP-HSP90C by ELISA. The values were present as mean ± SD.

group pcDNA3.1 ($p < 0.05$); it was also clear that IL-2 and IL-4 in mice immunized with PPSB were significantly higher than the mice immunized with PBS ($p < 0.05$). Moreover, considerable enhancements of IL-2 and IL-4 in group pD-HSP90C/PPSB were detected when compared with group pD-HSP90C ($p < 0.05$). The data indicated clearly that PPSB significantly augmented IL-2 and IL-4 in sera of mice immunized with pD-HSP90C.

3.6. PPSB can augment the protective efficacy against systemic C. albicans infection in mouse immunized with DNA vaccine pD-HSP90C

To determine the effect of pD-HSP90C combined with PPSB on protection against systemic *C. albicans* infection, the mice were challenged with living *C. albicans* cells after immunization with pD-HSP90C, pD-HSP90C/PPSB, PBS, pcDNA3.1, and PPSB. Both survival rate and CFU in their kidneys were assessed on the 15th day after infection. As shown in Fig. 7, 90% of the mice immunized with pD-HSP90C/PPSB survived. In contrast, only 70% of the mice immunized with pD-HSP90C survived, and survival rates of other groups were between 50 and 60%. The results demonstrated that no statistically significant difference was observed between pD-HSP90C-immunized group and PBS-injected group; however, the mice immunized with pD-HSP90C/PPSB was statistically significant different when compared to PBS-injected group ($p < 0.05$).

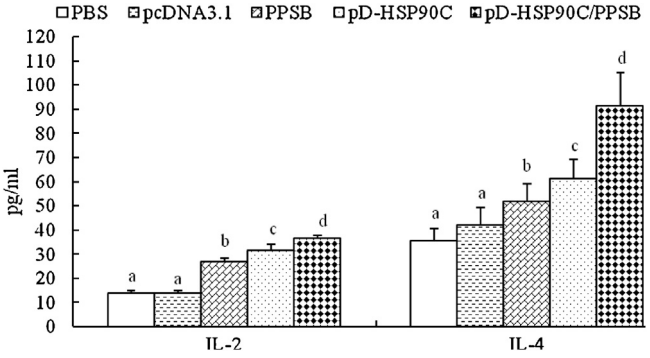

Fig. 6. Effect of DNA vaccine pD-HSP90C and its combination with PPBS on IL-2 and IL-4 in sera of mouse immunized with DNA vaccine pD-HSP90C. ICR mice (6 mice per group) were immunized intramuscularly followed by electroporation with pD-HSP90C in the absence or the presence of PPSB (pcDNA3.1, PPSB and PBS used as controls) twice at a 14-day interval. The mice were sacrificed two weeks after the second immunization. The immunized mice sera were collected to detect the quantities of IL-2, IL-4 by ELISA kits. The values were present as mean ± .

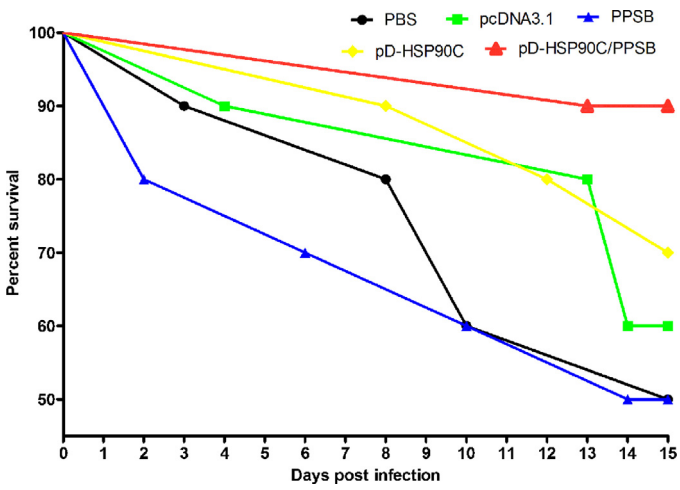

Fig. 7. Survival curves of immunized mice after systemic *C. albicans* infection. The male ICR mice (10 mice per group) were immunized twice with either pD-HSP90C alone or in presence of PPSB at a 14-day interval, meanwhile pcDNA3.1, PPSB and PBS were used as controls. The mice were immunized intramuscularly in tibialis anterior muscle followed by electroporation. The immunized mice were infected with live *C. albicans* cells (5×10^7) intravenously by the lateral tail vein two weeks following the last immunization, and the survival was monitored for 15 days. Survival rate of mice immunized with pD-HSP90C/PPSB was statistically significant difference compared to PBS-injected group ($p < 0.05$), however, no statistically significant difference was observed between pD-HSP90C-immunized group and PBS-injected group.

