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Research paper

A bispecific broadly neutralizing antibody against enterovirus 71 and coxsackievirus A16 with therapeutic potential

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ABSTRACT

Enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are the major pathogens of hand, foot and mouth disease (HFMD), which affects children worldwide and is often associated with neurological complications. At present, there is no vaccine or cure available for simultaneous EV71 and CA16 infection, posing a great need to develop novel strategies for the treatment of this disease. Here, we engineered four bispecific antibodies using variable fragments of monoclonal antibodies (mAbs) from EV71- and CA16-specific neutralizing antibodies. The engineered bispecific antibody Bs(scFv)4-IgG-1 exhibits remarkable cross-reactivity against EV71 and CA16 and has a more potent cross-neutralization than its parental antibodies. Furthermore, we showed that Bs(scFv)4-IgG-1 conferred 100% therapeutic efficacy against single or mixed EV71 and CA16 infections in mice. Our study provides important insights into bispecific antibody engineering against enterovirus and will inform new curative treatment options for HFMD.

1. Introduction

Hand, foot and mouth disease (HFMD) is an infectious illness that mainly affects infants and young children (Aswathyraj et al., 2016). The epidemic of HFMD is a serious public health problem, causing an enormous social and economic burden. Enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are the principal pathogenic agents of HFMD, which can cause severe central nervous system complications and death (Aswathyraj et al., 2016; Xu et al., 2012). In addition, EV71 and CA16 can cocirculate as the result of co-infection (Cabrerizo et al., 2014; Kapusinszky et al., 2010; Liu et al., 2014). Furthermore, the cocirculation of EV71 and CA16 can amplify the serious clinical symptoms of HFMD and lead to the emergence of genomic recombinant strains (Liu et al., 2014; Zhang et al., 2011; Zhang et al., 2010). There has been recent progress in the formulations of vaccines and antibody therapies for EV71 infection (Li et al., 2014a, 2014b; Wu et al., 2017; Xu et al., 2015; Yang et al., 2016). However, there are still no bivalent vaccines or effective bispecific antibodies (BsAbs) available against EV71 and CA16.

Currently, 44 monoclonal antibody (mAb)-based products are on the market, generating approximately $75 billion USD in total worldwide sales in 2013 (Ecker et al., 2015; Fan et al., 2015). By the end of 2017, the U.S. FDA had approved 70 new antibody drugs and 8 antibody analogues. That year, six of the ten top selling drugs in the world were antibody drugs. As targeted therapies, antibodies have been widely used against human diseases, including cancer, autoimmunity

Abbreviations: BsAb, bispecific antibody; cAb, chimeric antibody; CA16, coxsackievirus A16; CPE, cytopathic effect; EV71, enterovirus 71; FBS, fetal bovine serum; HFMD, hand, foot and mouth disease; HT, hypoxanthine and thymidine; mAb, monoclonal antibody; MEM, minimal essential medium; PEI, polyethylenimine; RD, human rhabdomyosarcoma

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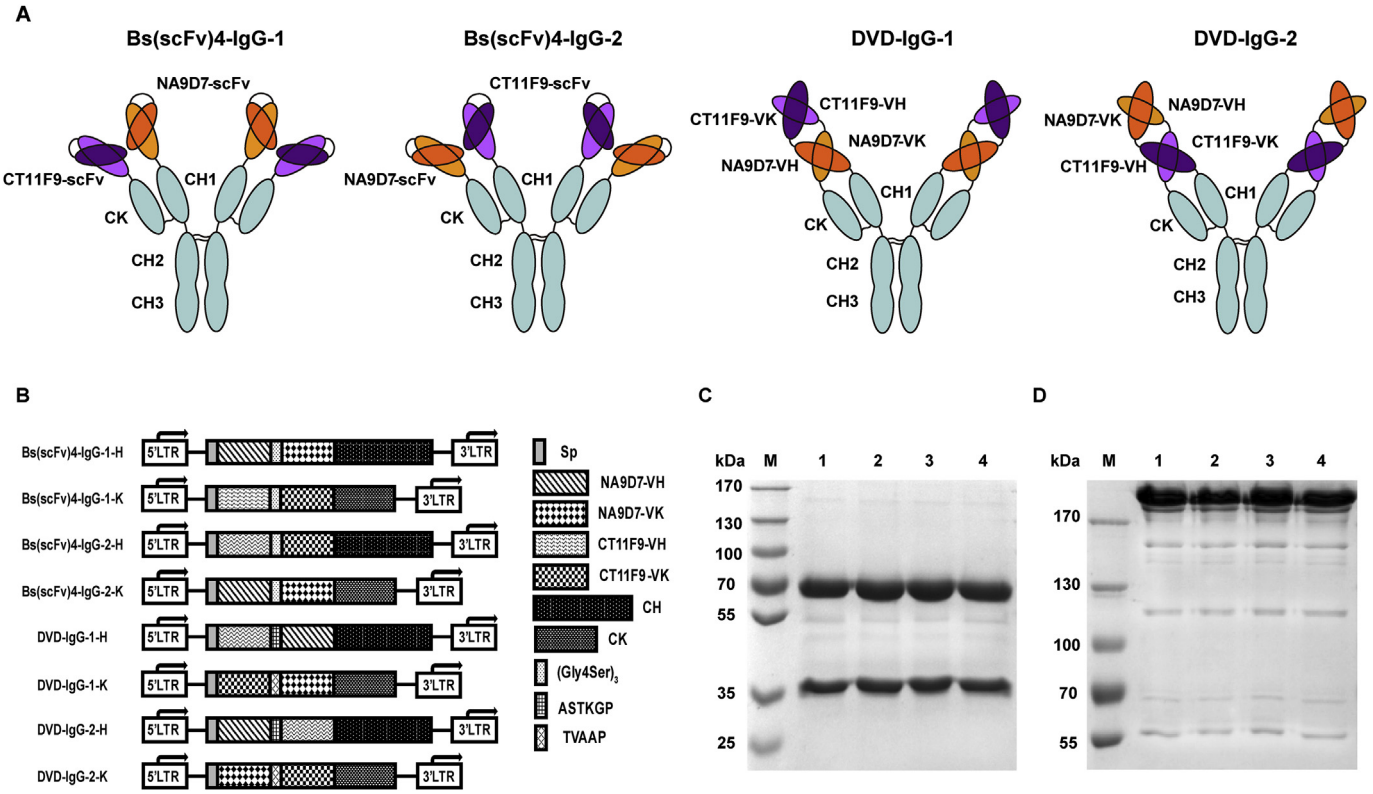

