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Novel recombinant chimeric virus-like particle is immunogenic and protective against both enterovirus 71 and coxsackievirus A16 in mice

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Hand-foot-and-mouth disease (HFMD) has been recognized as an important global public health issue, which is predominantly caused by enterovirus 71 (EV-A71) and coxsackievirus A16 (CVA16). There is no available vaccine against HFMD. An ideal HFMD vaccine should be bivalent against both EV-A71 and CVA16. Here, a novel strategy to produce bivalent HFMD vaccine based on chimeric EV-A71 virus-like particles (ChiEV-A71 VLPs) was proposed and illustrated. The neutralizing epitope SP70 within the capsid protein VP1 of EV-A71 was replaced with that of CVA16 in ChiEV-A71 VLPs. Structural modeling revealed that the replaced CVA16-SP70 epitope is well exposed on the surface of ChiEV-A71 VLPs. These VLPs produced in *Saccharomyces cerevisiae* exhibited similarity in both protein composition and morphology as naive EV-A71 VLPs. Immunization with ChiEV-A71 VLPs in mice elicited robust Th1/Th2 dependent immune responses against EV-A71 and CVA16. Furthermore, passive immunization with anti-ChiEV-A71 VLPs sera conferred full protection against lethal challenge of both EV-A71 and CVA16 infection in neonatal mice. These results suggested that this chimeric vaccine, ChiEV-A71 might have the potential to be further developed as a bivalent HFMD vaccine in the near future. Such chimeric enterovirus VLPs provide an alternative platform for bivalent HFMD vaccine development.

Hand-foot-and-mouth disease (HFMD) is a common infectious disease of infants and children younger than 5 years old[1]. Over the last decade, several large outbreaks of HFMD have been reported in countries of the Western Pacific Region, including China, Japan, Malaysia, Singapore, and Vietnam[2–6], occasionally associated with many death cases, and the incidence of HFMD appears to be increasing across the Region[7]. In China, 2.17 million cases of HFMD, including 567 deaths, were reported in 2012[8]. HFMD has now been recognized as an important global public health issue. This has prompted concerns that, effective prophylactic vaccines against HFMD are urgently needed.

HFMD is most commonly caused by coxsackievirus A16, which usually results in a mild self-limiting disease with few complications[9,10]. However, HFMD is also caused by enterovirus 71 (EV-A71), which has been associated with serious complications and may be fatal[11]. Both EV-A71 and CVA16 belong to *Picornaviridae* family, *Enterovirus* genus, and possess the similar biologic structure. Enterovirus genome contains a single open reading frame encoding a polyprotein, which is cleaved into at least 11 proteins: the four capsid proteins (VP1, VP2, VP3 and VP4), and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D), which together are responsible for essential processes during genome replication and viral assembly[12].

Currently, many monovalent vaccine candidates against HFMD have been developed. The inactivated EV-A71 vaccines have undergone phase III clinical trials with ideal efficacy[13,14], and several CVA16 vaccine candidates have showed promise for clinical use[15–18]. However, no effective cross-neutralization for EV-A71 and CVA16 has been observed yet. Moreover, co-circulation of the two viruses has been reported[10] which arouses significant public health concern[19,20]. Thus, for highly effective HFMD prevention, a bivalent vaccine against both EV-A71 and CVA16 is necessary.

Virus-like particles (VLPs) resembles the authentic virus in morphology, protein composition and capsid structure. The fact that VLP preserves the repetitive ordered arrangement of epitopes on the surface makes VLP candidates for potent immunogen and vaccine. The attention is increasingly being focused on VLP strategies for HFMD vaccine, due to the advantages of being highly immunogenic, non-infectious, and accessible to scaling-up during production. Recent studies showed that the cryo-electron microscopy reconstructions of EV-A71 and CVA16 VLPs highly resemble the crystal structures of EV-A71 natural empty particles and CVA16 135S-like expanded particles, respectively[21]. They both have quasi-T = 3 symmetry with 60 copies of each of the viral structural proteins VP1, VP2 (VP0), VP3[22], which provides meaningful information for the development of VLP vaccine against HFMD. To date, it has been shown that several VLP vaccine candidates for EV-A71 or CVA16 can induce effective immune protection responses in mice[18,23–25]. In our previous study, a yeast system has been developed for the production EV-A71 VLPs by co-expressing P1 and 3CD of EV-A71 in *Saccharomyces cerevisiae*, and immunization with these VLPs induced protective immune responses in mice[23].

Neutralizing antibodies against enterovirus have been demonstrated as the most important factors in limiting the severity of infection[26]. VP1 has been reported to be the main structural protein capable of inducing neutralizing antibodies[27,28]. Indeed, previous studies identified that the SP70 peptide (aa208-222 in VP1) of EV-A71 constitute a major neutralization epitope of EV-A71, which was capable of eliciting neutralizing antibodies and conferred protection against homologous and heterologous EV-A71 strains in neonatal BALB/c mice[29–31], and was usually applied for developing EV-A71 vaccine candidates based on epitope[32–35]. The high-resolution structure for the mature virus particles of EV-A71 showed that the SP70 peptide lies on the VP1 GH loop of the capsid surface[21,36]. In general, the most variable regions of enterovirus virion are the loops exposed on the virion surface that are also the most important neutralizing immunogenic sites[37]. The x-ray structure analysis on the CVA16 uncoating intermediate revealed that the corresponding SP70 epitope in CVA16 is also displayed on the virion surface[38]. Recently, Huang et al. identified six linear neutralizing epitopes of CVA16, among which the PEP71 located in the aa211–225 of VP1 with 80% overlap of amino acids in SP70. PEP71 was capable of eliciting neutralizing antibodies against homologous and heterologous CVA16 strains. Further, this epitope of different CVA16 subgenotypes are highly conserved, in particular, which is identical among all the different strains belonged to genotype B[39]. These data implied that SP70 might be a linear neutralizing epitopes of CVA16, suggesting a potential for developing a peptide-based, universal CVA16 vaccine. Our working hypothesis was that if the SP70 peptide in EV-A71 VLPs was replaced with the corresponding region in CVA16, a novel recombinant chimeric VLP vaccine against both EV-A71 and CVA16 might be produced.