Table 1 showed that there were significantly fewer CFU in the kidneys of mice vaccinated with pD-HSP90C than in those immunized with pcDNA3.1 or PBS-injected ($p < 0.05$). More importantly, PPSB significantly reduced the numbers of CFU in the kidneys of mice vaccinated with pD-HSP90C. It was clear that the PPSB significantly enhanced the protective immunity in mice immunized with pD-HSP90C.

4. Discussion

We have clearly shown in this study that PPSB from the fruit of *P. alkekengi* significantly enhanced humoral and cellular (Th1, Th2) immune responses in mice immunized with DNA vaccine pD-HSP90C; PPSB also greatly prolonged the survival time of immunized mice against the acute systemic candidiasis.

The prevalence of systemic candidal disease has been increasing in hospitalized patients over the past two decades (Ashman, Fulurija, & Papadimitriou, 1999; Cenci, Romani, Vecchiarelli, Puccetti, & Bistoni, 1990; Martin-Manso et al., 2012; Yuan, Casadevall, Spira, & Scharff, 1995). Administration of antifungal drugs such as amphotericin B and azoles has resulted in an increase of non-*albicans* species with higher resistance rates to anti-fungal drugs (Krcmery & Barnes, 2002; Pfaller et al., 2003). Hence, there is

Table 1
CFU in kidneys of the immunized mice.

Groups	CFU in kidney ($\log_{10}$ g of kidney)
PBS	4.63 ± 0.14^a
pcDNA3.1	4.49 ± 0.12^a
PPSB	4.22 ± 0.28^a
pD-HSP90C	4.05 ± 0.24^b
pD-HSP90C/PPSB	3.52 ± 0.33^c

The male ICR mice were immunized with either pD-HSP90C alone or in presence of PPSB twice at 14-day interval, meanwhile pcDNA3.1, PPSB and PBS were used as controls. The mice were immunized intramuscularly in tibialis anterior muscle followed by electroporation. The immunized mice were infected with live *C. albicans* cells (5×10^7) intravenously by the lateral tail vein two weeks after the second immunization. The CFU in the kidneys were evaluated on the 15th day after infection. The data were expressed as mean ± S.D.

an urgent need to develop measures of prophylactic immunointervention against systemic candidiasis. A particular interest is DNA vaccine, which gives a promising future for the prophylaxis of systemic candidiasis.

It has been reported that immunity against systemic Candida infections requires cell-mediated mechanisms. Defects in cell-mediated immunity cause patients, *e.g.* persons with AIDS, to have systemic *C. albicans* infections (Ashman, Vijayan, & Wells, 2010; Cenci, Romani, Vecchiarelli, Puccetti, & Bistoni, 1990; Coker, Mercadal, Rouse, & Moore, 1992; Levitz, 1992; Sun, 2006). However, there is growing evidence that antibodies in host defense against invasive Candidiasis are able to confer protection (Cassone & Casadevall, 2012; Han & Cutler, 1995; LeibundGut-Landmann et al., 2012; Romani, 1999; Wagner, Vazquez-Torres, Jones-Carson, Warner, & Balish, 1996;). Protective antibodies against invasive Candidiasis may be elicited by *C. albicans* cell wall polysaccharides, proteins, and peptides (Xin et al., 2012; Xin & Cutler, 2011). This shows that both humoral and cellular responses are fundamental to control the systemic Candida infections (Van de Veerdonk, Kullberg, & Netea, 2012).

