Immunological properties reveal the monovalent and bivalent recombinant dengue virus-like particles as candidate vaccine for dengue

Running title: Immunological properties of dengue virus

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Abstract

We aimed to systematically analyze the immunological properties of monovalent and bivalent dengue virus-like particles (DENV VLPs) prepared in our study and provided basis for the development of DENV VLPs as a promising non-infectious candidate vaccine for dengue infection. The DENV-1/2 VLPs were prepared in the GAP promoter-based P. pastoris expression system using α-factor secretion signal. Groups of mice were immunized by monovalent and bivalent VLPs compared with inactivated virions and PBS. Then, the humoral immune response in mice were tested by ELISA, immunofluorescence and neutralization assay. ELISA and Enzyme linked immunospot assay were performed for investigating cytokines production after virus challenge in vivo and in vitro, respectively. Besides, the protection ability of VLPs was assessed by passive protection assay in suckling mice. The recombinant DENV-1/2 VLPs were successfully expressed with α-factor secretion signal. Sera of mice immunized with monovalent and bivalent VLPs elevated the specific antibodies level comparable to inactivated virions group. The monovalent and bivalent VLPs induced specific cellular immune response as confirmed by ELISPOT. The PRNT 50 titers of monovalent and bivalent VLPs sera against DENV-1/2 were different ranging from 16 to 64. Moreover, anti-monovalent and anti-bivalent DENV VLPs sera displayed protective capacity in survival rate and morbidity. Monovalent and bivalent VLPs showed attracting vaccine effect for dengue virus. The multivalent VLPs may be promising for preventing different types of dengue virus.

Key words: dengue virus; humoral immune; cellular immune; passive protection in suckling mice
Introduction

Dengue virus (DENV) is characterized as the small, enveloped positive-stranded RNA viruses belonging to the genus *Flavivirus* of the *Flaviviridae* family (1). Based on the envelope protein, DENV can be classified into 4 known serotypes: DENV1, 2, 3 and 4. All types of DENV can cause the full spectrum of disease (2). It is estimated that around 390 million people are at risk of suffering dengue globally and 25,000 cases of 50-100 million DENV infections are dead annually. Additionally, there are about 50 to 100 million new cases of dengue infection all over the world (3). Dengue infection has been a potential threat to global public health.

Many contributions have been made to prevent and control the morbidity and mortality caused by DENV. Routine administration of vaccination is a proven method for controlling the DENV infection (4). Currently, numerous vaccines for DENV infection are in development and clinical evaluation, such as inactivated whole virus, live attenuated DENV, recombinant subunit protein, DNA and virus-vectored vaccine (5-7). However, a successful DENV vaccine that can protect against all the DENV types is not available by now. Therefore, the safe and effective DENV vaccine is in urgent need. In recent years, noninfectious virus-like particles (VLPs) formed in flavivirus replication have attracted many attentions. Many studies devoted to the rapid and effective construction of recombinant VLPs which possessed similar properties of native virions and lacked the viral genome (8, 9). But the evidence concerning the DENV VLPs as vaccine candidate for dengue is insufficient.

Currently, the DENV type 2 and type 1 are the most popular stains in Guangdong province, China (10, 11). In the present study, we systematically evaluated the immunological properties and protection ability of monovalent DENV VLPs and bivalent DENV VLPs against DENV-1/2 in vitro and in vivo. The purpose of our study was to provide the basis for the application of the DENV VLPs as attractive candidate vaccine in preventing the popular DENV infection in China.

Materials and methods

Cells and viruses

The C6/36 cell line derived from *Aedes aegypti* was used for virus propagation and the C6/36 cells were cultured as previously described (12). The DENV-1 GZ01/95 strain (GenBank accession No. EF032590) and DENV-2 ZS01/01 strain (GenBank accession no. EF051521) were propagated in C6/36 cells. Virus was purified by sucrose density gradient centrifugation (13) and the inactivation of virions were performed with 1:2000 β-propionolactone. Virions concentrations were detected by bicinchoninic acid (BCA) method (Biocolor, Shanghai, China) and stored at -80 °C for further studies.

