Japanese encephalitis virus prM-E antigen immunization conferred protection against challenge by four different serotypes of Dengue viruses in mice

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Abstract

Dengue virus (DENV) and Japanese encephalitis virus (JEV) are closely related mosquito-borne flaviviruses. Together, they caused most arthropod-borne diseases in the world. Previously, we had demonstrated that both live attenuated and inactivated JE vaccines elicited cross-protection against DENV infection, and a DNA vaccine candidate expressing JEV prM-E protein (named pCAG-JME) could provide effective protection against JEV infection in mice. In this study, we examined whether the same pCAG-JME could elicit cross-protection against DENV infection. Our results showed that pCAG-JME indeed induced cross-reactive antibodies and cross-protection against four different serotypes of DENV in mice. Interestingly, pCAG-JME-immunized mice also generated both Th1 and Th2 responses when stimulated by all four different serotypes of DENV antigens. Moreover, cross-primed CD8⁺ T cell response was also detected following the stimulation of DENV proteins using intracellular cytokine staining. In addition, sera from pCAG-JME-immunized mice significantly reduced the mortality caused by DENV2 infection in severe combination immunodeficiency mouse. These results suggest that both JE and DENV cross-reactive antibodies and cross-primed T cells might play important roles in the cross-protection. The findings of this study also indicate a possibility of developing novel multivalent genetically engineered vaccines against both JEV and DENV.

Keywords Japanese encephalitis virus · Dengue virus · Flaviviruses · DNA vaccine · Cross-protection

Introduction

Dengue virus (DENV) is a member of the genus *Flavivirus*, and a major cause of arthropod-borne diseases worldwide. An estimated 390 million individuals are infected with DENV globally per year, of which 96 million are clinically apparent cases (Bhatt

et al. 2013). DENV infection can result in asymptomatic infection, self-limited dengue fever (DF), or more severe and potentially fatal clinical manifestations including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Halstead 2007). Infection with one serotype stimulates an effective and possibly life-long immunity against the re-infection with the same serotype; however, it cannot elicit fully protection against other serotypes. Importantly, a secondary infection with heterotypic DENV serotype may increase the risk of severe dengue in patients due to antibody-dependent enhancement (ADE), which poses a major barrier to the development of successful vaccine against DENVs. Therefore, dengue vaccines should induce a balanced efficient immune response against all four DENV serotypes. Although the recombinant, live, attenuated, tetravalent dengue vaccine (CYD-TDV) produced by Sanofi Pasteur has been registered in over ten countries, the vaccine appears to increase the risk of severe dengue in younger vaccine recipients (2–5-year age group) and available only for 9- to 45-year-old in Asia and Latin America [with the exception of Paraguay (9–60-year age group) and Singapore (12–45-year age group)] (World

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Health Organization 2015; Capeding et al. 2014; Juraska et al. 2018). Recently, the World Health Organization (WHO) recommended that the vaccine should only be used in individuals who had been previously infected with DENV (World Health Organization 2018). Therefore, it is imperative to develop a more effective DENV vaccine, and several candidate vaccines are under investigation (Chiang et al. 2016; Gil et al. 2016; Sjatha et al. 2014; Swaminathan et al. 2016; Wang et al. 2015).

Japanese encephalitis virus (JEV), which also belongs to the genus *Flavivirus*, is genetically and antigenically closely related to DENVs. Different from DENV, several JE vaccines including an inactivated vaccine (INV, IXIARO®) (Schuller et al. 2011) and a live attenuated vaccine (LAV, SA14-14-2) have been available to control JEV infection (Yu 2010). In addition, a LAV IMOJEV® (previously known as ChimeriVAX-JE) created by inserting the prM-E genes of JEV into the yellow fever virus 17D vaccine was initially licensed in Australia in 2012 and now approved in a number of Southeast Asian countries (Chokephaibulkit et al. 2016). It has been demonstrated that all these JE vaccines can effectively decrease the morbidity and mortality of JE without any severe side effects. Moreover, our recent study demonstrated that JE-LAV and JE-INV could induce cross-protection against DENV infection in mice, and cross-reactive neutralizing antibodies (NAbs) were detected in sera of human recipients of JE vaccine (Li et al. 2016), suggesting there were common immunological antigens between JEV and DENVs, which could induce protective immune response against both viruses.