In the present work, based on the EV-A71 VLPs developed previously, we constructed a ChiEV-A71 VLPs, in which the SP70 of EV-A71 was replaced with that of CVA16. ChiEV-A71 VLPs were produced by co-expressing ChiEV-A71-P1 and 3CD of EV-A71 in *Saccharomyces cerevisiae*. ChiEV-A71 was evidenced to evoke potent humoral and cellular immune responses and passive transfer with anti-ChiEV-A71 sera conferred full protection against both EV-A71 and CVA16 challenges in mice, which represents a potential bivalent vaccine candidate for HFMD. Such chimeric VLPs provide an alternative platform for bivalent HFMD vaccine development.

Results

Production and purification of ChiEV-A71 VLPs. Previously, we have constructed the pYES2-P1 and pYES2-Trp-3CD vectors to produce EV-A71 VLPs[23]. In the present study, the DNA sequences coding for the SP70 epitope of CVA16 was rationally designed to replace the corresponding region of EV-A71 based on PCR mutagenesis, resulting in the desired plasmid pYES2-ChiEV-A71-P1 (Figure 1A). Then, a yeast transformant carrying both plasmid pYES2-ChiEV-A71-P1 and pYES2-Trp-3CD were generated. After galactose induction, both ChiEV-A71-P1 and EV-A71-3CD were expressed in the cytoplasm. The protease activity of the expressed EV-A71-3CD was demonstrated from the western blot analysis of cell lysates which indicated that VP0 of the right size was generated from the expressed ChiEV-A71-P1 (Figure 1C).

To test if the resulting VP0 and other proteins can assemble into VLPs, we performed sucrose gradient ultracentrifugation experiment as described previously[23]. Fractions of the gradient were subjected to SDS-PAGE analysis to examine the presence of capsid proteins VP0, VP3 and modified VP1. The co-sedimentation of these capsid proteins, at about a sucrose concentration of 30% and a density of 1.13 g/ml, suggested the assembly of higher-order structures from the capsid proteins (Figure 1B). Gradient fractions were subjected to western blot analysis to detect the presence of capsid protein VP0 (38 KDa). The sedimentation of VP0 is at a sucrose density of 1.11–1.16 g/ml, reminiscent of the protein distribution of EV-A71 VLPs produced from *Saccharomyces cerevisiae* reported previously (Figure 1C). Those fractions with capsid proteins co-sedimented (fractions 11–16 as shown in Figure 1B) were pooled together and concentrated by one more round of ultracentrifugation. SDS-PAGE analysis of the concentrated sample showed three obvious protein bands at about the same molecular sizes corresponding to capsid proteins VP0, VP1 and VP3 of EV-A71 (Figure 1D).

Structural modeling and characteristic of ChiEV-A71 VLPs. Structural modeling of ChiEV-A71 VLP was performed using the SWISS-MODEL server (http://swissmodel.expasy.org) to predict the location of CVA16-SP70 in VLP. The data showed that the replaced SP70 peptide from CVA16 protruded from the natural VP1 proteins (Figure 2A). The 60 copies of the CVA16-SP70 peptides were predicted to be uniformly displayed on the surface of VLP without affecting the original structure of the EV-A71 VLP (Figure 2B). Then, EM analysis was performed to examine the size and shape of the assembled higher-order structures. The EM image (Figure 2C) of negatively stained sample demonstrated the presence of intact VLPs, which were morphologically similar to EV-A71-VLPs obtained previously from yeast[23]. Further analysis with ELISA confirmed that the ChiEV-A71 VLPs could significantly react with mouse polyclonal antibodies against CVA16-SP70 compared with EV-A71 VLPs (Figure 2D). In contrast, the ChiEV-A71 VLPs had only a lower reactivity with mAbs targeting EV-A71-SP70 (Figure 2E.)These data demonstrated that CVA16-SP70 was functionally displayed in the ChiEV-A71 VLPs, and ChiEV-A71 was potential to be a CVA16 antigen as originally designed.

ChiEV-A71 VLPs induced humoral immunity response against both EV-A71 and CVA16. Humoral immunity responses against EV-A71 and CVA16 were evaluated in mice immunized with ChiEV-A71 VLPs. Group of mice immunized with EV-A71 VLPs was set for comparison. Serum samples were collected at two weeks after each immunization and detected for specific IgG antibodies against EV-A71 and CVA16 by Immunofluorescence assay as previously described[18,23]. The results revealed that IgG antibodies against EV-A71 were induced in ChiEV-A71 VLPs-immunized mice at two weeks after prime immunization, and IgG titer gradually increased along with boost immunization. After the third immunization, the total lgG titer in mice immunized was up to 1 : 12793. In EV-A71 VLPs-immunized mice, the similar IgG responses were elicited, and the total lgG titer was up to 1 : 11402 (Figure 3A). The data showed that levels of IgG responses against EV-A71 were similar between ChiEV-A71 and EV-A71 groups ($P = 0.765$). The same methods were used to detect the IgG antibodies against CVA16. As shown in Figure 3B, IgG antibodies against