The *Mesocricetus auratus* BHK-21 cells were cultured in DMEM (Gibco, Guangzhou, China) supplemented with 10% fetal bovine serum (Gibco, USA) at 37 °C in a 5% CO₂ atmosphere and used for plaque titration experiments (14).

Recombinant dengue virus-like particles (DENV VLPs) from *Pichia pastoris*
The wild-type *P. pastoris* strain X33 (Invitrogen, San Diego, CA) were served as the host strain. The expression vector was constructed with vector pGAPZaA (Invitrogen) according to a previous procedure with a slight modification (12). Briefly, the cDNAs coding prM/E from DENV-1 GZ 01/95 strain and DENV-2 ZS01/01 strain were synthesized by reverse transcription-polymerase chain reaction (RT-PCR) under optimized reaction conditions. The full-length prM/E gene was inserted into the downstream of GAP promoter with the α-factor secretion signal (Fig. 1 a). The recombinant plasmids of pGAPZa-PrME-D1 and pGAPZa-PrME-D2 were electroporated into *Pichia pastoris* to express recombinant DENV-1 VLPs and DENV-2 VLPs, respectively.

After the verified transformants were cultured, yeast cells were collected by centrifugation (10,000×g, 10 min, 4°C) and disrupted with glass beads in breaking buffer (50 mmol/L sodium phosphate, pH 7.4, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 1 mmol/L phenylmethyl sulfonylfluoride (PMSF), and 5% glycerol). The yeast lysates were subjected to ultracentrifugation at 153 000 ×g for 6 hours at 4°C (HATICH, P80AT rotor, Japan) using 5-50% sucrose density gradient. Then, the western blot analysis was performed to test the expression of recombinant VLPs. The expression of DENV-1 E proteins and DENV-2 E proteins were detected using the mAb D2-1F1-3 (specific mouse monoclonal antibody, the Center for Disease Control, USA) (15) and mAb 3H5 (specific mouse monoclonal antibody, American Type Culture Collection, USA) as the primary antibody, respectively and goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate as the secondary antibody (Santa Cruz, USA). Finally, protein concentrations were assessed using BCA method (Biocolor, Shanghai, China).

**Animal immunization and virus challenge**

All the animal studies were approved by Ethics Committee of Sun Yat-sen University and performed in accordance with the ethical standards. Specific pathogen-free female BALB/c mice (aging from 3 to 4 weeks old) were supplied by the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China) and were randomly divided into 6 groups: monovalent DENV-1 VLPs group (n=15; treated with DENV-1 VLPs followed by DENV-1 challenge), monovalent DENV-2 VLPs group (n=15; treatment with DENV-2 VLPs, followed by DENV-2 challenge), bivalent VLPs group (n=30; treatment with DENV-1/2 VLPs combination, followed by DENV-1 and DENV-2 challenge, separately), Inactivated DENV-1 group (n=15; treatment with inactivated DENV-1, followed by DENV-1 challenge), Inactivated DENV-2 group (n=15; treated with inactivated DENV-2 followed by DENV-2 challenge) and PBS group (n=30; treated by PBS solution, followed by DENV-1 and DENV-2 challenge, separately).

The mice in DENV-1 VLPs, DENV-2 VLPs and VLP combination groups were intraperitoneally injected respectively with monovalent DENV-1 VLPs (50 μg/dose), monovalent DENV-2 VLPs (50 μg/dose) and the bivalent DENV-VLPs (50 μg DENV-1 VLPs and 50 μg DENV-2 VLPs per dose) absorbed with Freund adjuvant (Sigma) at days...
0, 14 and 28 once a day for immunization. The DENV-1/2 virions (50μg/ dose) and PBS (with the same dose) were administered for mice as controls with the same procedure. Blood samples were collected on days 0, 13 and 27 before immunization through tail vein for serum IgG measurement. One week after the last immunization (at days 34), 1/3 mice (n=5) in each group were sacrificed to collect blood and spleens for further analysis. Additionally, 2/3 mice (n=10) in each group were intraperitoneally challenged with DENV-1 or DENV-2. On days 1, 2, 3, 4 post challenge, blood samples were collected through tail vein for serum cytokines measurement.