JEV genome comprises a single-stranded positive-sense RNA which encodes three structural proteins [the capsid protein (C), the precursor of membrane protein (prM), and the envelope protein (E)] and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Of these, the E protein contains immunologically important epitopes, and neutralizing antibodies (NAbs) directed against epitopes on E protein are key factors of protective immunity. The prM serves as a chaperone for the correct intracellular processing and secretion of E protein (Konishi and Mason 1993). Importantly, co-expression of prM and E protein in cells can produce particulate form antigen (subviral particle) (Saga et al. 2016), which have been proved as a useful vaccine construction strategy. Therefore, the prM and E genes are the principal molecular candidates for developing flavivirus DNA vaccines. It was reported that DNA vaccines have several advantages such as economy, stability, and inducing balanced Th1 and Th2 immune responses (Khan 2013). Our previous studies have shown that the inoculation of plasmid containing JEV prM-E genes could elicit considerable immune responses and protection against JEV infection in mice and pigs (Li et al. 2013a; Sheng et al. 2016).

In this study, we examined whether the same DNA vaccine (pCAG-JME), expressing the most essential proteins of prM-E of JEV, could also induce cross-protection against DENVs. Our results confirmed that prM-E proteins played an

important role in cross-protection between JEV and DENVs, and thus suggesting a novel strategy for developing a multi-valent vaccine against both JEV and DENV.

Material and methods

Cells, viruses, vaccines, and mice

Vero cells (ATCC CRL-1586) were grown in minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) at 37 °C. The *Aedes albopictus* C6/36 cells (ATCC CRL-1660) were grown in RPMI1640 supplemented with 10% FBS at 28 °C.

DENV1 (Hawaii), DENV3 (H87), and DENV4 (H241) were provided by the Guangdong Provincial CDC. DENV2 (Tr1751) was isolated from a patient with dengue fever. All of the viruses were propagated in either C6/36 cells or Vero cells. Virus titers and plaque reduction neutralization test (PRNT) were determined by plaque assay on Vero cells.

The DNA vaccine pCAG-JME expressing the prM-E gene of JEV was constructed as reported previously (Chen et al. 2012). The concentration of the purified plasmid DNA was adjusted to 1.0 mg/ml prior to use. In addition, the LAV was manufactured by Chengdu Institute of Biological Products (CDIBP, Chengdu, China), and the INV was produced in China by Liaoning Chengda Biotechnology, Co., Ltd. (CDBIO).

Six-week-old female BALB/c mice and severe combined immune deficient (SCID) mice were purchased from the Vital River Laboratories (Beijing, China). All mice were maintained in specific-pathogen-free facilities.

Mouse immunization and challenge

BALB/c mice were immunized three times with 100 µg of pCAG-JME intramuscularly at 3-week intervals. The control group was similarly immunized with the pCAGGSP7 vector. Three weeks after the last immunization, the mice were euthanized for evaluation of DENV cross-reactive antibody (IgG) titers, cross-reactive NAb titers in serum, and cross-reactive cytokine-secreting T cells in splenocytes. In addition, splenocytes were also harvested for intracellular cytokine staining.

For the protection test, mice were immunized as above and then challenged intracerebrally (i.c.) at 3 weeks after the last immunization with a lethal dose of DENV1 (~ 10⁵ PFU), DENV2 (~ 10² PFU), DENV3 (~ 10⁶ PFU), or DENV4 (~ 10⁴ PFU) individually. These mice were observed for mortality for 28 days following infection.

For LAV or INV vaccination, the mice were administered a dose corresponding to approximately one-tenth of the dose recommended for human use (three times at 2-week intervals).