Fig. 1. Schematic representation and characterization of bispecific antibodies. (A) Schematic representation of the structures of Bs(scFv)4-IgG and DVD-IgG molecules. The VH domains of CT11F9mAb and NA9D7mAb are colored in light purple and light orange. The VK domains of CT11F9mAb and NA9D7mAb are colored in dark purple and dark orange. (B) The constructions of Bs(scFv)4-IgG and DVD-IgG vectors. The N-terminal signal sequence (selected by our laboratory), the C-terminal CK or CH domains of a human IgG1 molecule, positions of the linker sequences and the arrangements of the variable domains are shown. Note: the diagram is not to scale. (C and D) SDS-PAGE analysis of the protein A purified antibodies under reducing conditions (C) and non-reducing conditions (D) Lane M, protein markers; lane 1, Bs(scFv)4-IgG-1; lane 2, Bs(scFv)4-IgG-2; lane 3, DVD-IgG-1; lane 4, DVD-IgG-2.

and infectious diseases (Ramsland et al., 2015; Tan et al., 2013). However, the pathogenesis of most diseases may involve multiple antigen epitopes or virus subtypes or pathogens acting in several independent steps (Shi et al., 2016). The utility of combining two or three mAbs (Uno et al., 2006; van den Berg et al., 1994) has been demonstrated in preclinical models. However, the option of using separate mAbs in combination therapy is limited, owing to regulatory hurdles and costs. In addition, the issues of efficacy and safety of mAb combinations have not been adequately addressed (Wu et al., 2007). Genetic engineering can convert any two mAbs into one IgG-like bispecific molecule that binds different antigens or epitopes simultaneously. Bispecific antibodies (BsAbs) have recently been described as effective potential treatments against DENV, HBV and ZIKA infections (Shi et al., 2016; Tan et al., 2013; Wang et al., 2017). BsAbs can bind two different epitopes simultaneously (Shatz et al., 2013; Spiess et al., 2015), building a bridge between target cells and effector molecules or cells, and then triggering an oriented immune response. This type of genetically engineered antibody has become a hot topic in the field of recombinant antibody technology. BsAbs have many potential advantages (Dao et al., 2015; Rossi et al., 2014; Sun et al., 2015), such as: (1) lower risk for immune complexes than multivalent bispecific antibodies due to monovalency for each target; (2) better effector cell recruitment to enhance killing; (3) greater synergistic potential than combination therapy; and (4) advantageous pharmaco-economics and lower cost than two single antibodies. BsAbs have been used to target viruses, viral-infected cells and bacterial pathogens. They have also been used to deliver thrombolytic agents to blood clots (Carter, 2001; Schanzer et al., 2011). In recent years, a large variety of recombinant methods and formats have been developed for the production of bispecific or bivalent and multivalent antibody fragments. These include BiTE (bispecific T-cell engager), DVD-IgG (dual variable domains IgG), scFv-IgG (scFv is fused to the termini of kappa light or heavy chain), CrossMab (CH1 domain of the heavy chain is swapped with the constant CL domain) and Bs(scFv)4-IgG (two different scFvs are fused to the N-terminal of CH or CL, respectively) (Fan et al., 2015; Metz et al., 2011; Scheuer et al., 2016; Spiess et al., 2013; Zuo et al., 2000).

In our previous study, we developed two conformational mAbs with high neutralizing activities. One, CT11F9mAb, targets EV71 (Li et al., 2014b). The other, NA9D7mAb, targets CA16 (Ye et al., 2016). Here, based on these two antibodies, we constructed two types of novel tetravalent bispecific IgG antibodies. Our strategy eliminates mispairing between the antibody heavy and light chains by applying the DVD-IgG and Bs(scFv)4-IgG building methods. The two approaches yielded a homogeneous bispecific IgG-like antibody product, with each molecule containing four antigen binding sites, two for each of its target antigens. We compared the biological activity of these two types of antibodies. The recombinant antibody constructed using Bs(scFv)4-IgG building methods has remarkable cross-reactivity with purified whole-virus particles and a potent cross-neutralizing capacity against EV71 and CA16. This antibody effectively protects neonatal mice from lethal EV71 and CA16 challenge.