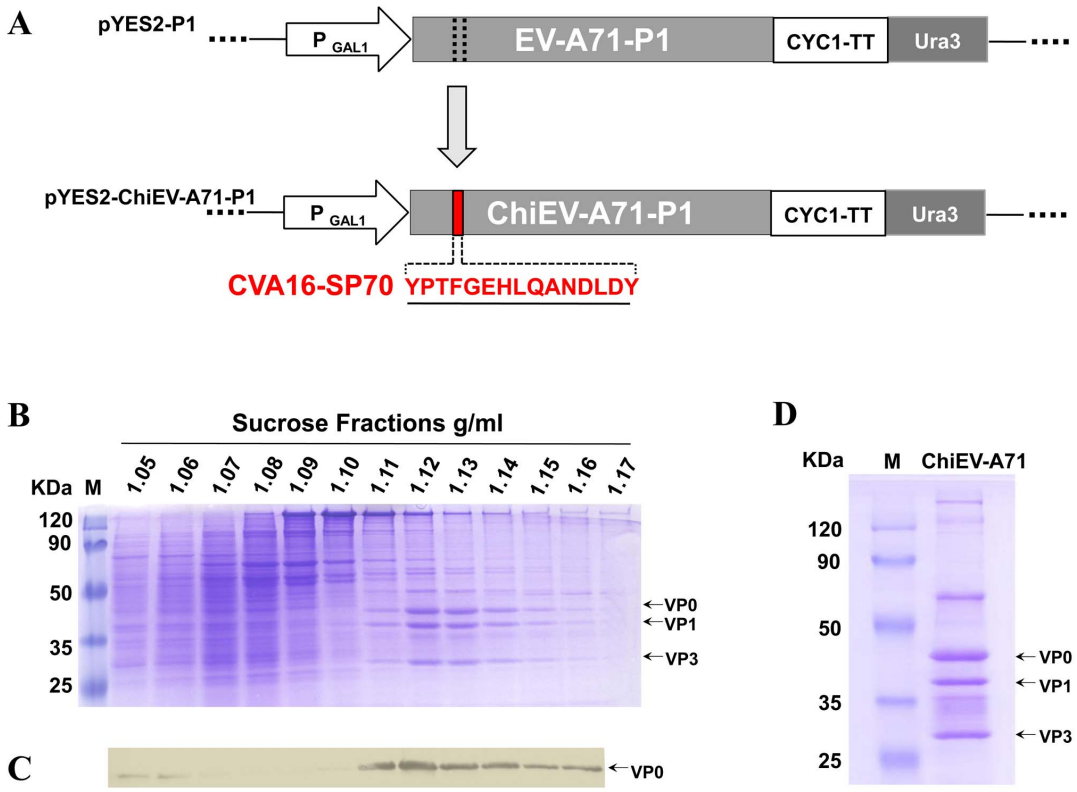

Figure 1 | Schematic representation of the ChiEV-A71-P1 constructs and expression and purification of ChiEV-A71 VLP. (A) The DNA sequences coding for the SP70 epitope in EV-A71-P1 was replaced with the corresponding region in CVA16-P1 to obtain ChiEV-A71-P1. The genes were under controls of GAL1 inducible promoters. (B) SDS-PAGE analysis indicated that fractions 11–16 contained enriched capsid proteins, i.e., VP0, VP1, and VP3, suggesting likely the presence of VLPs. The density of these fractions is about 1.13 g/ml. (C) Fractions were taken from top to bottom after sucrose gradient ultracentrifugation (10–40%). Samples of the fractions were probed with antibodies against VP0, indicating the expression of both ChiEV-A71-P1 and 3CD proteins and efficient protease activity of the expressed 3CD in the yeast cytoplasm. (D) SDS-PAGE analysis of purified ChiV71 VLPs. Fractions 11–16 were collected, diluted with PBS, and concentrated by ultracentrifugation. Coomassie blue staining of the purified ChiEV-A71 VLPs demonstrated the presence of three capsid proteins: VP0 (38 kDa), VP1 (36 kDa), and VP3 (27 kDa).

CVA16 were induced only in ChiEV-A71 VLPs-immunized mice, and the total lgG titer was up to 1 : 2540 at two weeks after third immunization. In contrast, there were no IgG responses in EV-A71 groups. These results demonstrated that ChiEV-A71 VLPs could elicit the IgG responses against both EV-A71 and CVA16. Further, the presence of different IgG subclasses in the immune sera was analyzed by ELISA. The data showed that the IgG induced by ChiEV-A71 VLP immunization include high levels of IgG1, following by IgG2a and IgG2b, but low levels of IgG3. In addition, the level of IgG subclasses in ChiEV-A71 VLPs-immunized mice was similar to that of EV-A71 VLPs group (Table 1).

Neutralizing antibody titers against EV-A71 and CVA16 were measured in RD or Vero cells by *in vitro* microneutralization assay. The data showed that high levels of neutralizing antibodies against EV-A71 were both induced by ChiEV-A71 VLPs and EV-A71 VLPs after the last boost immunization (Figure 4A), and there was no difference between the two groups ($P = 0.832$). All of the ChiEV-A71 VLPs immunized mouse sera could neutralize CVA16 and the geometric mean titers were 1 : 16. Although some of EV-A71 VLPs immunized mouse sera weakly neutralized CVA16, there was no significant difference compared with the sera of non-immunized mice (Figure 4B). Thus, the antisera of the ChiEV-A71 group showed potent neutralization activity against both EV-A71 and CVA16.

ChiEV-A71 VLPs induced cellular immunity responses. To examine the cellular immunity responses induced by ChiEV-A71 VLPs, cytokine profiles of mice immunized with VLPs were determined by measuring levels of IFN-γ, IL-2, IL-4 and IL-6 secreted by splenocytes stimulated with inactivated EV-A71. As shown in Figure 5, the production of IFN-γ, IL-2, IL-4 and IL-6 in splenocytes from ChiEV-A71 VLPs immunized mice was significantly higher than negative control, while no significant differences were observed in expression of cytokines between ChiEV-A71 and EV-A71 group. Taken together, ChiEV-A71 VLPs could induce potent cellular immunity responses against EV-A71.

Passive protection against lethal EV-A71 and CVA16 challenge. *In vivo* protective efficacy against lethal EV-A71 and CVA16 challenge was evaluated in an established neonatal mice model[18,23]. Groups of BALB/c neonatal mice were challenged with lethal EV-A71 (100 LD_{50} per mouse) or CVA16 (20 LD_{50} per mouse) followed by treatment with antisera from mice immunized ChiEV-A71 or EV-A71 VLPs. As shown in Figure 6A, when EV-A71-infected mice were administered with the anti-ChiEV-A71 or anti-EV-A71 antisera, all of them survived, while all mice that received PBS became sick at 7 days post-challenge and died within 10 days, which demonstrated that the antisera induced by ChiEV-A71 VLP immunization could provide full protection against EV-A71 infection in neonatal mice, and the protective efficacy against EV-A71 was similar between ChiEV-A71 and EV-A71 groups. *In vivo* protective assay against lethal CVA16 challenge showed that anti-ChiEV-A71 antisera treatment completely protected mice from CVA16 infection, while all mice that received PBS began to develop clinical symptoms at 8 days post-challenge and died within 11 days (Figure 6B). Although two mice in the anti-EV-A71 antisera group ($n = 8$) were live, which had limb paralysis, by Log-rank test, anti-EV-A71 antisera could not