Antibody assay

The mouse serum samples were collected 3 weeks after the last immunization and analyzed for the presence of DENV cross-reactive antibodies (IgG) by enzyme-linked immunosorbent assay (ELISA) as described previously (Gao et al. 2010). Briefly, 96-well plates were coated with 10 µg/well of inactivated DENV1 or 2 or 3 or 4 viral particles that had been resuspended in 100 µl of carbonate-bicarbonate buffer (45.3 mM NaHCO₃, 18.2 mM Na₂CO₃, pH 9.6) at 4 °C overnight. Following washing and blocking, 100 µl of twofold serially diluted of serum samples (from 1:100) was added to each well and incubated at 4 °C overnight. Then, 100 µl of diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was added to each well, followed by color development with the addition of the substrate solution of orthophenylenediamine. The absorbance was measured at 492 nm on a microplate reader (Thermo). The endpoint titers of anti-DENV antibodies were determined as the reciprocal of the highest dilution, giving an optical density twice that of the non-immune serum.

Plaque reduction neutralization test (PRNT)

To determine the NAb in the sera of vaccinated BALB/c mice, heat-inactivated (at 56 °C for 30 min) mouse sera were twofold serially diluted (from 1:10 to 1:5120) with MEM containing 2% FBS. Then, 500 µl of the serial dilutions of sera was incubated with an equal volume of virus solution (DENV1, 2, 3, or 4) containing about 500 plaque focus units (PFU) at 37 °C for 1 h. Then 200 µl of the mixture was added to duplicate wells of Vero cell monolayer in a 24-well plate, so that each well contains approximately 100 PFU of the virus. The plate was incubated at 37 °C for 1 h. After washing, the infected Vero cells were overlaid with MEM containing 5% FBS and 1.2% methylcellulose. The plates were incubated at 37 °C for 7–9 days before count viral plaques. The NAb titer was computed as the reciprocal of the maximum dilution of serum that yielded a 50% plaque reduction (PRNT50) (Gao et al. 2010).

Cytokine-enzyme linked immunospot (ELISPOT) assay

Splenocytes of immunized mice were harvested after the last immunization. The splenocytes secreting cytokines including interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, and IL-10 were enumerated by ELISPOT assay according to the manufacturer's instructions (Li et al. 2013a; Li et al. 2016). Briefly, 96-well filtration plates (Millipore, USA) were coated with 50 µl (10 µg/ml) of anti-mouse IFN-γ, IL-2, IL-4, or IL-10 capture antibodies at 4 °C overnight.

The next day, the plates were blocked with sterile RPMI 1640 containing 10% FBS and 1% BSA at 37 °C for 2 h. Then a total of 5×10^5 splenocytes were added to each well of the plates and stimulated with 50 µg/ml of either DENV1, or DENV2, or DENV3, or DENV4 viral particles concentrated from the media of infected C6/36 cells at 37 °C for 48 h. After stimulation, the cells were incubated with biotin-conjugated anti-cytokine Abs and then streptavidin-HRP. The cells incubated with ConA (5 µg/ml, Sigma, USA) were used as a positive control, and cells cultured in RPMI 1640 medium alone were used as a negative control. The number of cytokine-secreting cell was determined by an automated ELISPOT reader and analyzed by ImmunoSpot image analyzer software (version 4.0, CTL Ltd., USA).

Intracellular cytokine staining (ICS)

For intracellular cytokine staining, after being blocked with rat anti-mouse CD16/CD32 mAb, 2×10^6 splenocytes from pCAG-JME immunized mice were respectively stimulated with 2 µg of individual concentrated viral particles (JEV or DENV1–4) for 24 h, at the last 5 h, Brefeldin A was added. Splenocytes from pCAGGSP7 immunized mice were stimulated with pooled viral antigens. Then the cells were incubated with anti-CD3e-FITC, anti-CD8-APC-H7 and anti-CD11a-APC. After fixation and permeabilization, the cells were subsequently stained with anti-IFN-γ-PE. Finally, the samples were processed on a DxFLEx flow cytometer (Beckman Coulter, USA) and analyzed using CytExpert software (version 2.0, USA).

In vivo neutralizing activity in SCID mice

The neutralizing activity of immune sera was further detected in vivo as described previously (Tang et al. 2015; Zhao et al. 2014). Three weeks after the final vaccination, pooled sera from pCAG-JME, pCAGGSP7, JE-LAV, or JE-INV groups (Li et al. 2013a; Li et al. 2016) were mixed with DENV2, respectively, and incubated at 37 °C for 1 h. Then 20 µl of each type of sera/virus mixture (10^3 PFU of DENV2) was i.c. inoculated to each SCID mouse. A total of five mice were used per group. The mice were monitored daily for morbidity and mortality for 28 days. Sera from pCAGGSP7 group were used as negative control.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Data were considered statistically significant if $P < 0.05$.