Despite the extensive investigations of DNA vaccines, none has yet been approved by the US Food and Drug Administration (FDA) for either prophylactic or therapeutic applications in humans (Donate, Coppola, Cruz, & Heller, 2011; Saade & Petrovsky, 2012; Sardesai & Weiner, 2011). DNA vaccines' poor immunogenicity is the main reason behind their failure on human applications (Klinman, Klaschik, Tross, Shirota, & Steinhagen, 2010). Therefore, selecting the adjuvant or delivery system is as important as selecting the vaccine candidates. Although some strategies attempted to enhance DNA vaccine potency, a promising approach to improve immunogenicity of DNA vaccine is the incorporation of immunologic adjuvants into a vaccine formulation. It has been shown that polysaccharides of herbal plants can enhance the humoral and cell-mediated immune responses (Ooi & Liu, 2000; Sun, 2006; Wang et al., 2005; Wasser, 2002). More importantly, most polysaccharides from herbal plants are typically less immunogenic, more tolerable, non-toxic, and biodegradable; they are an unlimited natural resource and low cost to manufacture (Schepetkin & Quinn, 2006; Sun, Xie, & Ye, 2009; Xie, Schepetkin & Quinn, 2007). Moreover, formulation of a DNA vaccine with polysaccharide adjuvant is very simple, as it is only the mixture of the two components. Hence, the use of herbal immuno-modulators may be helpful in overcoming the limitation of Alum or QuilA, and polysaccharide can be one of the most ideal adjuvant candidates. *P. alkekengi* is an edible and medicinal plant in East Asia, and it is commonly used as a traditional Chinese herbal plant. *P. alkekengi* is used broadly to treat inflammation, cold, cough, and fungal infections (Vessal, Mehrani, & Omrani, 1991; Vessal, Rasti, & Kooshesh, 1996;). Our lab has previously reported that PPSB, which is a purified polysaccharide isolated from *P. alkekengi*, can enhance both humoral immunity and cellular immunity in OVA-immunized mice (Li et al., 2011). In this study, we constructed DNA vaccine pD-HSP90C containing epitope C (LKVIRK) from HSP90 of *C. albicans*, and investigated adjuvant effect of PPSB in DNA vaccine pD-HSP90C.

It is well known that Th1 immune response is a requisite for CTLs, which are necessary for subunit vaccines, vaccines directed against intracellular pathogens, and therapeutic cancer vaccine. In comparison, Th2 response is effective for protection against most bacteria as well as some viral infections (Cox & Coulter, 1997). The major cytokines associated with the Th1 cell subsets are IL-2, TNF-α, IFN-γ *etc.*, promoting the production of IgG2a, IgG2b, and IgG3; the main role of these cytokines is to enhance Tc cellular cytotoxicity and cell-mediated immune responses (Yang et al., 2005). Th2 cell subsets can secrete IL-4, IL-5, and IL-10, promoting the production of IgG1 and IgE; the main role of these cytokines is to promote antibody production and mediate humoral immune responses (Chiarella, Massi, Robertis, Signori, & Fazio, 2007; Guy, 2007; Marciani, 2003). Thus, IgG2b, IgG1, IL-2, and IL-4 in sera of immunized mice were detected as an indicator of Th1 and Th2 responses in order to characterize the immune response mediated by DNA vaccine pD-HSP90C combined with PPSB. The results indicated that PPSB significantly promoted the production of IgG2b, IgG1, IL-2, and IL-4 in sera of mice immunized with pD-HSP90C; meanwhile, IgG titer in mice immunized with pD-HSP90C containing PPSB was higher than pD-HSP90C-injected mice. More importantly, it was found that the mice immunized with pD-HSP90C in presence of PPSB not only had fewer CFU in the kidneys than mice immunized with pD-HSP90C, but also a statistically significant higher survival rate over PBS-injected group ($p < 0.05$). To further investigate mechanism of immunologic enhancement mediated by PPSB, mice were immunized with PPSB alone. The data showed PPSB augmented the production of IL-2 and IL-4 remarkly compared to PBS-injected mice. The results revealed that PPSB strongly enhanced Th1 and Th2 responses in addition to humoral response in DNA vaccine pD-HSP90C; meanwhile PPSB greatly improved the protection against systemic candidiasis in pD-HSP90C-immunized mice.

Our data confirmed that polysaccharide PPSB from herbal plant formulated with DNA vaccine enhanced not only humoral responses, but also Th1and Th2 responses. In conclusion, PPSB may be an ideal candidate of adjuvant in vaccines.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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