2. Materials and methods

2.1. Cells and viruses

Human rhabdomyosarcoma (RD) cells were obtained from the American Type Culture Collection (ATCC) and maintained in minimal essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Chinese hamster ovary (CHO-S) cells were

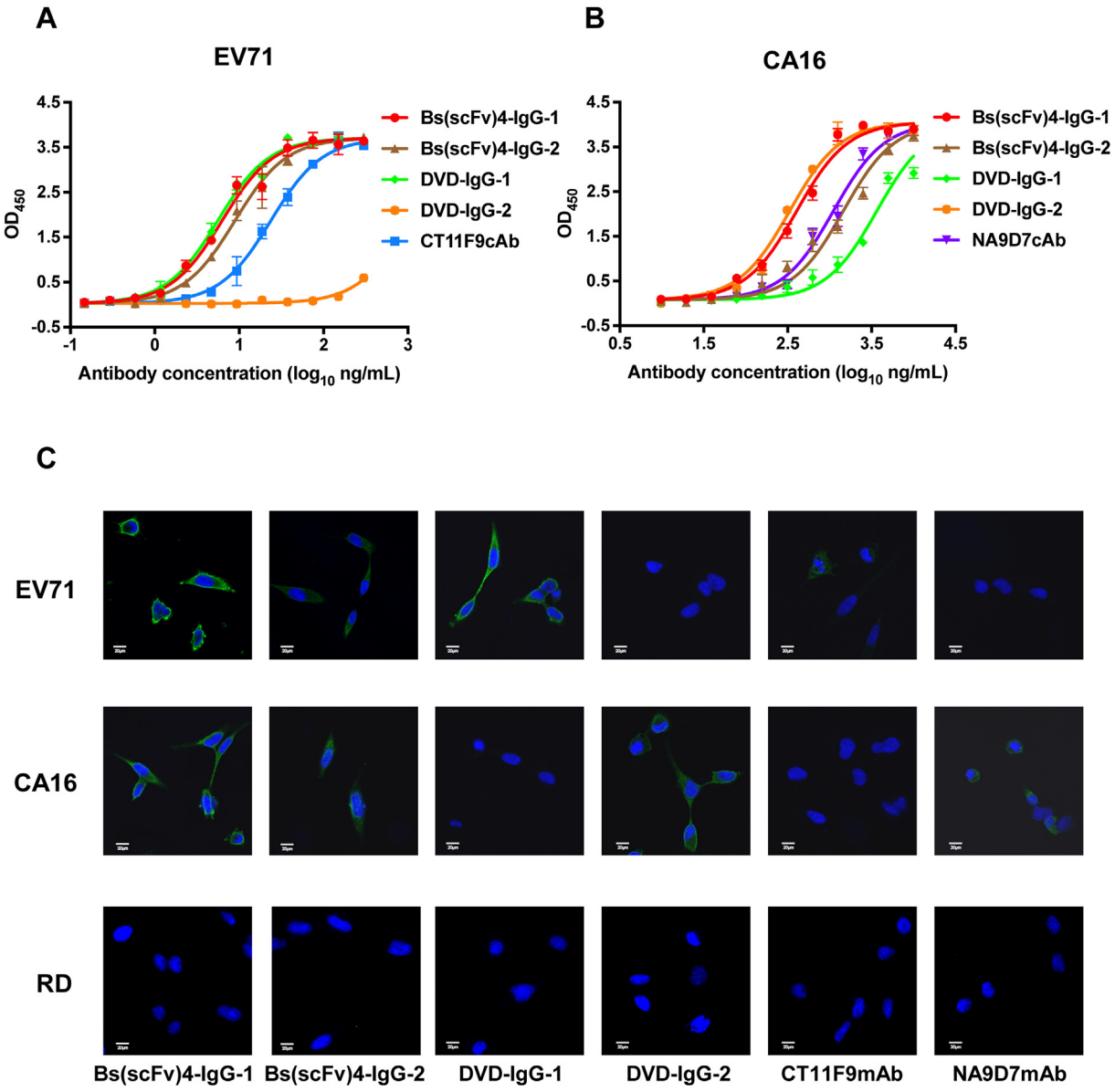

Fig. 2. Enterovirus binding activity of the bispecific antibodies. (A and B) ELISA analysis of antibodies Bs(scFv)4-IgG-1, Bs(scFv)4-IgG-2, DVD-IgG-1, DVD-IgG-2, CT11F9cAb and NA9D7cAb binding to EV71 or CA16. Data shown as mean ± SD. (C) Immunofluorescence assay of the bispecific antibodies Bs(scFv)4-IgG-1, Bs (scFv)4-IgG-2, DVD-IgG-1 or DVD-IgG-2 against EV71- or CA16-infected RD cells. The uninfected RD cells were used as negative controls. The secondary antibody was FITC-conjugated (green). The nuclei were stained with DAPI (blue).

Table 1
The Neutralization efficacy of antibodies.