Results

The levels of cross-reactive antibodies to DENV1–4

The pCAG-JME induced DENV cross-reactive antibody levels were shown in Fig. 1. Endpoint titers of IgG cross-react with DENV2 and DENV3 were 1:1493 and 1:1481, higher than those against DENV1 (1:872) and DENV4 (1:459). These demonstrate that pCAG-JME immunization elicited high level cross-reactive IgG antibodies to each of the four dengue serotypes. Additionally, the IgG titer against JEV was up to 1:6400 (Li et al. 2013a).

The levels of cross-neutralizing-antibodies to DENV1–4

The serum samples collected after final immunization were used for determining cross-NAb titers. As shown in Table 1, cross-NAb titer of the empty vector was less than 1:10. In contrast, the pCAG-JME induced cross-NAb against DENV1–4, with the highest against DENV2 (1:592), slightly lower against DENV1 (1:160), even lower against DENV3 (1:60) and DENV4 (1:53). Notably, these cross anti-DENV NAb titers were much higher than that elicited by JE-LAV or JE-INV vaccination (Li et al. 2016). Additionally, the NAb titer against JEV was 1:1280 (Li et al. 2013a).

Cross-reactive cell-mediated immune response to DENV1–4

Having demonstrated cross-reactive antibody responses, we next sought to determine whether cross-primed T cell immune responses were also generated by the pCAG-JME vaccine. As

Fig. 1 DENV cross-reactive antibodies were elicited by JEV DNA vaccine. The endpoint titers of cross-reactive antibodies were determined by ELISA and presented as the reciprocal of the highest dilution, giving an optical density twice that of the pCAGGSP7-immune sera ($n = 10$). Sera were collected from immunized mice 3 weeks after the final immunization. The results showed above are geometrical mean titers

shown in Fig. 2, when stimulated with DENV1 and DENV3 antigen, splenocytes from vaccinated mice generated significant amounts of IFN- γ , IL-2, IL-4, and IL-10 as compared with the control group (Fig. 2A-a and A-c, $P < 0.001$). In comparison, DENV2 stimulation only activated increased IFN- γ and IL-4 secretion (Fig. 2A-b); DENV4 only stimulated significant elevation of IFN- γ and IL-10 secretion in comparison with control (Fig. 2A-d). Despite the discrepancy in the type of cytokines activated, all four DENV viral antigens elicited both Th1- and Th2-type immune responses. These results suggest that balanced Th1- and Th2-type immune responses could be contributable to cross-reactivity between DENV and JEV.

Moreover, cross-reactive IFN- γ -secreting CD8 $^{+}$ T cells in the spleen from pCAG-JME immunized mice were detected by ICS assay. The percentages of CD3 $^{+}$ CD8 $^{+}$ CD11a $^{+}$ IFN- γ $^{+}$ T cells in the spleen after stimulation with JEV and DENV1–4 antigen were $1.62 \pm 0.38\%$, $1.15 \pm 0.37\%$, 1.07 ± 0.0026 , $1.20 \pm 0.09\%$, and $1.00 \pm 0.22\%$, respectively (Fig. 3, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. pCAGGSP7). As expected, T cell from pCAGGSP7 immunized mice did not significantly respond to the pooled viral antigens ($0.53 \pm 0.07\%$). The results mentioned above imply that pCAG-JME-immunized T cell also mediates cross-reactive response to DENVs.

