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Engineering a novel IgG-like bispecific antibody against enterovirus A71

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ABSTRACT

Frequent outbreaks of enterovirus A71 (EVA71) occur in the Asia-Pacific area, and these are closely associated with severe neurological symptoms in young children. No effective antiviral therapy is currently available for the treatment of EVA71 infection. The development of monoclonal antibodies (mAbs) has demonstrated promise as a novel therapy for the prevention and treatment of infectious diseases. Several medical conditions have been treated using bispecific or multi-specific antibodies that recognize two or more distinct epitopes simultaneously. However, bispecific or multi-specific antibodies often encounter protein expression and product stability problems. In this study, we developed an IgG-like bispecific antibody (E18-F1) comprising two anti-EVA71 antibodies: E18 mAb and llama-derived F1 single-domain antibody. E18-F1 was demonstrated to exhibit superior binding affinity and antiviral activity compared with E18 or F1. Additionally, E18-F1 not only improved survival rate, but also reduced clinical signs in human SCARB2 receptor (hSCARB2) transgenic mice challenged with a lethal dose of EVA71. Altogether, our results reveal that E18-F1 is a simple format bispecific antibody with promising antiviral activity for EVA71.

1. Introduction

Antibody-based therapeutic modalities have demonstrated clinical success for the management of infectious states: raxibacumab and obiltoxaximab are used for the treatment of inhalational anthrax caused by *Bacillus anthracis*, bezlotoxumab for the prevention of recurrent *Clostridium difficile* infection, palivizumab for the prevention of pediatric respiratory tract infections caused by respiratory syncytial virus, and ibalizumab for the management of multidrug-resistant HIV-1 infection [1,2]. However, RNA viruses may escape most antibodies with single or combined multi-mutations due to the high mutation rate, highlighting bispecific- or multi-specific antibodies targeting to at least two targets might reduce drug resistance [3–7]. Recently, several state-of-the-art technologies have been applied in the engineering of bispecific or multi-specific antibodies through combining two or more functional antigen-recognizing domains into a single construct after tremendous efforts [8–11]. For instance, we previously engineered a tetravalent, bispecific antibody based on single-chain variable fragments (scFv) targeting EGFR and Her2/neu with theranostics application [12].

Enterovirus A71 (EVA71) is a prevalent neurotropic pathogen in the Asia-Pacific region for which no specific drug has been approved to date. EVA71 not only induces viral encephalitis, but also causes severe hand, foot, and mouth disease (HFMD) in young children aged less than 5 years [13]. Numerous potent broadly neutralizing antibodies that recognize different epitopes on the viral capsids of EVA71 have been reported in the past decade [14–16]. Although only one study reported a bispecific broadly neutralizing antibody against EVA71 and coxsackievirus A16 by using scFv technology, scFv-based bispecific fusion proteins often exhibit manufacturability and clinical development limitations such as aggregation and subsequent stability problems [14,

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17]. In contrast to similar smaller scFv antibody formats with some limitations, single-domain antibodies (sdAbs) derived from llama were used for therapeutics against numerous viral diseases due to their flexibility and stability [18]. In addition, the bispecific antibody (BiSAb) based on IgG light-chain C-terminal fusion has been observed to prevent steric hindrance effects that may disrupt individual antibody-targeting effects of each other [19,20].

We present a novel BiSAb, E18-F1 design as a C-terminal fusion of an EVA71-specific sdAb clone F1 to the light chain of an EVA71-specific IgG clone E18 [21,22]. E18-F1 BiSAb can be expressed in the mammalian cell culture and purified through protein A chromatography. E18-F1 BiSAb exhibits not only enhanced binding activity to EVA71 but also improved antiviral activity compared with original antibodies. These data suggest a potential use of E18-F1 BiSAb in EVA71 treatment.