Antibodies	Neutralization (IC_{50}, μg/mL)	
	EV71/52-3	CA16/00190
Bs(scFv)4-IgG-1	1.01	0.59
Bs(scFv)4-IgG-2	1.36	1.44
DVD-IgG-1	0.87	–
DVD-IgG-2	–	0.43
CT11F9cAb	1.33	–
NA9D7cAb	–	1.64
CT11F9mAb	1.52	–
NA9D7mAb	–	2.57

– refers to the antibody cannot neutralize the virus, at a concentration of 125 μg/mL.

obtained from the Gibco and maintained in SFM 4 medium (HyClone), supplemented with 4 mM L-glutamine, 0.1 μM HT (hypoxanthine and thymidine, Sigma) and 1 g/L Pluronic F68 (Sigma) and without FBS. EV71/pSVA-MP4/B3 was a mouse-adapted strain that was generated by four passages in newborn mice using an infectious clone of the EV71 strain SK-EV006 (GenBank No. AB469182) (Li et al., 2014b). EV71/05535/B5 (GenBank No. JN964686) was isolated from a HFMD clinical specimen in Fujian Province. EV71/52-3/C4 (GenBank No. FJ600325) was isolated from a HFMD clinical specimen in Jiangsu Province. EV71/03149/C2 (GenBank No. JF420552) and EV71/02969/C5 (GenBank No. JF420554) were isolated from HFMD clinical specimens in Taiwan Province. CA16/00190/B1b (GenBank No. JF420555) was isolated from HFMD clinical specimens in Taiwan Province. CA16/213a/B1b (GenBank No. JX127259) and CA16/4430/B1b (GenBank No. JX127274) were isolated from HFMD clinical specimens in Fujian Province.

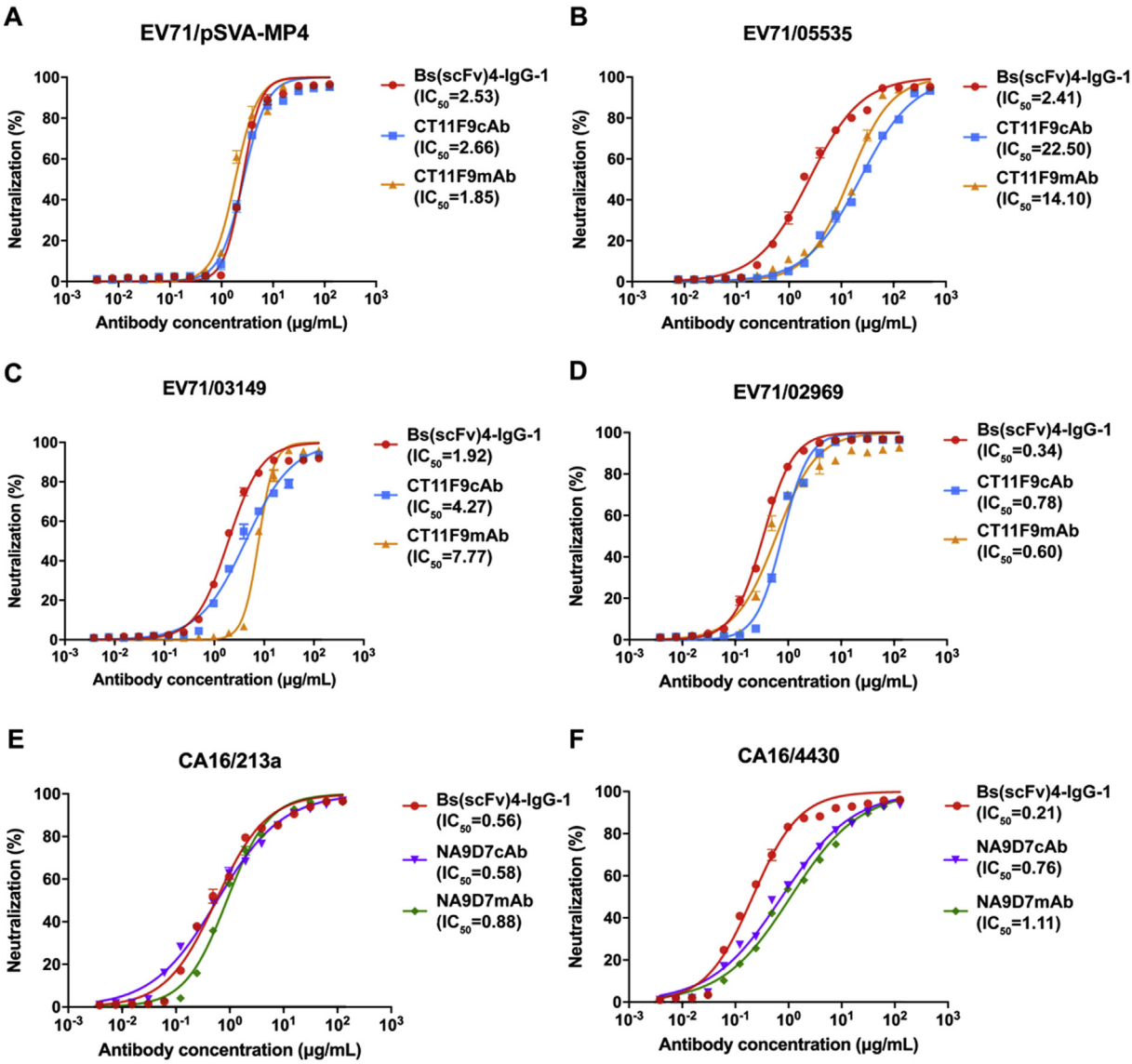

Fig. 3. The Cross-neutralization efficacy of Bs(scFv)4-IgG-1. The neutralizing efficacy of Bs(scFv)4-IgG-1 against EV71/pSVA-MP4 (A), EV71/05535 (B), EV71/03149 (C), EV71/02969 (D), CA16/213a (E) and CA16/4430 (F) were evaluated by an *in vitro* micro-neutralization assay using RD cells. The neutralization efficacy are plotted as a function of the antibody concentration and the IC_{50} value was noted.