**Enzyme-linked immunosorbent assay**

The DENV VLPs and DENV specific antibodies were tested by enzyme-linked immunosorbent assay (ELISA) with the serum samples collected at days 0, 13, 27 and 34. Briefly, polystyrene plates with 96 wells (Costar, Cambridge, MA) were coated with inactivated DENV antigen or DENV VLPs antigen over night at 4°C and blocked in coating buffer containing 5% fat-free milk powder for 1 h at 37°C. After washing with PBS-T (PBS containing 0.05% Tween 20), serum samples (dilutions at 1:100; 100μl per well) were incubated for 1 h at 37°C. Subsequently, 100μl of 1:5000 diluted goat anti-mouse IgG-peroxidase conjugate (Santa Cruz, USA) was added per well and incubated for 1 h at 37°C, followed by addition of 100μl/well 3, 3, 5, 5'-tetramethyl benzidine substrate at 37°C for 15 min. After the reaction was stopped with 2M H₂SO₄, absorbance was measured at 450 nm using an automated ELISA reader (ELx800 BioTek). The results were considered to be positive if the absorbance exceeded 2 times the mean absorbance of serum pre-immunization.

**Immunofluorescence assay**

Anti-monovalent VLPs antibody, anti-bivalent VLPs antibody, anti-inactivated DENV antibody raised in sera were tested by immunofluorescence assay with PBS treatment sera as control. The slides with C6/36 cells monolayer infected with DENV-1 strain GZ01/95 and DENV-2 strain ZS01/01 were fixed in acetone for 15min at -20°C. Then, the 1:80 diluted antisera were added onto the slides and incubated for 1 h at 37°C. After the slides were washed 3 times with PBS, Alexa-Fluor-488-conjugated goat anti–mouse IgG (1:200; Invitrogen, Carlsbad, CA) was added and incubated for 45 min at 37°C. Cells were stained with 4’,6-diamidino-2-phenylindole (DAPI). The staining images were photographed and observed under the fluorescent microscope.

**Cytokines production in mice after virus challenge**

To determine the levels of IFN-γ, TNF-α and IL-10 in sera after virus challenge, commercial ELISA kits (R&D, USA) were used according to the manufacturer introduction. Blood samples collected on days 1, 2, 3, 4 post challenge and days 34 post immune were analyzed. The limits of detection for these cytokines were 2 pg/ml (IFN-γ), 2 pg/ml (TNF-α) and 2 pg/ml (IL-10).
**Enzyme linked immunospot assay for cytokines**

The enzyme linked immunospot assay (ELISPOT) were performed used ELISPOT kits (Ucytech, NLD), following the manufacturer’s instructions. Briefly, the ELISPOT 96-well plates (Millipore, USA) were coated overnight at 4 °C with 100μl of anti-mouse IFN-γ or with anti-mouse IL-10 or TNF-α (5μg/ml). Plates were washed twice and blocked with blocking solution for 2 h. Then, 100 μl freshly isolated splenocytes (2×10⁵ cells) from the immunized mice were transferred to each well and stimulated with inactivated dengue virus at 37 °C for 24 h. Cells were washed away and the secondary biotinylated anti-cytokine mAb was added to each well, followed by streptavidin-HRP and AEC substrate solution. The spots were counted by ImmunoSpot® Analyzer (Cellular Technology Ltd.).

**Neutralization test**

Sera from immunized mice at days 34 were used for Plaque Reduction Neutralization Test (PRNT) as previous description (16). Briefly, the *Mesocricetus auratus* BHK-21 cells were grown to 80% confluence in 24-well plates. The immunized mice serum samples with twofold serial dilutions of the serum (1:4-1:64) were mixed with equal volume of DENV-1 or DENV-2 (150–200 PFU per milliliter) at 37 °C for 1 h to neutralize the infectious virus. The virus/serum mixture was aspirated and added with 0.8mL medium to infect the BHK-21 cells at 37 °C with 5% CO₂ for 7 days. Finally, the dengue virus plaques were counted with naked eye and observed by scanning the cluster plate into Adobe Photoshop CS for further analysis. PRNT50 titer was defined as the reciprocal of maximum serum dilution to show 50% reduction of the plaque count based on the controls.