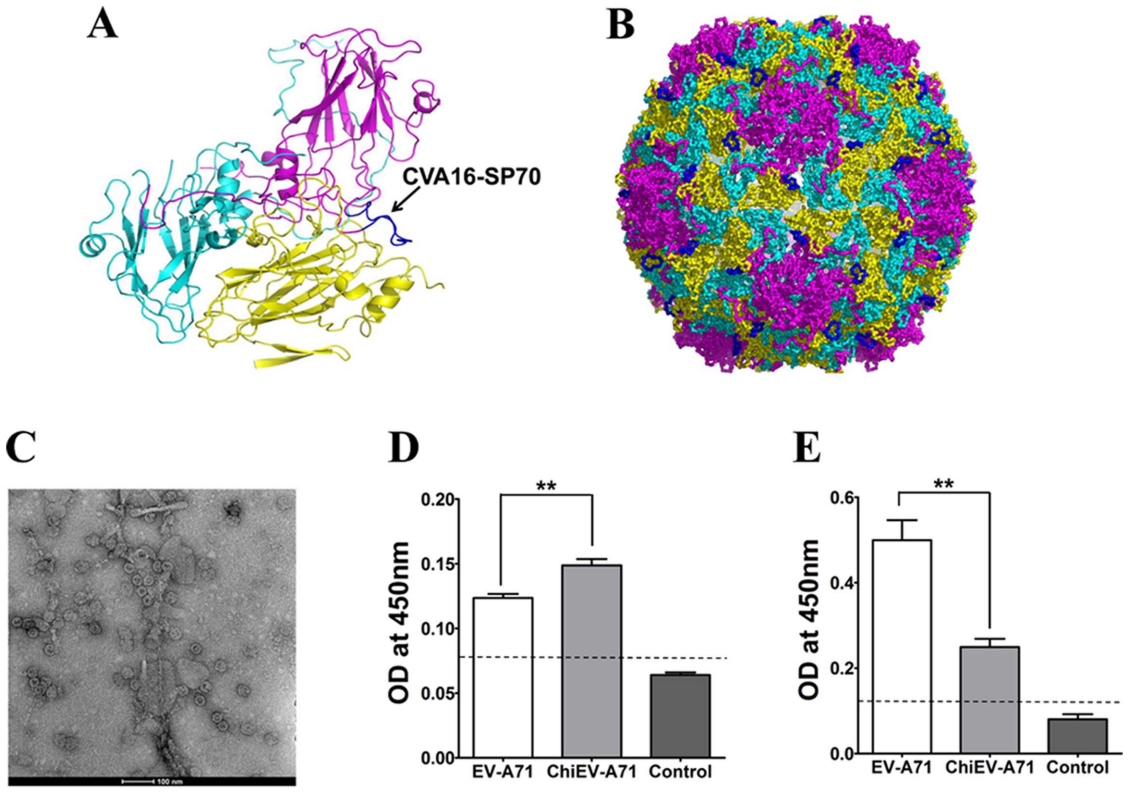

Figure 2 | Structural modeling and identification of ChiEV-A71 VLPs. (A) Structural modeling using the SWISS-MODEL server (http://swissmodel.expasy.org). A cartoon representation of a protomer with VP1, VP2, VP3 and SP70 epitope colored in magenta, yellow, cyan and blue, respectively, showed that the replaced SP70 peptide from CVA16 protruded from the natural VP1 proteins. (B) A cartoon representation of the capsid with VP1, VP2, VP3 and SP70 epitope colored in magenta, yellow, cyan and blue, respectively. The 60 copies of CVA16-SP70 peptides were predicted to be uniformly displayed on the surface of EV-A71 VLP without affecting the original structure. (C) Electron micrograph of negatively stained ChiEV-A71 VLPs. These VLPs are about 30 nm in diameter and are morphologically similar to EV-A71-VLPs. ELISA of purified ChiEV-A71 VLPs was performed using polyclonal antibody against CVA16-SP70 peptide (D) and monoclonal antibody targeting EV-A71-SP70 epitope (E), respectively. Yeast antigen was control. The cut-off for the ELISA is shown by a dotted line. **$P < 0.01$ for ChiEV-A71 vs. EV-A71 group, by one-way ANOVA.

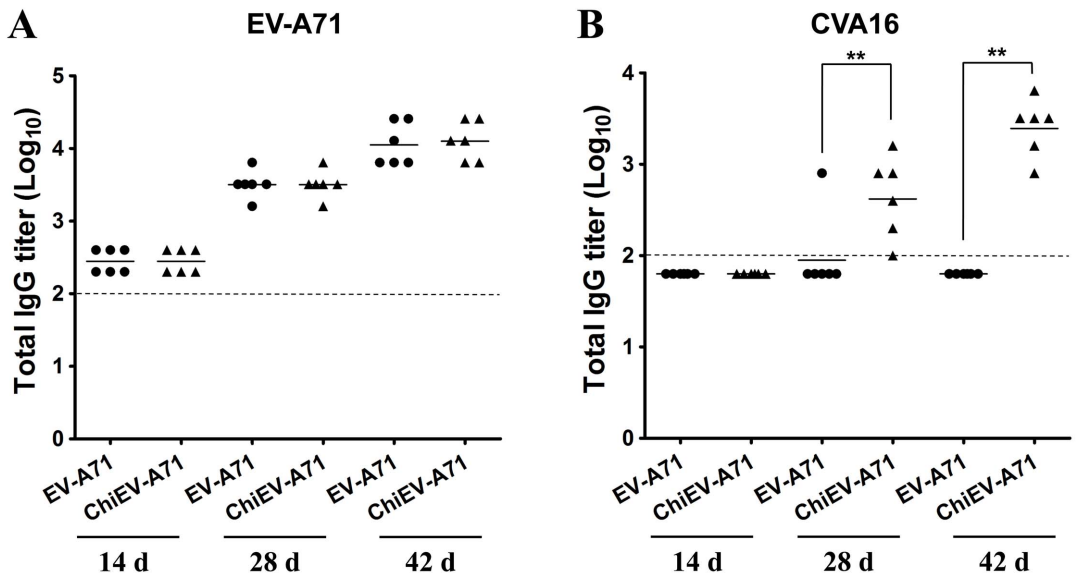

Figure 3 | IgG antibodies responses in mice immunized with ChiEV-A71 VLPs. (A) The total anti-EV-A71 IgG titers (A) and anti-CVA16 IgG titers (B) of immune sera from mice immunized with VLPs at two weeks after each immunization were measured by IFA. Dotted lines represent limits of detection. **$P < 0.01$ for ChiEV-A71 sera group vs. EV-A71 sera group, by *t* test.