2. Materials and methods

2.1. Cell lines and materials

Rhabdomyosarcoma (RD) cells (American Type Culture Collection [ATCC] accession no. CCL-136) were purchased from ATCC. RD cells were cultured in Dulbecco's Modified Eagle Medium with 10% FBS and Pen-Strep (Thermo Fisher) at 37 °C with 5% CO2. CHO–S cells were purchased from Thermo Fisher Scientific Inc. CHO–S cells were cultured in serum-free HyClone CDM4PERMAb medium (GE Healthcare) with Pen-Strep (Thermo Fisher) at 37 °C with 5% CO2. The Cell Line Nucleofector Kit V is used for the transfection of CHO–S cell lines in the Nucleofector 2b Device (Lonza). The selection antibiotic Hygromycin B was purchased from Thermo Fisher Scientific Inc. Enzyme-linked immunosorbent assay (ELISA) plates and blocking buffer were purchased from Thermo Fisher Scientific Inc. The EVA71 TW/73/12 (genotype C4) used in this study was isolated from Chang Gung Memorial Hospital in Taiwan in 2012. Clinically isolated EVA71 genotype C2 strain Tainan/4643/98, GenBank accession number AF304458, was kindly provided by Dr. Jen-Ren Wang, National Cheng Kung University, Tainan, Taiwan [23]. EVA71 was propagated in RD cells, and formalin-inactivated EVA71 was obtained through sucrose gradient ultracentrifugation, as described elsewhere [22]. The pSecTag2/Hygro mammalian expression vector used for human Fc fusion protein expression was purchased from Thermo Fisher Scientific Inc. The pFUSE2ss-CLIg-hK and pFUSEss-CHIg-hG1 gene expression vector system used for IgG expression was purchased from InvivoGen.

2.2. General construction of recombinant proteins

Specific constructs were made to create by with overlap extension PCR polymerase chain reaction and DNA synthesis service from GenScript. In preparation for the anti-EVA71 sdAb clone F1-human Fc fusion (F1-hFc), we first inserted sdAb clone F1 DNA into a human IgG1 Fc containing the pSecTag2/Hygro expression vector as previously described (Fig. 1A) [22]. Heavy- (VH) and light-chain (VL) sequences of E18 antibody were obtained from the Protein Data Bank (VH: AZK29196.1; VL: AZK29195.1). In preparation for the anti-EVA71 IgG antibody clone E18 (E18), CHO cell codon optimized nucleotide sequences were synthesized and then cloned into expression vectors (pFUSE2ss-CLIg-hK for VL and pFUSEss-CHIg-hG1 for VH) (Fig. 1A). In preparation for the IgG-like bispecific antibodies (E18-F1), the C-terminus of the E18 IgG light chain was extended by a nine-amino-acid G_4SG_3S linker (GGGGSGGGS) and an sdAb clone F1 (Fig. 1A).

2.3. Production and purification of recombinant proteins

Expression and purification of recombinant proteins were also implemented using the same strategy based on the CHO cell expression system and protein A–derived affinity medium (GE Healthcare) as previously described [22]. In brief, expression vectors transfecting CHO cells through an electroporation-based transfection method was used in the selection of stable expression cells. After several runs of Hygromycin B treatment, a stable cell line expressing the target protein was obtained. Stable expression cells were grown in flasks on an orbital shaker platform rotating at 130 rpm at 37 °C under 5% CO_2 and subcultured following the manufacturer's protocol. Purified proteins obtained from MabSelect SuRe™ LX were analyzed through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in nonreducing (without dithiothreitol [DTT]) and reducing conditions (with DTT), followed by staining with InstantBlue (Expedeon).