**Passive protection assay in suckling mice**

The 1-day old BALB/c mice (purchased from the Experimental Animal Center of Sun Yat-sen University) were assigned into 6 groups: group 1 (n=14; treated by DENV-1 VLP sera with DENV-1), group 2 (n=10; treated by DENV-2 VLP sera + DENV-2), group 3 (n=14; treated by divalent DENV VLPs sera + DENV-1), group 4 (n=13; treated by divalent DENV VLPs sera + DENV-2), group 5 (n=15; treated by PBS treating sera + DENV-1) and group 6 (n=10; treated by PBS treating sera + DENV-2). In each group, the pups were confirmed to be from the same litter. The 1:10 dilution sera were incubated with 20 LD₅₀ DENV-1 or DENV-2 (4×10⁴ PFU/ml) for 1 h at 37 °C. The suckling mice were intracerebrally injected with a 20μl of sera-virus mixture. The manifestations of the animals post challenge were recorded daily for 3 weeks, including the morbidity of paralysis, ruffling, slowing of activity, kyphoscoliosis and mortality.

**Statistical analysis**

All the data were analyzed using SPSS software (version 13.0) and displayed as mean ±standard deviation (SD). Statistical differences among groups were analyzed by one-way ANOVA. For survival analysis, Kaplan-Meier survival curves were analyzed by the log rank test. P<0.05 was defined as significant.
Results

Expression and purification of DENV VLPs

Yeast lysates were analyzed for the expression of DENV-1 E proteins and DENV-2 E proteins by Western blotting using mAb D2-1F1-3 and 3H5. About 50 kDa band of recombinant E protein band was detected in the lysates of yeast clones (Fig1.b). After purified by 5-50% sucrose density gradient centrifugation, the expressed DENV-1 E proteins and DENV-2 E proteins were present in the faction of 20%-25% sucrose density gradient (Fig1.c). After the fractions containing DENV VLPs were harvested and mixed, the recombinant protein concentration was determined to be 0.5mg/ml.

DENV VLPs elicited antibody detected by ELISA

To evaluate the immunogenicity induced by recombinant VLPs, blood samples from immunized mice with purified DENV-1/2 VLPs were collected at days 0, 13, 27 before immunization and at 34 day after the last immunization. For control, blood samples of the mice immunized with heat-inactivated DENV-1 and DENV-2 were collected at the same time point. As shown in Fig. 2 a and b, mice immunized with either DENV VLPs or inactivated virions showed high levels of antigen specific serum IgG. There was an increasing trend for the production of antibodies among all groups. At days 27 after immunization, the inactivated DENV-1/2 induced the highest levels of antibodies. However, at days 34, the highest levels of antibodies were observed in DENV-1/2 VLPs groups. Bivalent-VLPs and inactivated DENV induced the comparable level of antibodies at days 34 post immunization.

DENV VLPs elicited antibody detected by immunofluorescence assay

Studies of immunofluorescence assay showed that the sera from mice immunized with DENV-1 VLPs or DENV-2 VLPs were able to react with the native DENV-1 or DENV-2 antigens (Fig 3). Similar results can be observed in sera from mice immunized with bivalent DENV VLPs and inactivated DENV. The immunofluorescence staining was observed in the cytoplasm of C6/36 cells for the acetone permeability. The studies showed that immune sera produced from mice immunized with DENV VLPs could recognize native DENV structure proteins. No immunofluorescence staining was observed in normal sera suggesting no viral antigens were detected in mice treated with PBS.