2.2. Ethics statement

The animal use protocol was approved by the Xiamen University Laboratory Animal Center (XMULAC). All animal experiments were carried out in strict compliance with the guidelines of the Xiamen University Institutional Committee for Care and Use of Laboratory Animals. The Animal Ethics Committee approval code is XMULAC20160049.

2.3. Construction of expression vectors for Bs(scFv)4-IgG and DVD-IgG

The variable regions of NA9D7mAb and CT11F9mAb were replaced by scFv genes that were constructed by combining the antibody variable domains with a $(Gly4Ser)_3$ linker. The modified variable regions were subcloned into the human IgG1 expression vector pTT5 (NRC) with heavy chain and kappa light chain constant regions to create the Bs(scFv)4-IgG heavy chain (Bs(scFv)4-IgG-H) and Bs(scFv)4-IgG kappa light chain (Bs(scFv)4-IgG-K). The variable domains of two mAbs were fused in tandem using a short linker (the heavy chain linker was ASTKGP, the kappa light chain linker was TVAAP) and were ligated into the expression vector mentioned above to create the DVD-IgG heavy chain (DVD-IgG-H) and DVD-IgG kappa light chain (DVD-IgG-K). Heavy chain and kappa light chain variable regions of NA9D7mAb and CT11F9mAb were subcloned into the same vectors to construct cAb (chimeric antibody) heavy chain (cAb-H) and kappa light chain (cAb-K). All constructs were verified by dideoxy nucleotide sequencing.

2.4. Mammalian cell antibody expression and purification

The recombinant vectors were cotransfected into CHO cells at a 1:1 ratio for transient expression by using polyethylenimine (PEI, Polyscience) and cultured for 7 days. The molecular weight of PEI is 25 kDa and the mass ratio of the antibody to PEI was 1:2. Bs(scFv)4-IgGs, DVD-IgGs and cAbs were purified from the culture supernatants by protein A affinity chromatography. The size, homogeneity and composition of all antibodies were identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