2.4. ELISA

Nunc MaxiSorp high protein-binding capacity 96-well ELISA plates

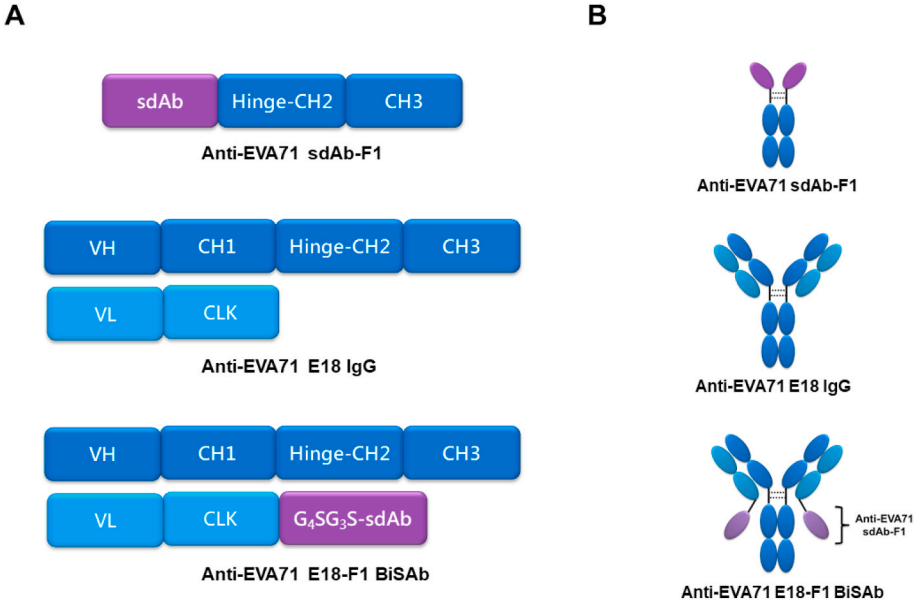


Fig. 1. Design and generation of anti-EVA71 antibodies. (A) Bivalent anti-EVA71 sdAb-F1, anti-EVA71 E18 IgG, and anti-EVA71 IgG-like bispecific antibody E18-F1. (B) Recombinant proteins.

were coated with 10 μg/mL formalin-inactivated EVA71 (in 0.1 M sodium carbonate buffer, pH 9.5) overnight at 4 °C and then blocked with StartingBlock™ T20 Blocking Buffer (Thermo Fisher). Binding of recombinant proteins was revealed with a goat anti-human IgG-Fc fragment cross-adsorbed antibody (Bethyl Laboratories, Inc.) conjugated with horseradish peroxidase (HRP) and HRP substrate (United States Biological).

For the ELISA-based competitive binding assay, 100 nM E18-F1 BiSAb was premixed with different concentrations of E18 IgG (50, 100, and 200 nM), the reaction mixtures were then used for ELISA assay basically as described above. To avoid the false-positive signals, we used the HRP-conjugated MonoRab™ rabbit anti-camelid VHH antibody (GenScript) that is only specific for camelid sdAb but no cross-reactivity with human immunoglobulins for the detection of E18-F1 BiSAb.

2.5. Cell-based neutralization assay

Ninety-six-well tissue culture plates were seeded with 3×10^4 RD cells/well in Dulbecco's Modified Eagle Medium with 10% FBS and were incubated at 37 °C overnight. Recombinant proteins at various concentrations were incubated with hundred 50% tissue culture infective doses ($TCID_{50}$) of EVA71 for 1 h at 37 °C. After incubation, RD cells were infected with the mixture of recombinant proteins and virus. After adsorption, infected cells were covered with medium containing 2% FBS. Infected cells were further incubated at 37 °C for at least 64 h. The plates were fixed with formaldehyde and then stained with crystal violet. The density of the well at OD 570 nm was measured, and analysis was performed as previously described [22].

2.6. Plaque reduction neutralization test

Different concentrations of E18-F1 BiSAb were incubated with infectious EVA71 (4643 strain) (100 PFU/mL) at 37 °C for 1 h. These virus-antibody mixtures were used to inoculate monolayers of RD cells cultured in 6-well plates (2×10^5 cells/well; n = 3). The medium was aspirated from the adherent cultures before inoculation with 500 μl of the antibody-virus mixtures. The plates were incubated in a CO_2 incubator at 35 °C for 2 h during viral adsorption, after which 2 mL of DMEM supplemented with 2% FBS and 0.3% agarose was overlaid in each well. Plates were incubated at 35 °C with 5% CO_2 for an additional 2 days and stained with 0.5% crystal violet solution (Sigma) after 10% formalin solution (Sigma) fixation. Plaques were determined from manual-based visual counting by inverting the transparent plate. Percentages of inhibition of infection were calculated by comparing the number of plaques observed from wells inoculated with antibody-virus mixtures with those of control groups (virus only).

2.7. Prophylactic efficacy of E18-F1 BiSAb in hSCARB2 transgenic mice

The hSCARB2 transgenic mice kindly provided from Professor Satoshi Koike (Tokyo Metropolitan Institute of Medical Science) were bred and maintained as previously described [24]. Groups of hSCARB2 transgenic mice, 3 weeks old, were infected with EVA71 at the indicated dose of 10^5 plaque-forming units (pfu)/mouse via intraperitoneal (i.p) injection and observed for survival rate and clinical signs daily. Disease score was tallied as follows: 0, normal movement; 1, jerky movement; 2, paralysis of one hind leg; 3, paralysis of both hind legs; and 4, death. To evaluate the prophylactic efficacy of E18-F1 BiSAb, hSCARB2 transgenic mice were intraperitoneally injected with 200 μg E18-F1 BiSAb one day before EVA71 infection. All procedures were approved by the Institutional Animal Care and Use Committee of Chang Gung University, Taiwan.