Neutralizing antibodies elicited by immunization with DENV VLPs

To evaluate the specific DENV neutralizing antibody in sera from immunized mice, the PRNT was performed. Fig.4 showed that DENV1/2-VLPs could exhibit comparable levels of homotypic neutralizing antibodies with inactivated DENV1/2. The PRNT50 titers of bivalent VLPs sera against DENV-1 and DENV-2 were 16 and 64, respectively. The PRNT50 titers of DENV-1VLPs and DENV-2 VLPs against homologous DENV were 32 and 64, respectively. Both monovalent and bivalent formula of DENV VLPs could effectively induce antigen specific IgG and produce protective
neutralizing antibodies in immunized mice.

*The cytokines profiles by DENV-VLPs immunization*

To assess the cellular immune response to DENV-1 and DENV-2 in vitro, we isolated spleen cells at day 34 after immunization by VLPs and inactivated virions. The levels of IFN-γ, TNF-α, and IL-10 in spleen cells after stimulated by DENV-1 or DENV-2 in vitro were analyzed by ELISPOT. As shown in Fig. 5 a, splenocytes in monovalent VLPs, bivalent VLPs and inactivated virions groups secreted higher levels of IFN-γ after stimulation with DENV-1 and DENV-2 in comparison with PBS group (P<0.05). Similar results were observed in TNF-α and IL-10 levels (Fig.5 b, c).

The IFN-γ level was elevated significantly in monovalent VLPs group after DENV-1/2 challenge (P<0.05), which suggested that the monovalent VLPs predominantly induced the Th-1 response in splenocytes both stimulated by inactivated DENV-1 and DENV-2. The spleen cells produced the most TNF-α in bivalent VLP group by DENV-1 simulation. The level of IL-10 was significantly accumulated in inactivated virions group with DENV-2 challenge. Among the 3 cytokines, the mean TNF-α levels in splenocytes were the highest after DENV virions stimulation. In contrast, the mean IL-10 levels were the lowest in all of the groups.

*Levels of serum cytokines elicited by immunization with DENV VLPs*

To explore the cytokines profiles after virus challenge in vivo, we harvested sera at days 1, 2, 3, 4 after DENV-1/2 challenge. The changes in the levels of the TNF-α, IFN-γ and IL-10 of sera detected by ELISA assays were shown in Fig. 6. After challenging with DENV-1/2, the monovalent, bivalent and inactivated DENV groups showed high levels of 3 cytokines. The IFN-γ and IL-10 levels were higher at day 1 post challenge and declined dramatically at days 2-4. For the TNF-α, bivalent VLPs group showed the highest level at 1-4 days after virus challenge.

*Suckling Mice Protection Assay*

To evaluate protective capability of sera from mice immunized with monovalent or bivalent VLPs in vivo, we performed protection assays in BALB/c suckling mice. As shown in Fig.7, anti-bivalent VLPs sera showed great ability of protecting sucking mice against DENV-1/2. All mice still survived at day 20 after treated with bivalent VLPs sera + DENV-1/2 (Fig. 7a, b). The anti-monovalent VLPs sera prolonged the survival time of mice compared with PBS group. All animals died at day 15 after DENV-1 VLPs sera + DENV-1 treatment (Fig. 7a), and 20% mice still survived at day 20 in anti DENV-2 VLPs sera + DENV-2 group (Fig. 7b). In PBS groups, all animals died at day 11 after infected by DENV-1 (Fig. 7a), and day 16 (Fig. 7b) after infected by DENV-2 (Fig. 7b). In addition, as shown in Fig. 8, there was no complication in mice treated with bivalent VLPs sera + DENV-1/2. The onset of complications was observed earlier in mice of normal sera group than those in DENV-1/2 VLPs sera groups. The efficacy of anti-bivalent VLPs sera to protect
animals against DENV-1/2 infection was greater than DENV-1/2 VLPs sera. DENV-1/2 VLPs sera inhibited the onset of complications compared with normal sera.