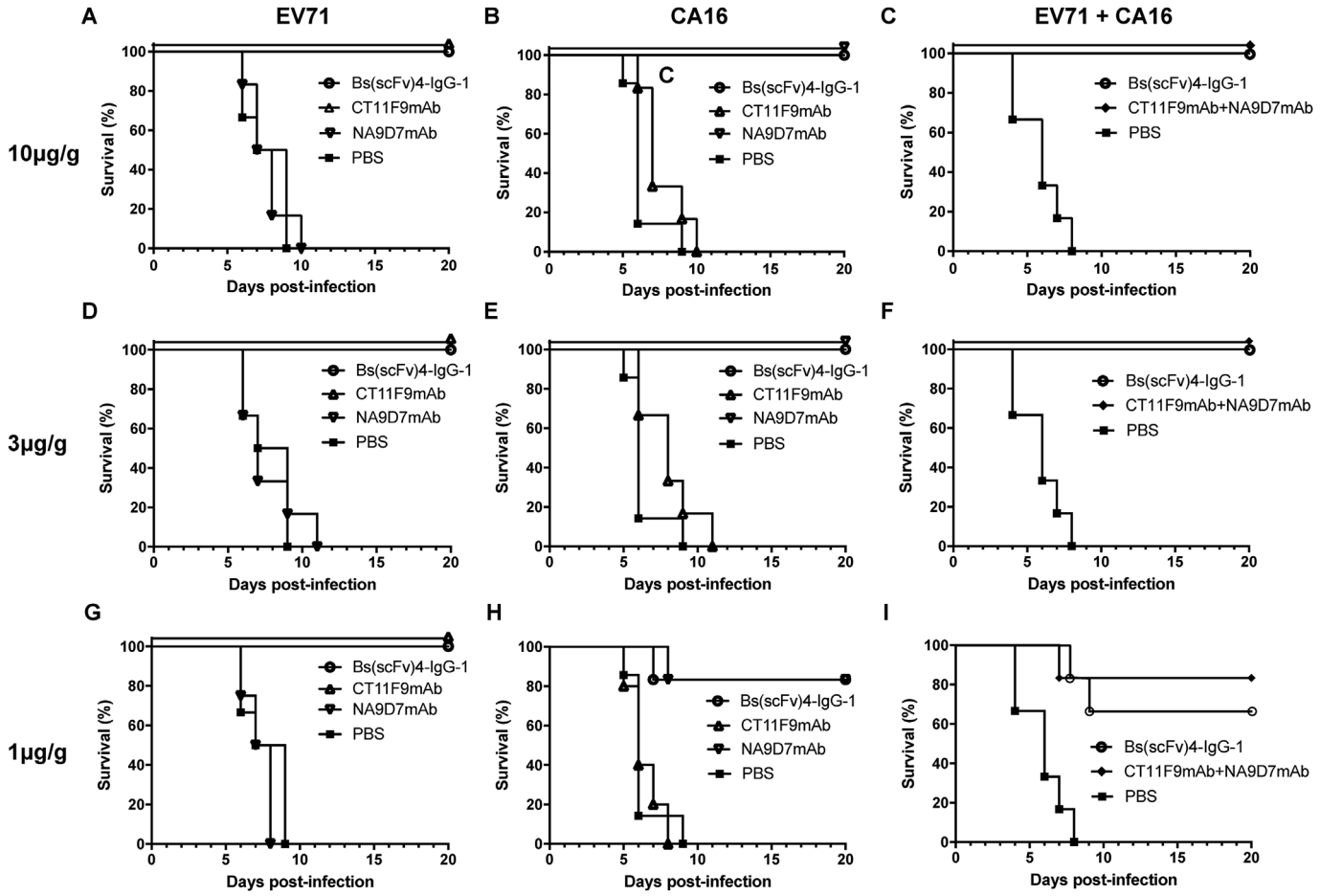

Fig. 4. ***In vivo*** **protective efficacy of the Bs(scFv)4-IgG-1 at different doses against EV71 and CA16**. One-day-old BALB/c mice were challenged with EV71 (A, D and G), CA16 (B, E and H) or EV71 + CA16 (C, F and I), and then treated with different doses of antibodies ranging from 10 μg/g, 3 μg/g to 1 μg/g at 24 h after infection. The control mice were treated with PBS. The survival rates of mice were monitored and recorded daily for 20 days.

2.5. Determining the binding activities of bispecific antibodies

ELISA: 96-Well ELISA plates were precoated with EV71 or CA16 at 50 ng per well and blocked with PBS containing 2% bovine serum albumin (BSA) and 10% sucrose for 2h at 37 °C. Different concentrations of antibodies were added into the wells. After 1 h of incubation at 37 °C, GAH-HRP (goat anti-human IgG labeled with horseradish peroxidase, Wantai) (1:5000 dilution) was added and incubated for 30 min at 37 °C. Finally, o-phenyl-diamine-2HCl (10 μg/mL in 5 mM Tris-HCL, pH 7.0) was added as a substrate for 15 min. The reaction was stopped by adding 50 μL of 2 M H_2SO_4, and the absorbance was measured at 450 nm. The ELISA plates were washed with PBST (0.05% Tween-20 in PBS) between every step.

Immunofluorescence Assay (IFA): RD cells were plated on 24-well plates containing glass slides and were infected with EV71 or CA16. After 12 h, the cells were fixed with 4% paraformaldehyde, followed by 0.3% Triton X-100 for 10 min and were then blocked with goat serum for 15 min at 37 °C. The cells were incubated with antibodies (2 μg/mL) for 1 h, followed by incubation with GAH-FITC or GAM-FITC (goat anti-human IgG or goat anti-human IgG labeled with fluorescein isothiocyanate, Sigma-Aldrich) (1:500 dilution) for 30 min in the dark. Finally, after washing, nucleus staining was performed by incubation with DAPI (1:2000 dilution) for 5 min. Image data were captured using a confocal microscope (MRC-1024, Bio-Rad, Hercules, CA).

2.6. In vitro neutralization assay

An *in vitro* neutralization assay was performed as previously described (Hou et al., 2015), with the following modifications. Briefly, RD cells were resuspended in MEM supplemented with 2% FBS and seeded into 96-well plates (NUNC) at 2×10^4 cells per well. The antibodies (initial concentration 1 mg/mL) were serially diluted two-fold with PBS buffer and incubated in a 1:1 vol ratio with 10^4 $TCID_{50}$ EV71 or CA16 at 37 °C for 1 h. Each antibody-virus mixture was then added to the preseeded RD cells in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h. After incubation, an enzyme-linked immunosorbent spot (Elispot) assay was performed. The inhibition rate of antibody was calculated with the following formula: Neutralization % = $(1-(N_{test}-N_{cell\ control}))/(N_{virus\ control}-N_{cell\ control}) \times 100\%$. In this equation, N_{test}, $N_{cell\ control}$ and $N_{virus\ control}$ are the average number of spots in the test wells, virus control wells and cell control wells, respectively. The neutralization titers were defined as the highest dilution that inhibits > 50% of the spots. The IC_{50} was calculated by nonlinear regression fitting curves using GraphPad Prism version 7.0. The experiments were repeated in triplicate.

2.7. In vivo protection assay

An *in vivo* protection assay was performed as previously described (Li et al., 2014b; Mao et al., 2012; Wu et al., 2017; Xu et al., 2015). Groups of one-day-old mice (n = 8–10) were challenged intraperitoneally (i.p.) with 10^7 $TCID_{50}$ EV71/pSVA-MP4 or intracranially (i.c.) with 10^5 $TCID_{50}$ CA16/190 24 h before i.p. injection with antibodies (each antibody at the concentration of 10 μg/g, 3 μg/g or 1 μg/g). All mice were monitored and recorded daily for body weight, clinical illness and death until 20 days post-infection (dpi).