Mouse challenge experiments

To determine whether the above-described cross-reactive immune responses could confer protection, we examined the survival rates in each vaccinated group following challenge with a lethal dose of DENVs (Fig. 4, Table 1). Consistent with the results of ELISA, PRNT, and ELISPOT assays, pCAG-JME-immunized mice were partly protected from DENV1, DENV2, or DENV3 challenge, with 60%, 60%, or 87.5% survival rates, respectively. Notably, mice immunized with pCAG-JME were completely protected from DENV4 challenge, with a 100% survival rate. In mice immunized with a control pCAGGSP7 DNA, 90% of mice died after being challenged with DENV1, 2, and 4; 75% of mice died after being challenged with DENV3. These results demonstrate that pCAG-JME vaccine candidate was able to induce cross-protective immunity against lethal challenge with DENV1–4. It is noting that survival rates observed in this study were greater than those induced by JE-LAV and JE-INV (Li et al. 2016).

pCAG-JME-immunized sera showed neutralizing activity in SCID mice

To further confirm the DENV cross-protection conferred by pCAG-JME vaccines, sera pooled from immunized mice were mixed with DENV2 and the mixture was i.c. inoculated into SCID mouse for detecting in vivo neutralizing activity. In

Table 1 The levels of cross-reactive NAb and survival rates in JEV DNA vaccine-immunized BALB/c mice after challenged with different serotypes of DENV

Types	Groups	Neutralizing antibody titers ^a	<i>P</i> ^c	Survival % ^b (no. of survivors/no. of challenged)	<i>P</i> ^d
DENV1	pCAG-JME	1:160	0.000	60(6/10)	0.002
	pCAGGSP7	< 1:10		1(1/10)	
DENV2	pCAG-JME	1:592	0.000	60(6/10)	0.003
	pCAGGSP7	< 1:10		1(1/10)	
DENV3	pCAG-JME	1:61	0.000	87.5(7/8)	0.032
	pCAGGSP7	< 1:10		25(2/8)	
DENV4	pCAG-JME	1:53	0.001	100(10/10)	0.000
	pCAGGSP7	< 1:10		10(1/10)	

^a The geometrical mean of neutralizing titers was assayed^b Mice were challenged i.c. with different serotypes of DENV at 3 weeks after the final immunization, and then monitored daily for 28 days. The survival rate was determined as $100 \times (\text{number of survivors})/(\text{numbers of challenged})$ ^{c, d} *P* value of NAb and survival rate between pCAG-JME and pCAGGSP7, respectively

addition, the neutralizing activity of sera derived for LAV- and INV-immunized mice were also evaluated in the present study. As shown in Fig. 5, all mice in the control group died during days 12 to 15 after the inoculation, only 20% of mice survived in JE-INV group, and unexpected, all mice in the JE-LAV group also died. In comparison, 60% of survival rate was observed in pCAG-JME group, significantly better than all other groups ($P < 0.05$). Survival rates observed in each group were consistent with the level of cross-reactive IgG titers to DENV2, which were 1:920, 1:311, and 1:1493 in JE-INV, JE-LAV, and pCAG-JME groups, respectively (Li et al. 2016); it was also correlated with the cross-NAb titers to DENV2, which were 1:403, 1:74, and 1:592 in JE-INV, JE-LAV, and pCAG-JME groups, respectively (Li et al. 2016). Taken together, these data highlight the importance of cross-reactive antibodies, especially NABs, against to DENV2 infection. Since pCAG-JME induced the highest cross-IgG and cross-NAb titers against DENV2 in the present study, we only performed the in vivo assay with DENV2 in SCID mice.

Discussion

DENV consists of four distinct serotypes. Individuals infected with DENV could induce protective immune responses to the same serotype through lifetime, but those afflicted by secondary infection with heterogenous serotype tend to have higher virus loads, aggravated symptoms, and greater risk of suffering severe dengue such as DHF and DSS, possibly due to ADE and cytokine storm linked to the “original antigenic sin” (Balsitis et al. 2010; Flipse et al. 2013; Halstead et al. 1983; Wahala and Silva 2011). Therefore, much effort had been placed on developing an ideal DENV vaccine that could induce a balanced immunity to all four serotypes of DENV

using various strategies (Coller and Clements 2011; Heinz and Stiasny 2012; Thomas and Endy 2011).

Based on high antigenic homology among flaviviruses, the viral chimerization technique has been employed for DF vaccine development. For example, CYD-TDV is the chimeric vaccine comprising four recombinant, live, attenuated viruses (Sanofi-Pasteur, France), using the YFV 17D vaccine as the backbone (Guirakhoo et al. 2004; Morrison et al. 2010). Although the first DENV vaccine CYD-TDV was licensed in more than ten countries, it showed the risk of severe and hospitalized disease in non-immunes and those under 9 years old, which prompted a change in the vaccine label and a re-review by the WHO (World Health Organization 2018), indicating it is imperative to develop a safer and more effective preventative strategy.