Discussion

Dengue viruses that can be transmitted among humans by mosquitoes (17), are popular throughout tropical areas of the world including China (18). For the high mortality and morbidity resulting from dengue infection, dengue has been a serious healthy concern (19). VLP vaccine has shown great potential in preventing viral infection (20). At present, many studies have been conducted to explore the effect of recombination VLPs in preventing dengue infection. In this work, we constructed the recombination VLPs according to a previous method with slight modifications and systematically analyzed the immunization properties of monovalent and bivalent DENV in humoral immune responses, cellular immune responses in vivo and in vitro.

In the present study, we successfully generated recombinant DENV-1 and -2 VLPs in *Pichia pastoris* by optimizing the expression plasmids. *P. pastoris* yeast cells characterized by tolerance, were found to the most economic expression systems for vaccine development (21) and GAP promoter was successfully used in expressing large amount of HBsAg in yeast cells (22). The GAP promoter-based *P. pastoris* expression system with the signal peptide of prM has been determined to be safe and stable for constitutive and effective production of DENV-2 VLPs (23). In contrast, we applied α-factor secretion signal in the GAP promoter-*P. pastoris* expression system and obtained high-level production of DENV-1 VLPs and DENV-2 VLPs. Thus, α-factor secretion signal can be recognized by the yeast secretory apparatus and lead to the effective expression of different types of DENV VLPs.

As outlined in previous study, VLPs vaccine showed considerable promise in preventing various virus challenge (20, 24). VLPs can be efficiently taken up, internalized and processed by antigen presenting cells (APCs) (25-28). Previous evidence indicated that VLP based vaccine was able to elicit strong humoral and cellular immune responses against viruses (29-31). Data of ELISA and indirect immunofluorescence assay revealed that DENV VLPs, similar with inactivated dengue virions, induced increasing levels of antigen specific IgG after immunization and the antisera from mice immunized by monovalent and bivalent DENV VLPs could bind to natural DENV specific antigen. It is reported that DENV E protein has many B-cell-specific epitopes that can stimulate B cells to produce neutralization antibodies against virus infection (32, 33). In this work, the DENV E protein was successfully expressed in the recombinant DENV VLPs and stimulated the production of neutralizing antibodies that are particularly important to block dengue virus entry into target cells. The DENV VLPs expressed in our paper induced humoral immune response in mice infected by DENV. In this work, the humoral response was characterized by high PRNT50 titers of monovalent or bivalent DENV VLPs in neutralizing assays. These findings are consistent with the humoral response induced by VLPs in other studies (34). All
these results confirmed that the dengue virus type 1 and 2 VLPs prepared in this study preserved the antigenicity of prM and E proteins and effectively induced virus specific humoral immune responses.

In addition, another marked advantage of VLPs is their ability to induce cellular immunity (35, 36). Tumor necrosis factor alpha (TNF-α), is a modulator of cell immunization involved in systemic inflammation, and has been reported to have the ability to inhibit viral replications (37). Interferon gamma (IFN-γ), known as immune interferon, is also found to be a critical antiviral mediator (38). Moreover, IL-10 (interleukin-10), an anti-inflammatory cytokine, plays key role in immune response. These cytokines (TNF-α, IFN-γ and IL-10) are all closely associated with the immunopathogenesis of virus infection (39). Therefore, in this study, the levels of these cytokines were investigated to explore the cellular immune response induced by VLPs in vitro and in vivo. The production of helper T cell (Th1) immune response cytokine (IFN-γ) and Th2 immune response cytokine (IL-10) and inflammation cytokines (TNF-α) from virus-stimulated splenocytes in vitro were analyzed by ELISPOT quantitatively. Results showed that compared with PBS group, the inactivated DENV-1/2 antigen induced the secretion of 3 cytokines significantly in monovalent, bivalent DENV VLPs and inactivated virions group. It revealed that monovalent and bivalent VLPs had the ability to induce a potent immune response comparable with inactivated virions. The monovalent VLPs predominantly induced the Th-1 response in splenocytes both stimulated by inactivated DENV-1 and DENV-2 (Fig. 5). Similar cytokines profiles were found in the blood of mice challenged by virus in vivo. Results showed that expressions of IFN-γ, TNF-α and IL-10 were elevated by VLPs compared with PBS group, suggesting that monovalent and bivalent VLPs could effectively induce cellular immune response. It has been reported that the levels of TNF-α, IFN-γ and IL-10 are elevated in patients with dengue virus infection (40). The serum levels of these cytokines is a potential predictor of disease severity (40). Patients who died for DENV infection showed higher IL-10 levels in comparison with survivals. Besides, TNF-α is elevated in the later phase of illness, while IFN-γ was elevated in early phase of Dengue haemorrhagic fever (41). In our results, the levels of IL-10 and IFN-γ in mice treated with monovalent, bivalent DENV VLPs were raised at day 1 after DENV-1/2 challenge and declined gradually at day 2-4 (Fig.6), which might indicate that DENV VLPs inhibited the development and progression of virus infection effectively.