3. Results

3.1. Construction, expression and purification of Bs(scFv)4-IgG and DVD-IgG

Enterovirus antibodies with high neutralizing activity, CT11F9mAb against EV71 and NA9D7mAb against CA16, were used for the construction of Bs(scFv)4-IgG and DVD-IgG. We engineered four bispecific antibodies, Bs(scFv)4-IgG-1, Bs(scFv)4-IgG-2, DVD-IgG-1 and DVD-IgG-2 (Fig. 1A and B). In Bs(scFv)4-IgG1, the scFv genes of CT11F9 mAb were fused to the N-terminal of CK, and the scFv genes of NA9D7 mAb were fused to the N-terminal of CH. The construction of Bs(scFv)4-IgG2 was in contrast to the Bs(scFv)4-IgG1. DVD-IgG-1 is in V_{CT11F9}-V_{NA9D7}-constant orientation, and DVD-IgG-2 is in V_{NA9D7}-V_{CT11F9}-constant orientation. The co-expression of Bs(scFv)4-IgG-H and Bs(scFv)4-IgG-K or DVD-IgG-H and DVD-IgG-K yielded an IgG-like tetravalent molecule, Bs (scFv)4-IgG or DVD-IgG, with dual specificity. The four BsAbs were transiently expressed in CHO suspension cells and were purified by protein A chromatography. The purity and size of the four BsAbs were confirmed by SDS-PAGE. After purification, the purity of the BsAbs was greater than 95%. Under reducing conditions, the Bs(scFv)4-IgGs and DVD-IgGs yielded two obvious bands with molecular masses of ~65 kDa (heavy chain) and ~35 kDa (kappa light chain) (Fig. 1C). Under nonreducing conditions, the Bs(scFv)4-IgGs and DVD-IgGs gave rise to a predominant band with a molecular mass of ~200 kDa (Fig. 1D).

3.2. Dual specificity of Bs(scFv)4-IgG and DVD-IgG

The specificity binding abilities of Bs(scFv)4-IgG and DVD-IgG were determined by ELISA and IFA (Fig. 2). ELISA was used to directly compare the binding activity of the four BsAbs to the virus. As shown in Fig. 2A, the EV71 binding activities of Bs(scFv)4-IgG-1, Bs(scFv)4-IgG-2 and DVD-IgG-1 were stronger than that of CT11F9cAb, while DVD-IgG-2 showed a weaker EV71 binding activity. In contrast, targeting CA16, the binding activities of the Bs(scFv)4-IgG-1 and DVD-IgG-2 antibodies were better than that of NA9D7cAb and Bs(scFv)4-IgG-2 was similar to NA9D7cAb. However, CA16 binding levels of the other BsAb, DVD-IgG-1, was slightly lower than that of NA9D7cAb (Fig. 2B). At the whole-cell level, IFA was used to test the reactivity of the four BsAbs with EV71- or CA16-infected cells. As shown in Fig. 2C, Bs(scFv)4-IgG-1 and Bs(scFv)4-IgG-2 showed good reactivity with both EV71 and CA16. On the other hand, DVD-IgG-1 and DVD-IgG-2 were similar to their parental mAbs, which only reacted with one of viruses. Taken together, these results show that Bs(scFv)4-IgG-1 has a better binding activity to both EV71 and CA16.

3.3. Cross-neutralizing activity of the bispecific antibodies in vitro

To investigate the cross-neutralizing activity of the bispecific antibodies against EV71/52-3 and CA16/190, we used an *in vitro* microneutralization assay on RD cells. The chimeric antibodies (CT11F9cAb and NA9D7cAb) and their parental mAbs (CT11F9mAb and NA9D7mAb) were used as controls. The results showed that, similar to the controls, the bispecific antibodies DVD-IgG-1 and DVD-IgG-2 can only neutralize one of the viruses, EV71/52-3 or CA16/190 (Table 1). In contrast, Bs(scFv)4-IgG-1 and Bs(scFv)4-IgG-2 had potent cross-neutralizing activity against EV71/52-3 and CA16/190, as demonstrated by IC_{50} values ranging from 0.5 to 1.5 μg/mL (Table 1). Bs(scFv)4-IgG-1 showed a higher neutralizing activity for CA16/190, which was 3 times higher than that of Bs(scFv)4-IgG-2 (Table 1). In addition, we evaluated the cross-neutralizing ability of Bs(scFv)4-IgG-1 against additional EV71 (pSVA-MP4/B3, 05535/B5, 03149/C2 and 02969/C5) and CVA16 (213a/B1b and 4430/B1b) strains. As shown in Fig. 3, Bs (scFv)4-IgG-1 could effectively neutralize EV71 strains covering the B3, B5, C2 and C5 subgenotypes and the other two B1b subgenotypes of the CVA16 strains, with IC_{50} values ranging from 0.21 to 2.53 μg/mL. Taken together, these results indicate that Bs(scFv)4-IgG-1 is a promising cross-neutralizing agent against EV71 and CA16.

3.4. Protective efficacy of Bs(scFv)4-IgG-1 against EV71 and CA16 in vivo

The protective efficacy of the bispecific antibody Bs(scfv)4-IgG-1 was assessed using a neonatal mouse model infected with EV71, CA16, or EV71 + CA16. Groups of one-day-old BALB/c mice were challenged with 10^7 $TCID_{50}$ EV71 via the i.p. route or with 10^5 $TCID_{50}$ CA16 via i.c. routes. After 24 h, the mice were inoculated with diluted antibodies (10 μg/g, 3 μg/g or 1 μg/g). In the EV71 challenge test, the neonatal mice in the NA9D7mAb and PBS groups started to show illness at 4 dpi, and all died within 10 dpi. In contrast, all mice treated with 10 μg/g, 3 μg/g or 1 μg/g doses of Bs(scFv)4-IgG-1 or CT11F9mAb survived (Fig. 4A, D, G). In the CA16 challenge test, the control mice that received CT11F9mAb or PBS died within 10 dpi. In contrast, the 10 μg/g and 3 μg/g doses of Bs(scFv)4-IgG-1 or NA9D7mAb could fully protect the mice against CA16 infection, with no visible clinical symptoms. However, only 80% of the mice were protected from infection in both of the 1 μg/g dose groups compared to the control group (Fig. 4B, E, H).