Table 1 | IgG subclasses induced by ChiEV-A71 VLPs[a]

Group	OD_{450} of ELISA for EV-A71 specific IgG			
	IgG1	IgG2a	IgG2b	IgG3
EV-A71	0.318 (0.01)*	0.229 (0.03)*	0.191 (0.02)*	0.113 (0.02)*
ChiEV-A71	0.327 (0.05)*	0.211 (0.01)*	0.209 (0.03)*	0.104 (0.01)*
Control	0.042 (0.02)	0.026 (0.02)	0.019 (0.02)	0.039 (0.01)

[a]Profile of IgG subclasses in immune sera from mice immunized with ChiEV-A71 VLPs or EV-A71 VLPs. Serum samples were collected at two weeks after the last immunization and analyzed for presence of IgG1, IgG2a, IgG2b and IgG3 subclass antibodies by ELISA, and sera from non-immunized mice was control. ChiEV-A71 VLPs predominantly induced IgG1, following by IgG2a and IgG2b, while low levels of IgG3. *$P < 0.05$ for ChiEV-A71 or EV-A71 sera group vs. control sera group, by *t* test.

significantly prolonged the percentage of survival compared with the PBS group ($P = 0.108$). These results demonstrated that the neonatal mice receiving the ChiEV-A71 antisera were protected from both the EV-A71 and CVA16 infection.

Discussion

Vaccine development against HFMD has become an active research area. The causative agents responsible for HFMD include EV-A71, CVA16 and some other enteroviruses. Thus a desirable vaccine need confer protective effects against different causative agents. In the present study, a novel recombinant chimeric enterovirus VLP (ChiEV-A71) was developed by replacing the neutralizing epitope SP70 of EV-A71 with that of CVA16, which was rationally designed to develop a bivalent HFMD vaccine.

We previously demonstrated that VLPs for EV-A71 or CVA16 produced from yeast exhibited similar morphology and protein composition as empty particles from EV-A71 or CVA16-infected cells. Further, we showed that these VLPs from yeast can elicit potent immune responses and confer protection against respective viral infections in neonatal mice[18,23]. In this study, we showed that VLPs were assembled by co-expression of EV-A71-3CD and ChiEV-A71-P1 which has the SP70 peptide of EV-A71 replaced from that of CVA16. Structural modeling of ChiEV-A71 VLP demonstrated that CVA16-SP70 located on the surface of the particle (Figure 2B), which was further confirmed by the ELISA data (Figure 2D). Recent report based on the cryo-electron microscopy reconstruction have demonstrated that PEP71 epitope of CVA16 was in the GH loops of VP1, exposed on the surface of CVA16 VLPs[40], which further supported our results. Our immunization experiments demonstrated that these VLPs can elicit potent immune responses and confer protection in neonatal mice against both EV-A71 and CVA16 infections. Our study also revealed that the corresponding SP70 peptide in CVA16 also constitute a major neutralization epitope in CVA16. Interesting, immunity protection response of ChiEV-A71 VLPs against EV-A71 was not attenuated by deletion of EV-A71-SP70 (Figure 4A and 6A), which suggested that EV-A71-SP70 is not a critical epitope in the process of interaction between EV-A71 infection and host immune system.

In general, neutralizing ability of antisera is important for protection against virus infection. In the present study, ChiEV-A71 VLPs immunization induced robust neutralizing antibody responses against EV-A71 similar to that of EV-A71 VLPs, and provided full protection to neonatal mice against lethal EV-A71 challenge. However, the geometric mean of neutralizing antibody titers induced by ChiEV-A71 was only 1 : 16 against CVA16. Recent reports showed that a CVA16 experimental inactivated vaccine was reported to elicit neutralizing antibody titers of 1 : 256[41], and CVA16 VLPs vaccine was about 1 : 128[18]. It is apparent that the efficacy of single epitope for protecting the infection of viruses is limited. Hence, more effective antigenic epitopes of CVA16 should be further screened and might be considered to display in the surface of recombinant VLPs and enhance the immunogenicity of VLPs in the future. In addition, an optimal system is underway to improve the immunogenic and protective potential of the SP70 epitope of CAV16, including dose, adjuvant and prime-boost strategy. Nevertheless, our data clearly demonstrated that ChiEV-A71 VLP-immunized sera elicited neutralizing antibodies and conferred full protection to neonatal mice against lethal CVA16 challenge (Figure 6B).

In addition to the humoral responses, immunization with the ChiEV-A71 VLPs also induced cellular immune responses as evidenced by the production of cytokines (Figure 5). The data demonstrated that the production of IFN-γ, IL-2, IL-4 and IL-6 were

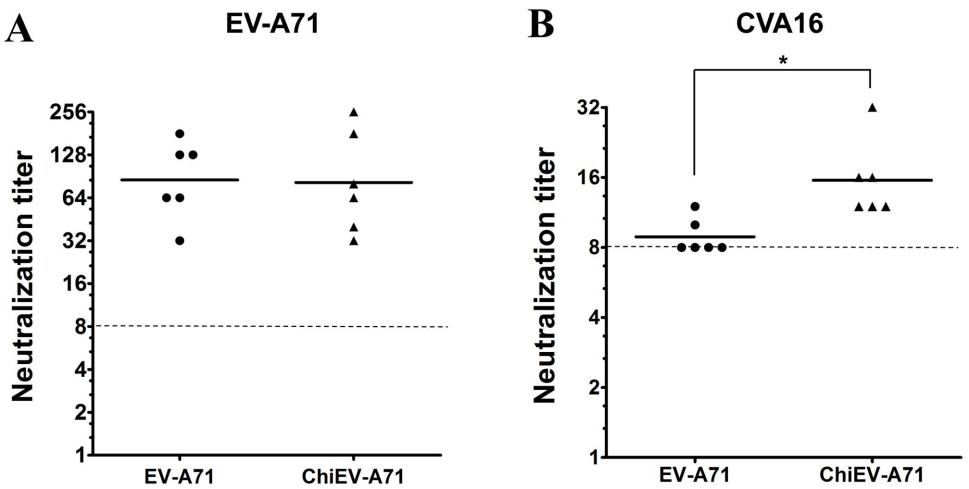

Figure 4 | Neutralizing antibodies responses in mice immunized with ChiEV-A71 VLPs. Anti-EV-A71 neutralization titer (A) and anti-CVA16 neutralization titers (B) of VLPs immune sera at two weeks after the last immunization were measured by microneutralization assay. Dotted lines represent limits of detection. *$P < 0.05$ for ChiEV-A71 sera vs. EV-A71 sera group, by *t* test.