Furthermore, we tested the protective efficacy of the DENV VLPs in suckling mice model. There were obvious differences in the protective effect in recipients with monovalent VLPs sera and bivalent VLPs sera. All the sucking mice were survived with bivalent VLPs sera after the challenge with both DENV-1 and DENV-2. Monovalent VLPs sera prolonged the survival period of infected mice compared with PBS treated group. The highest protection rates (100%) observed in the sulking mice immunized with bivalent VLPs sera were possibly due to synergistic effect between DENV-1 VLPs and DENV-2 VLPs sera. Alternatively, the high concentration of cross-reactive antibodies forming
macromolecules compounds by cross-linking numerous virions, may also prevent virus entering into cells. Although our data of protective experiment showed the significant protective effect of bivalent DENV VLPs against different types of dengue virus, it is well documented that DENV infection commonly lacks antibody cross-protection among serotypes (9). The phenomenon of antibody dependent-enhancement (ADE) may be occur in VLPs-immunized mice, which is known as that different types of virus infection can increase the risk of development serious disease (17). The ADE activity of the monovalent and bivalent DENV VLPs was not assayed and this is a limitation in this paper. Subsequently, a large number of studies should be conducted to determine whether the bivalent DENV VLPs is a promising candidate vaccine against different DENV serotypes.

In summary, we successfully expressed the recombinant DENV-1/2 VLPs with the α-factor secretion signal. The monovalent and bivalent DENV VLPs induced the humoral immune responses and cellular immune response specific for DENV-1 and DENV-2 serotypes. Bivalent VLPs sera showed excellent protective ability for suckling mice infected by DENV-1/2. The monovalent and bivalent DENV VLPs showed attracting vaccination effect for prevention of dengue virus. However, a large amount of studies is needed to verify whether multivalent DENV VLPs are more promising vaccine candidate for dengue infection.

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Disclosure There is no conflict of interest.

List of abbreviations

antibody dependent-enhancement (ADE)
bicinchoninic acid (BCA)
Dengue virus (DENV)
dengue virus-like particles (DENV-VLPs)
ethylene diamine tetraacetic acid (EDTA)
fluorescein isothiocyanate (FITC)
houserdish peroxidase (HRP)
immunoglobulin G (IgG)
Interferon gamma (IFN-γ)
virus-like particles (VLPs)
reverse transcription-polymerase chain reaction (RT-PCR)
standard deviation (SD)

Tumor necrosis factor alpha (TNF-α)
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Figure legends

Fig. 1 Expression of recombinant dengue virus-like particles (DENV VLPs) from Pichia pastoris

a. Construction strategy of recombinant plasmid pGAPZaA-prME-D1 and pGAPZaA-prME-D2. The full length prME-D1 and prME-D2 genes were cloned into the downstream of GAP promoter with the α-factor secretion signal to generate the recombinant pGAPZaA-prME-D1 and pGAPZaA-prME-D2 expression vector, respectively.

b. DENV-1/2 E protein expressed in yeast lysates. Lane1: DENV-1 E protein, lane2: DENV2 E protein.

c. Sucrose gradient sedimentation analysis for DENV-1/2 E protein expression. Arrow heads indicate E antigen. The size of molecular weight marker is shown in kDa.