To further evaluate the ability of the bispecific antibody to protect against co-infection, we also developed an *in vivo* co-infection model, in which neonatal mice were challenged with a mixture of the EV71 and CA16 strains. As a control, mice in the PBS control group developed illness as early as 3 dpi, and all died within 8 dpi. As shown in Fig. 4C, F, I, following co-infection, the mice receiving Bs(scfv)4-IgG-1 (10 μg/g or 3 μg/g) or a mixture of CT11F9mAb and NA9D7mAb (the concentration of each antibody was 10 μg/g or 3 μg/g) were well protected, with a survival rate of 100%, and a 1 μg/g dose of Bs(scfv)4-IgG-1 or the mixture of CT11F9mAb and NA9D7mAb could protect 70% or 80% of the mice from death, respectively, indicating a significant treatment effect compared to the control group. Moreover, the dose of Bs(scFv)4-IgG-1 required to protect against co-infection was half that of a mixture of CT11F9mAb and NA9D7mAb. Overall, these results indicate that the Bs(scfv)4-IgG-1 has a significant treatment effect against single or mixed infections with EV71 and CA16.

4. Discussion

In our study, we described an efficient method to develop tetravalent, IgG-like bispecific antibodies with a considerable therapeutic potential against enterovirus. The original VH or VK domains of the parental antibody (NA9D7 and CT11F9) were replaced by two scFv genes or were used in series (VH or VK) with different specificities to create the expression vectors Bs(scFv)4-IgG-H and Bs(scFv)4-IgG-K or DVD-IgG-H and DVD-IgG-K. This arrangement made a bispecific antibody that also constituted an independent antigen-binding unit (Fig. 1). These BsAbs could be produced using transient transfection in a CHO cell line with yields > 15 mg/L. SDS-PAGE showed that both DVD-IgGs and Bs(scFv)4-IgGs were functionally expressed and assembled in mammalian cells. Since both forms of DVD-IgGs and Bs(scFv)4-IgGs had unique heavy and kappa light chains, mispaired antibodies were rarely produced. In addition, the four BsAbs were purified only by protein A chromatography. Very few proteins that were not assembled into complete antibodies have been purified, but this doesn't affect the activity of bispecific antibodies (Fig. 1C and D). Binding assays revealed that the four bispecific antibodies were each capable of binding both EV71 and CA16. The affinities of each antibody for its targets were different (Fig. 2). In addition, the binding and neutralization activity of the Bs(scFv)4-IgG-1 antibody against both EV71 and CA16 were significantly better than those of other bispecific antibodies. This may be due to the difference of flexibility or conformation of the reformed antibodies (Wu et al., 2007). Because the light chain and the heavy chain of the natural IgG molecule are connected by disulfide bonds, the flexibility and distances are limited. However, we introduced two

$(Gly4Ser)_3$ linkers into the variable region of the Bs(scFv)4-IgG-1, which endowed the bispecific antibody with better plasticity, to some extent. As a result of the differences between Bs(scFv)4-IgG-1 and Bs (scFv)4-IgG-2, we also suspect that optimization of the location or orientation of the two scFvs or variable domains is important to ensure that each V_H and V_L domain best retain its original virus-binding ability.

EV71 and CA16 are the two major pathogens of HFMD, which has a wide variety of clinical manifestations, including fever, viremia, skin and mucosa lesions, serious complications and even death (Mao et al., 2014; Yi et al., 2017). Vaccines are the most cost-effective clinical tools to control HFMD. Three inactivated EV71 vaccines are currently commercially available in China and have good safety and a high efficacy (Yi et al., 2017). However, a monovalent EV71 vaccine cannot effectively prevent CA16 infection (Aswathyraj et al., 2016). Neutralizing antibodies are considered to be effective and necessary for protection against viral infectious diseases. Therefore, bivalent and multivalent HFMD vaccine candidates and antiviral drugs continue to be developed. The work described here represents the first use of antibody engineering to develop cross-neutralizing antibodies against both EV71 and CA16 simultaneously. The *in vivo* protective efficacy of the bispecific antibody Bs(scFv)4-IgG-1 was assessed in EV71 and CA16 virus challenge models (Fig. 4). Importantly, Bs(scFv)4-IgG-1 could protect neonatal mice against lethal EV71 and CA16 challenge. The effectiveness of other combinations of neutralizing antibodies targeting other epitopes of EV71 or CA16, and whether they provide high levels of cross-protection, remains to be determined.

In summary, the present study describes an approach for the generation of Bs(scFv)4-IgG-1, an engineered bispecific antibody targeting different epitopes of EV71 and CA16, and it has great potential as a novel antiviral treatment option for HFMD. Our study also provides important insights into bispecific antibody engineering for use against enterovirus infections.

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