All members of the genus *Flavivirus* are antigenically related but distinctive serocomplexes, having cross-reactivity to each other. Previously, the cross-reactivity among *Flaviviruses* had been investigated by several groups. Tesh et al. found that immunization with the live attenuated JEV (SA14-2-8 strain) protected against West Nile virus infection in hamster (Tesh et al. 2002). Lobigs et al. reported that the live chimeric JE vaccine (ChimeriVAX) could induced better cross-protection than JE-INV (JE-VAX) following Murray Valley encephalitis virus challenge, which also belongs to the JEV serocomplex (Lobigs et al. 2009). However, a few studies have observed the opposite results. Saito et al. demonstrated that the JE Vero cell-derived INV could induce cross-reactive DENV IgG antibodies but not NABs in human, and part of serum samples displayed ADE activity to DENV (Saito et al. 2016). The different results mentioned above might associated with the following reasons: 1) immune schedule, 2) vaccine doses, and types as well as 3) different sampling times post vaccination. Recently, we have demonstrated that the JE-INV and the JE-LAV elicited partial cross-

Fig. 2 Detection of the Th1 and Th2 responses. Splenocytes were stimulated with DENV1–4 antigens, cytokine secretion was detected by ELISPOT ($n = 5$). **A**, The numbers of cytokine-positive cells were expressed as spot-forming units (SFU)/5 × 10⁵ cells after background subtraction. **a**, DENV1 antigen stimulation; **b**, DENV2 antigen

stimulation; **c**, DENV3 antigen stimulation; **d**, DENV4 antigen stimulation (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. pCAGGSP7). **B**, **A** representative raw ELISPOT data in the format of scanned image, **a-d**: stimulated by DENV1–4 antigen, respectively

Fig. 3 Cross-reactive CD8⁺ T cell response to DENVs. Following the stimulation with JEV and DENV1–4 antigens, the percentage of CD3⁺CD8⁺CD11a⁺IFN-γ⁺ T cells in spleen of pCAG-JME immunized mice was detected by ICS assay, respectively ($n = 5$). Splenocytes from pCAGGSP7 immunized mice were stimulated with pooled viral antigens. A, Representative data from the ICS analysis of CD3⁺CD8⁺CD11a⁺IFN-

γ⁺ T cells from the pCAG-JME immunized mice. The percentage of double-positive cells is shown in the top right corner. B, The bar graph shows the mean percentage with a SD of CD3⁺CD8⁺CD11a⁺IFN-γ⁺ T cells in different antigen stimulation (* $P < 0.05$, ** $P < 0.01$ vs. pCAGGSP7)

protection against DENV1–4 in mice and cross-reactive NAb were also detected in sera of human JE vaccine recipients (Li et al. 2016).

Among the several viral proteins of flaviviruses, it is well known that the E protein, exposed on the virion surface, contains immunological epitopes that are not only able to induce

Fig. 4 Protection against DENV challenge after immunization with JEV DNA vaccine. The immunized mice were challenged intracerebrally (i.c.) with a lethal dose of DENV1–4. The results were expressed as survival rates. A–D, Challenged with DENV1–4 respectively

Fig. 5 In vivo neutralizing activity of immune sera in SCID mice ($n = 5$). Three weeks after the final vaccination, the sera from immunized mice receiving pCAG-JME, pCAGGSP7, LAV, or INV groups were mixed with DENV2, and then the sera/virus mixture (10^3 PFU of DENV2) was i.c. inoculated to each mouse. The mice were monitored daily for morbidity and mortality for 28 days. The survival rates were shown as the percentage of survivors

protective NAb but also prime-specific T cell responses (Aberle et al. 2018), and the prM is necessary for correct folding of E protein. Our previous studies have shown that the inoculation of plasmid containing JEV prM-E genes could elicit considerable immune responses and protection against JEV infection in mice (Li et al. 2013a). In the present study, we evaluated the cross-reactive humoral and cellular immunity mediated by pCAG-JME, and demonstrated that the cross-protection between JEV and DENVs.