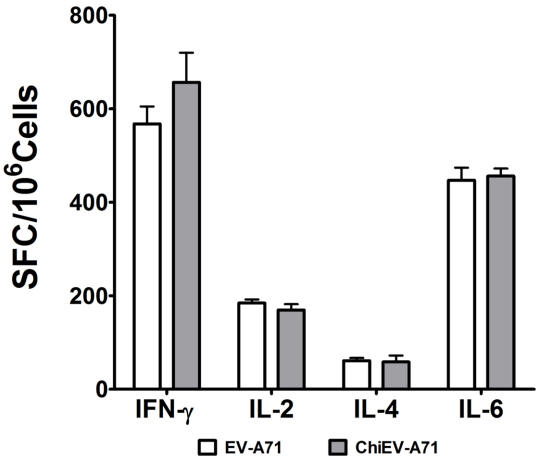

Figure 5 | Cytokine profiles of mice immunized with ChiEV-A71 VLPs. Cytokines (IFN-γ, IL-2, IL-4 and IL-6) generated by splenocytes collected from mice immunized with VLPs were measured by ELISPOT, following re-stimulation with inactivated EV-A71 (inEV-A71). The number of spot-forming cells (SFC) detected after 48 h of stimulation are shown.

enhanced in splenocytes from ChiEV-A71 VLP-immunized mice, similar as that from EV-A71 VLP-immunized mice, indicating that the Th1 and Th2 responses were both induced by immunization with these VLPs. This result was consistent with the observation by Li et al[23]. In addition, we used the CVA16-SP70 peptide as stimulus in ELISpot assay, and found that there were no differences between stimulus group and blank group in the production of cytokines (data not shown), which suggested the CVA16-SP70 peptide might be a B-cell epitope. The previous study showed the EV71-SP70 was also a B-cell epitope[32]. In the present work, evaluation of IgG subclasses demonstrated that VLPs predominantly induced IgG1, IgG2a and IgG2b (Table 1), which further suggested a mixed Th1/Th2 dependent antibody responses.

In this study, we found that EV-A71 VLP immunization induced weak neutralizing antibodies (Figure 4B) and protection against CVA16 (Figure 6B). Such weak cross-neutralization between EV-A71 and CVA16 has also been observed by others[41–43]. Several work demonstrated that EV-A71 and CVA16 shared some common epitopes, and showed cross-reactivity with each other[44], due to intertypic recombination between EV-A71 and CVA16[20,45,46], which is likely correlated with this phenotype.

Serotype-specific neutralizing antibodies are critical for long-term protection against virus infection[47–49]. Sequence comparison showed that there are only 4 amino acid variations in the SP70 peptide between EV-A71 and CVA16, and it remains poorly understood whether these different sites determined the serotype specificity of epitope SP70, which warrants further investigation. Moreover, for many viruses, the presence of cross-reactive non-neutralizing antibodies from the first infection has been reported to lead to increased susceptibility to heterologous viral infection, due to antibody-dependent enhancement (ADE)[50–53]. Thus, the epitopes responsible for eliciting serotype-specific neutralizing antibodies against CVA16, and not the cross-reactive antibodies with potential for disease enhancement, should be determined in the future, which is important for the design of a recombinant chimeric vaccine with enhanced safety against HFMD.

In previous studies, CVA16 linear neutralizing epitopes were identified using mice as an infection model[39]. However, in many cases, such identified epitopes of virus failed to react with human antisera[54,55]. Therefore, epitopes responsible for eliciting neutralizing antibodies that can protect human against CVA16 infection should be further identified, which is crucial for the development of an epitope-based chimeric vaccine against HFMD for human.

During the time of our study, others reported the development of HFMD vaccine by combining inactivated EV-A71 and CVA16 virions together, which induced a balanced protective immunity against both EV-A71 and CVA16[56]. Our approach has also led to generation of a bivalent vaccine against both EV-A71 and CVA16, however with much less workload and significantly enhanced biosafety. Importantly, to our knowledge, this is the first chimeric EV-A71 VLP carrying foreign epitopes, and such chimeric VLPs provide an alternative platform for bivalent HFMD vaccine development. Viruses within *Enterovirus* genus share the similar genome organization and virion structure, which suggests that this novel chimera strategy may be applied universally among other enteroviruses and can be readily adapted in either recombinant VLP or recombinant virus production.

In conclusion, we demonstrated that immunization with the ChiEV-A71 VLPs induced similar immune responses against EV-A71 in mice compared with the inactivated virus and VP1 subunit, and conferred full protection to neonatal mice against lethal CVA16 challenge. These results suggested that this chimeric vaccine,