3. Results

3.1. Design and production of E18-F1

In this study, we designed a BiSAb, E18-F1, by fusing two anti-EV71 antibodies into one human antibody backbone. The backbone antibody was an EVA71-specific E18 mAb reported to exhibit a novel mechanism of action for inducing viral genome release from EVA71 [21]. Fused to the light chain of the E18 backbone antibody was a novel anti-EVA71 specific F1, an sdAb selected from the llama phage display library and with neutralizing activity as described elsewhere (Fig. 1B) [22]. The sdAb F1 was fused to the light chain of E18 at its C terminus through a G_4SG_3S (GGGGSGGGS) linker.

Recombinant E18-F1 BiSAb was produced and purified in CHO cells. The molecular weights and antiviral activity of these recombinant proteins are summarized in Table 1 and Fig. 2. E18-F1 BiSAb formed an expected dimer as E18 IgG because the nonreducing SDS-PAGE of purified E18-F1 BiSAb displayed a species with a molecular weight of 190 kDa (Fig. 2A). In reducing conditions, purified E18-F1 BiSAb gave rise to two bands, one of approximately 50 kDa, reflecting the size of the E18 heavy chain, VH-CH1-Hinge-CH2-CH3 (Fig. 1A), and the other approximately 40 kDa, reflecting the size of the light-chain-F1 fusion, VL-CLk-G_4SG_3S–F1.

3.2. Characteristics of various anti-EV71 antibodies

For the enhancement of antibody binding affinity, a BiSAb against two adjacent epitopes in EVA71 virion—one located between VP4–VP2–VP3–VP1 protomers and the other in VP3—was prepared using a recombinant fusion strategy. The tetravalency of E18-F1 BiSAb might have been responsible for its improved EVA71 targeting compared with the original bivalent E18 antibody. The binding signals of E18-F1 BiSAb were greater by more than two-fold as measured using an ELISA assay (Fig. 3A), indicating that sdAb-F1 exhibited improved EVA71-targeting selectivity. Moreover, E18-F1 BiSAb remained same binding to EVA71 even in the presence of different concentrations of E18 IgG as a binding competitor in a dose-dependent manner, indicating that E18-F1 BiSAb harbors functional antibody activity of sdAb-F1 (Fig. 3B). In addition, E18-F1 BiSAb is more potent than the original E18 IgG in terms of antiviral activity; EC_{50}'s are 5.2 nM for E18, and 1.6 nM for E18-F1 (Table 1). In plaque reduction test, E18-F1 BiSAb showed improved antiviral activity as compared with that of E18 IgG (Fig. 4A). As shown in Fig. 4C, more than 99.5% viral load of EVA71 was reduced when the virus was pre-incubated with E18-F1 BiSAb at 10 nM. Consequently, these *in vitro* data highlights that E18-F1 BiSAb bears promising functions to combat EVA71 (see Fig. 4).

3.3. Prophylactic efficacy of E18-F1 BiSAb in hSCARB2 transgenic mice

To further investigate the prophylactic efficacy, hSCARB2 transgenic mice were administered with E18-F1 BiSAb one day before EVA71 infection at lethal dose (10^5 pfu/mouse). On day 14 after infection, the

Table 1
Characterization of EVA71 specific antibodies, including sdAb-F1, E18 IgG, and E18-F1 BiSAb.

Antibody name	sdAb-F1	E18 IgG	E18-F1 BiSAb
Characterization			
Structure	Human IgG1 Fc fusion (dimer)	Chimeric IgG1 mAb (dimer)	Single domain antibody (sdAb) armed chimeric IgG1 bispecific mAb (dimer)
Molecular weight	~95 kDa	~150 kDa	~190 kDa
Neutralization activity to EVA71 (EC_{50})	~406.3 nM	~5.2 nM	~1.6 nM