High titers of cross-reactive IgG and NAb to DENVs were observed in pCAG-JME vaccinated mice. Moreover, cross-protection to each serotype of DENVs was induced by pCAG-JME. Also worth noting that there was no significant difference in survival rates after challenged with DENV1–4, indicating that pCAG-JME induced a relatively balanced cross-protection to DENV1–4. Furthermore, the highest in vivo neutralizing activity against to DENV2 in SCID mice was also induced by pCAG-JME.

The elevated productions of IFN- γ , IL-2, and IL-4, IL-10, markers of the Th1- and Th2-immune responses, respectively, were also observed when stimulated with DENV1 and 3. Whereas increased cytokine level of IFN- γ , IL-4 were stimulated by DENV2, and IFN- γ , IL-10 by DENV4. The divergent pattern of cytokine secretion upon stimulation with different serotype of DENV is unexpected. The exact reason for this waits for further investigation. Moreover, cross-primed CD8 $^+$ T cell response was also detected following the stimulation of DENV proteins using ICS. These results indicated that cross-reactive cellular immunity induced by pCAG-JME also involved in the cross-protection against DENVs. More recently, the cross-protection mediated by cross-reactive CD8 $^+$ T cells was also reported between DENV and Zika virus (ZIKV), another antigenically related member of genus *Flavivirus*

(Wen et al. 2017). Additionally, Grifoni et al. found that memory T cell responses elicited by prior infection with DENV or immunization with a tetravalent DENV vaccine (TV005) recognized the ZIKV-derived peptides and modulated earlier and greater magnitude of T cell responses to the following ZIKV infection (Grifoni et al. 2017). Thus, further exploration of the cross-protective T cell epitopes between JEV and DENV in the future may help us to understand the mechanism underlying the cross-protection among flaviviruses.

The pCAG-JME expresses both prM and E proteins. Our data do not perform dissection of the relative role of these two proteins in protection. DENV E protein contains 12 strictly conserved cysteine residues forming six disulfide bridges. It exists as a heterodimer with the prM before the maturation of the virion. X-ray crystallographic studies of the ectodomain of E protein have revealed three distinct domains: DI, DII, and DIII. Previous studies have demonstrated that domain II of E protein (EDII) is able to induce cross-reactive immune responses to members of the flavivirus group (Crill et al. 2007; Lai et al. 2008; Stiasny et al. 2006; Trainor et al. 2007). Therefore, highly conserved epitopes at the fusion loop of EDII might be responsible for the cross-reactivity between JEV and DENV. Additionally, antibodies to prM (anti-prMs) also formed one of the major components of the host response to DENV infection, and they are highly cross-reactive among the DENV serotypes. However, anti-prMs can only induce weak neutralization activity even at high concentrations but potentially promote ADE (Dejnirattisai et al. 2010). Thus, it is advisable to minimize the anti-prM response for future vaccine design. There is relatively low sequence conservation between DENV and JEV amino acid sequences from prM (35% DENV versus JEV) as compared with that of E (50%), and the majority of antibodies to DENV prM do not cross-react with JEV (Dejnirattisai et al. 2010). Actually, the homology of E between JEV and DENV1, DENV2, DENV3, and DENV4 are 50.4%, 48.4%, 48%, and 47.8%, respectively. Therefore, our study may indicate some possibilities: (1) vaccination with JE vaccine may reduce the severity of dengue disease or prevent DENV infection; (2) identification of the cross-immunological epitopes and then construction of a chimeric vaccine using JEV cDNA as a backbone may be an important way for developing a novel multivalent vaccine against both DENV and JEV. Indeed, a chimeric vaccine of DENV using JE vaccine strain SA14-14-2 has been developed and tested in several groups (Li et al. 2013b; Li et al. 2014).

Taken together, our results demonstrated that a novel DNA vaccine (expressing JEV prM-E protein) could induce relatively balanced cross-immunity and cross-protection against all four serotypes of DENVs in mice. The findings provide an alternative insight into the development of the next generation of DENV vaccines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal experiments were approved by the animal ethics committees of Capital Medical University, Beijing, China.

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