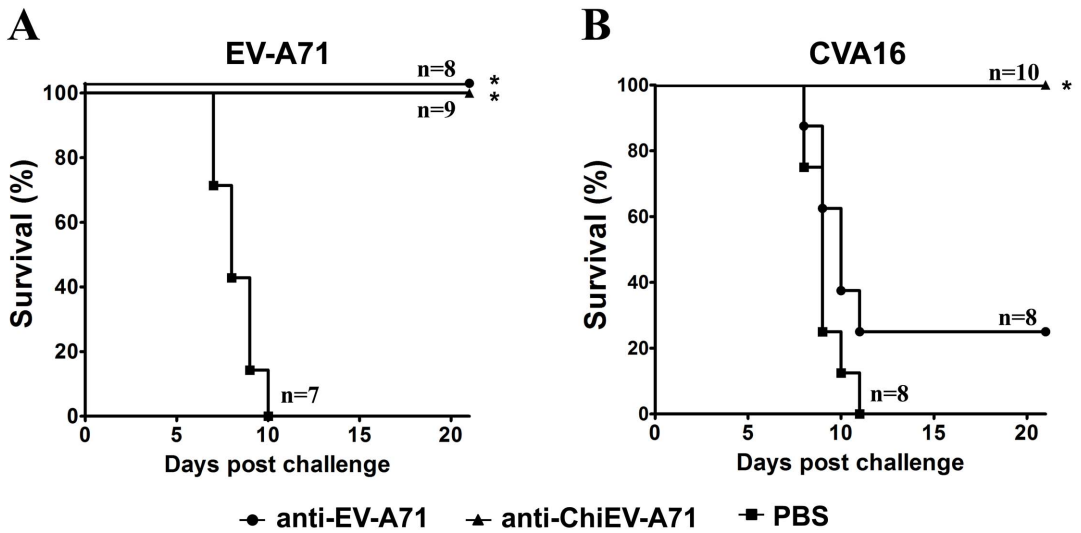

Figure 6 | Passive protection conferred by ChiEV-A71 VLPs immune sera. Groups of one-day-old BALB/c mice were inoculated i.p. with 100 LD_{50} of EV-A71 (A) or 20 LD_{50} of CVA16 (B), followed by administration of PBS, heat-treated anti-EV-A71, or anti-ChiEV-A71 sera, respectively, at 1 h post inoculation. The mortality were monitored and recorded daily for 21 days. *$P < 0.05$ for anti-ChiEV-A71 sera or anti-EV-A71 sera group vs. PBS group.

ChiEV-A71 might have the potential to be further developed as a bivalent HFMD vaccine in the near future. More broadly, this study establishes a template for similar approaches to develop multivalent vaccine against other clinically important viral disease.

Methods

Cell lines and viruses. A Chinese endemic genotype C4 strain EV-A71 AH08/06 (GenBank No.HQ611148) was propagated in RD cells using Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) as previously descried[57]. A Chinese endemic CVA16 strain CVA16/GD09/119 (GenBank No.KC117318.1), isolated from a severe HFMD patient, was propagated in Vero cells, using DMEM containing 10% FBS. The virus titers were determined based on typical cytopathic effect developing in the infected RD or Vero cells, and are expressed in 50% tissue culture infectious dose ($TCID_{50}$).

Preparation of mouse polyclonal antibody against synthetic peptide CVA16-SP70. Keyhole limpet hemocyanin-conjugated synthetic peptide representing the amino acid sequence of CVA16-SP70 was synthesized by Shanghai Bootech Bioscience & Technology Co., Ltd (Shanghai, China). Groups of 6-week-old female BALB/c mice were subcutaneouly (s.c.) immunized with a 50% emulsion of aluminum hydroxide (Imject Alum from Thermo Scientific) containing 50 μg of conjugated synthetic peptide CVA16-SP70. Two boosters with the same dose were given at two weekly intervals. Two weeks after immunization, serum samples were collected and stored frozen until use.

Plasmid construction for expression of ChiEV-A71-P1 and EV-A71-3CD genes. The viral RNA of both EV-A71 and CVA16 were isolated and subjected to RT-PCR experiments to obtain the cDNA sequences of both viral genomes. The derived coding sequences of EV-A71-P1 were then optimized to the codon usage of *Saccharomyces cerevisiae* using the JCat bioinformatic tool (http://www.jcat.de/). In order to replace the SP70 epitope (YPTFGEHKQEKDLEY) in VP1 of EV-A71-P1 with the corresponding region from CVA16-P1 (YPTFGEHLQANDLDY), two primers denoted as SP70-F (5′-ttgcaagctaacgacttggactacggagcttgcccaaacaac-3′) and SP70-R (5′-gtccaagtcgttagcttgcaagtgctcgccgaaggtaggg-3′) were designed and PCR mutagenesis experiments were then performed with the optimized cDNA sequences of EV-A71-P1 as the template. The derived modified EV-A71-P1 sequences, denoted as ChiEV-A71-P1, were cloned into pYES2 expression vector (Invitrogen), which is labeled with the auxotrophic complementary gene for uracil, to obtain pYES2-ChiEV-A71-P1 (Figure 1A). In order to co-express EV-A71-3CD, the vector pYES2-Trp-3CD was constructed as previously described[23]. Expression of ChiEV-A71-P1 and EV-A71-3CD were under the control of the GAL1 promotor which can be activated by galactose.

Generation of yeast expression strains. The INVSc1 strain of yeast (Invitrogen) was transformed with both pYES2-ChiEV-A71-P1 and pYES2-Trp-3CD. The transformed yeast cells were then plated onto a SC minimal plate lack of tryptophan and uracil. All yeast transformation procedures were performed according to the pYES2 user manual (Invitrogen). A single colony of yeast transformant was then picked and inoculated into the SC minimal liquid medium lack of tryptophan and uracil. The protein expression was induced in YPG liquid medium when OD_{600} reached 1, whereas the yeast cells were collected when OD_{600} reached 5. All yeast culture experiments were performed at 30°C.

Fractionation and Purification of ChiEV-A71 VLPs. The yeast cells were pelleted and resuspended in PBS buffer containing 1 mM PMSF and 2 mM β-mercaptoethanol. NP-40 (Genebase) was then added into the cell lysate to a final concentration of 1% (v/v) followed by stirring at 4°C for 30 min and high-pressure cell breaking at 4°C under a pressure of 1600 Bar for 4 times in a JN3000 Plus cell breaker (JNBIO). Cell debris was then pelleted by centrifugation at 12000 g for 10 min at 4°C. The resulting supernatant was added with NaCl to a final concentration of 200 mM and polyethylene glycol 8000 (Genebase) to a final concentration of 10% (w/v) and mixed well at 4°C overnight. The precipitate was then collected by centrifugation at 12000 g for 1 hour at 4°C and resuspended in PBS buffer. Insoluble impurity was removed by centrifugation at 8000 g for 30 min at 4°C. The cleared supernatant was then loaded onto a 15–45% (w/v) non-continuous sucrose gradient in the same buffer, and spun in a SW 28 rotor (Beckman) for 3 hour at 27000 rpm without brake. The resulting fractions were collected from top to bottom, and then subject to western blot analysis. Fractions containing VLPs were collected, diluted in PBS and pelleted by ultracentrifugation at 26000 rpm. Concentrated VLPs were resuspended in PBS and the concentration was measured using the DC Protein Assay Kit (Bio-Rad).