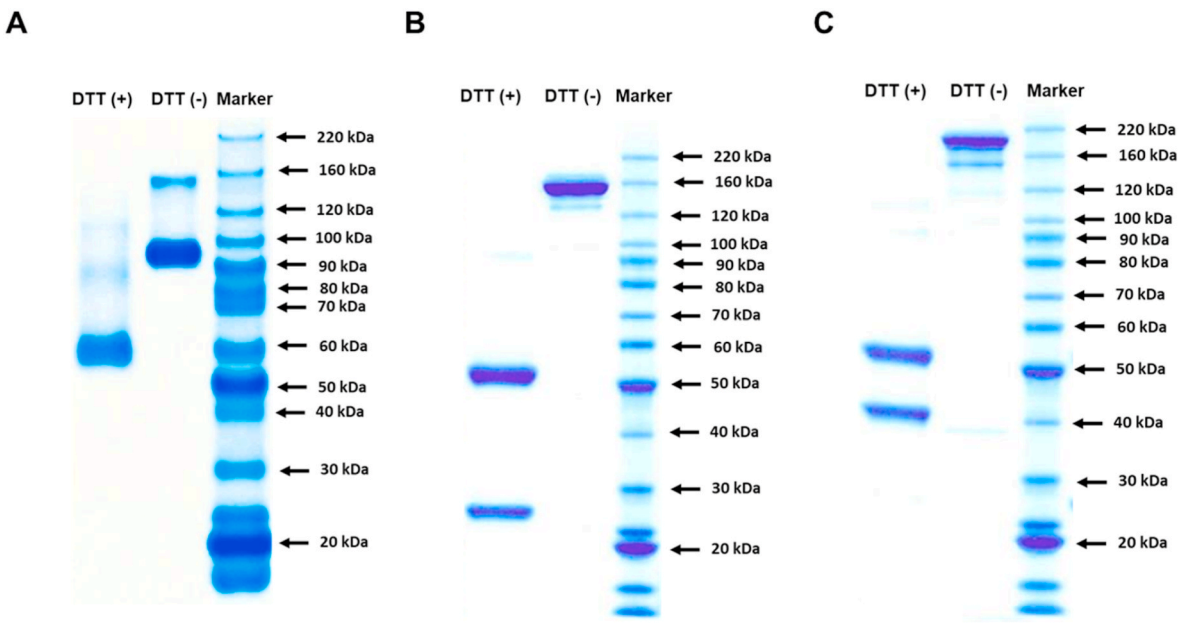


Fig. 2. Characterization of recombinant proteins. Anti-EVA71 antibodies were electrophoresed on SDS-PAGE in nonreducing conditions (DTT (−)) and reducing conditions (DTT (+)). SDS-PAGE analysis of Protein A purified anti-EVA71 sdAb-F1 (A), anti-EVA71 E18 IgG (B), and anti-EVA71 E18-F1 BiSAb (C).

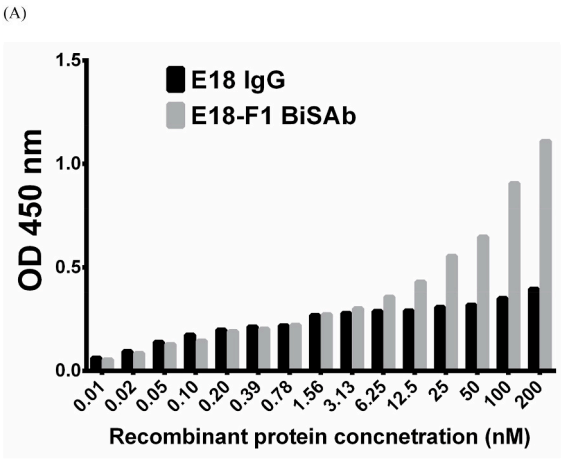


Fig. 3. Analyses of binding of E18 or E18-F1 to EVA71. (A) Indirect ELISA was performed by coating ELISA plate wells with formalin-inactivated EVA71. Various concentrations of E18-F1 or E18 were added in duplicates. The antibodies were detected by HRP assay and the data are presented as average OD_{450} value. (B) Effects of E18 on E18-F1 binding to EVA71. Various concentrations of E18 IgG, as a binding competitor, were premixed with 100 nM E18-F1 BiSAb for competitive binding assay. A different secondary antibody, as compared to the one used in Fig. 3(A), was employed in this assay.

survival rates were 0% and 71.4% in the PBS-treated group and E18-F1 BiSAb treated groups, respectively (Fig. 5A). Moreover, only mice receiving E18-F1 BiSAb treatment were free from paralysis (Fig. 5B). Ultimately, all these results indicate that E18-F1 BiSAb not only improved survival rate, but also reduced clinical signs in hSCARB2 transgenic mice challenged with a lethal dose of EVA71.