Fig. 2 Detection of antigen specific IgG antibody by ELISA

BALB/c mice were intraperitoneally immunized with 50ug monovalent DENV-1/2 VLPs, inactivated DENV-1/2 virions, or bivalent VLPs for three times at two-week intervals. At day 0, 13, 27, 34, sera were collected and indirect ELISA was performed to test for antigen specific IgG.

a. Absorbance of antigen specific IgG antibody after immunized by DENV-1 VLPs, bivalent VLPs and inactivated DENV-1.

b. Absorbance of antigen specific IgG antibody after immunized by DENV-2 VLPs, bivalent VLPs and inactivated DENV-2. The baseline (dash line) indicates specific antibody level in serum treated by PBS.

Fig. 3 Indirect immunofluorescence of the C6/36 cells infected with DENV-1/2

A. C6/36 cells infected with DENV-1 (GZ01/95) B. C6/36 cells infected with DENV-2 (ZS01/01).a. Anti-monovalent VLPs antibody in sera; b. Anti-bivalent VLPs antibody in sera; c. Anti-inactivated DENV antibody in sera; d. normal Balb/c mouse sera. Alexa-488 was used for DENV-1/2 virus staining (green); DAPI staining was used to label cell nucleus (blue); magnification: ×400.

Fig. 4 Serum neutralizing antibody titer of vaccinated mice. Balb/c mice were immunized with 50ug monovalent VLPs or virions, bivalent VLPs (50ug of each VLPs) for three times at two weeks interval. At day 7 post 3rd immunization, neutralizing antibodies against DENV-1 (a, c) and DENV-2 (b, c) were assessed using PRNT. 150-200PFU DENV-1/2 was reacted with serially diluted mouse antisera applied to BHK-21 cells in 24-well plates. Data of each group was expressed as mean of plaque reduction percent with an S.D bar (n=3).
**Fig. 5** ELISPOT assay. BALB/c mice were immunized with DENV-1 VLPs DENV-2 VLPs or virions in either monovalent or bivalent VLPs formula. The immunized mice were sacrificed at day 7 post 3rd immunization and the collected spleen cells were isolated and stimulated in vitro with each of DENV-1/2 inactivated virions. Spleenocytes (2×10^5) were stimulated in vitro by partially purified DENV-1 or DENV-2 virus and IFN-γ(a), IL-10(b) and TNF-α(c) producing lymphocytes were enumerated by ELISPOT assay. The mean number of spot forming cells (SFCs)/2×10^5 splenocytes was shown as virions-stimulated with an S.D bar.

* and ** mean p<0.05, p<0.01, respectively.

**Fig. 6** Detection of cytokine production after challenge. Sera were harvested from immunized Balb/c mice at day 0-4 post-challenge for quantification, day 0 represent the sera collected at day 34 after immunization. a. IFN-γ; b. IL-10; c. TNF-α. The dash line indicates the limits of detection.

**Fig. 7** Protective assay in suckling mice. Protective effects of mouse antisera were performed by preincubation of the different sera at 1:10 dilution and 400PFU DENV-1(a) /DENV-2(b) at 37°C for 1 h, then intracerebrally inoculated in test suckling mice, and the survivors were counted within 15 days post virus challenge. *** and ** mean P<0.001 and P<0.01, respectively.

**Fig. 8** The morbidity in suckling mice after DENV-1 (a) and DENV-2 (b) challenge. Protective effects of mouse anti-DENV-1/2 VLPs and anti-bivalent VLPs sera at 1:10 dilution were performed by preincubation of the sera and 400PFU DENV-1/2 at 37 °C for 1 h, then intracerebrally inoculated in suckling mice, and clinical signs of infection, mainly hind leg paralysis, alterations in spinal column and deaths, were monitored during 20 days post challenge. ***P<0.001 compared with normal serum group.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

(a) IFN-γ
(b) IL-10
(c) TNF-α

Legend:
- Black: Monovalent VLPs
- Clear: Bivalent VLPs
- Grey: Inactivated virions
- Cross: PBS
Figure 7
Figure 8