SDS-PAGE and western blot analysis. Protein samples collected from sucrose gradient fractions were first treated with 1× SDS gel-loading buffer (50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) for 5 min at 100°C and then separated on 15% SDS-PAGE gels. For western blot analysis, protein samples already separated on SDS-PAGE were transferred onto BioTrace NT membranes (PALL). The membranes were then blocked with 5% skimmed milk dissolved with TBST buffer (pH7.5). To detect the presence of VP0, mouse monoclonal antibody for VP0 (Chemicon MAB979) were used. The secondary antibody is anti-mouse IgG (H + L) AP conjugate antibody (Promega S372B). All the membranes were washed with TBST buffer (pH7.5) and visualized by Western Blue stabilized substrate for alkaline phosphatase (Promega).

Electron microscopy of negatively stained VLPs. Concentrated VLPs (0.5 mg/ml) in suspension were adsorbed to carbon-Formvar-coated copper grids and negatively stained with 0.5% uranyl acetate aqueous solution for 30 secs. The grids were then examined with a Tecnai F20 electron microscope (FEI) at 200 kV.

Enzyme-linked immunosorbent assay (ELISA). The 96-well microtiter plates were coated with bicarbonate coating buffer (pH 9.6) containing 1 μg/ml of ChiEV-A71 VLPs, EV-A71 VLPs or similarly prepared yeast antigen (Control group) overnight at 4°C followed by 10% skim milk blocking. The coated plate was then incubated with anti-EV-A71-SP70 monoclonal antibody (mAb, prepared in our lab) or anti-CVA16-SP70 antibody at 1 : 100 dilution at 37°C for 1 h, and followed by incubating HRP-conjugated secondary antibody at 37°C for 30 min. A total of 100 μl of tetramethyl benzidine dihydrochloride (TMB) was added for incubation for 15 min at room temperature. Finally, the absorbance at 450 nm was recorded using an ELISA plate reader.

Mice immunization. All animal experimental procedures were carried out in strict accordance with and approved by the Animal Experiment Committee of Beijing Institute of Microbiology and Epidemiology. Groups of 6-week-old female BALB/c mice (n = 6) were inoculated with 10 μg of ChiEV-A71 VLPs (ChiEV-A71 group) or 10 μg of EV-A71 VLPs (EV-A71 group) by intraperitoneal route with aluminum hydroxide at a volumetric ratio of 1 : 1, respectively. All mice were then boosted twice with the same dose in aluminum hydroxide adjuvant at an interval of two weeks. Two weeks after each immunization, serum samples were prepared and stored frozen until use. Mice were euthanasiaed and bleeding at week 4 after the second boost injection.

Immunofluorescence assay (IFA). The titer of IgG antibody in the sera from immunized mice was detected by IFA as previously described[18,23].

Antibody subclasses analysis. The profiles of specific IgG subclasses in the mice antisera were determined by ELISA. In brief, ELISA plates were coated with heat-inactivated EV-A71 (10^6 $TCID_{50}$ in DMEM containing 10% FBS) in bicarbonate coating buffer overnight at 4°C. After blocking, sera were added at 1 : 50 dilution in PBS and incubated for 1 h. Primary antibody was reacted with isotype specific antibodies (goat anti-mouse IgG1, IgG2a, IgG2b and IgG3, purchased from Invitrogen) at 1 : 500 dilution for 1 h at 37°C. After washing, the HRP-conjugated secondary antibodies were added. Finally, the plate was incubated in dark with 100 μl/well of TMB at 37°C for 20 min. The reaction was stopped by addition of Stop Solution and the absorbance was read at 450 nm in an ELISA reader.

Neutralization antibody assay. The titer of neutralizing antibodies against EV-A71 or CVA16 was determined in RD or Vero cells according to a standard protocol[18,23].

Cytokine analysis. The production of cytokine from stimulated T cells was detected by enzyme-linked immunospot (ELISPOT) analysis using BD™ ELISPOT Set as previously described[18,23]. In brief, the 96-well plates were coated respectively with IFN-γ, IL-2, IL-4, IL-6 capture antibody overnight at 4°C. Spleen cells (10^6/well for IFN-γ and IL-6 or 2×10^5/well for IL-2 and IL-4) from immunized mice were added to wells and cultured at 37°C for 48 h, with total proteins of heat-inactivated EV-A71 (100 PFU/well) or ConA (250 ng/well, Sigma). Following procedures were made according to the manufacturer's instructions.

***In vivo* protection assay.** The *in vivo* challenge experiments were performed as previously described[18,23]. Briefly, for protection assay against EV-A71, three groups of one-day-old BALB/c neonatal mice were inoculated intraperitoneally (i.p.) with 100 LD_{50} of EV-A71 (40 μl in sterile PBS buffer), followed by i.p. administration of 40 l of PBS or antisera from mice immunized with ChiEV-A71 or EV-A71, respectively. All mice were monitored daily for clinical symptoms and death until 21 days after inoculation. For protection assay against CVA16, 20 LD_{50} of CVA16 was used, and other procedures were made as the same to protection assay for EV-A71.

Statistics. Statistical significance was determined by the Student's two-tailed *t*-test or one-way ANOVA using GraphPad Prism version 5, and $p < 0.05$ was considered as significance. For *in vivo* protection assay, the surviving curve was compared using Log-rank test by GraphPad Prism version 5.

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Author contributions

C.F.Q., R.C. designed the study; H.Z., H.Y.L. and H.Q.Y. performed the experiments; H.Z., H.Y.L., C.F.Q. and R.C. analyzed the data; J.F.H., Y.Q.D., S.Y.Z., X.F.L., Y.X.L., Y.Z. and E.D.Q. contributed reagents/materials/analysis tools; H.Z., C.F.Q. and R.C. wrote the paper.

Additional information

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