4. Discussion

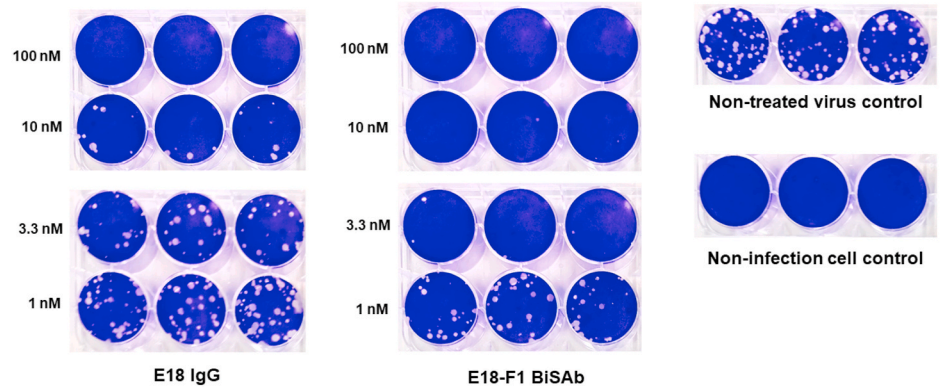
Currently, two BiSAbs, blinatumomab and emicizumab, have been approved by the US FDA for the treatment of cancers and hemophila A, respectively. Although almost 100 BiSAbs are used in clinical studies, new types of BiSAb are required for many as-yet untreated medical conditions [2]. However, technical challenges exist that necessitate sizeable engineering advances. For instance, heavy chain heterodimerization strategies, such as knobs-into-holes technology, and specific light-chain associations, such as CrossMab technology, all require more than three expression vectors to produce target proteins [25,26]. The findings of this work can be used to alleviate expression and stability problems encountered in many BiSAb designs. In this study, we engineered a simple and novel IgG-like BiSAb construct, E18-F1, by fusing an F1 sdAb to the C terminus of the light chain of E18 mAb. Fusion of the F1 sdAb in this manner might minimize the steric hindrance effect resulting from traditional N-terminal fusion to the heavy or the light chain that otherwise interferes with simultaneous binding of both target antigens. In addition, the use of structurally stable sdAb as a fusion partner instead of scFv not only reduces total molecular weight, but also prevents aggregation and instability resulting from scFv fusion.

To the best of our knowledge, no such fusion strategy has yet been reported, and the binding and antiviral properties of E18-F1 BiSAb promising function both *in vitro* and *in vivo*. Particularly, one single dose of E18-F1 BiSAb (200 μg/mouse) can protect mice from paralysis in hSCARB2 transgenic mice challenged with EVA71 at a lethal dose. In summary, our strategy warrants further development as an effective design to combat emerging diseases caused by viruses such as EVA71.

CRediT authorship contribution statement

Hsiang-Ching Wang: Conceptualization, Methodology, Investigation, Writing - original draft. **Hui-Chen Hung:** Methodology,

(A)



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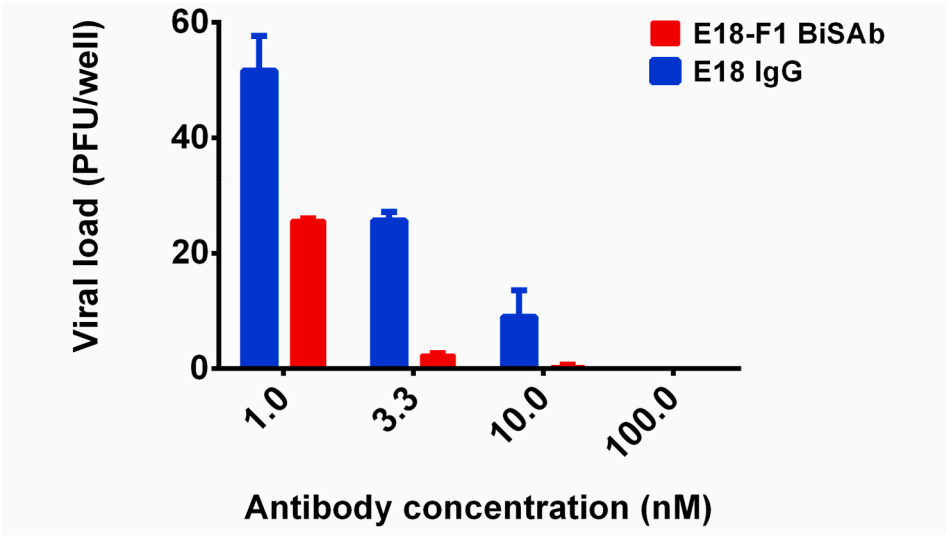


(C)

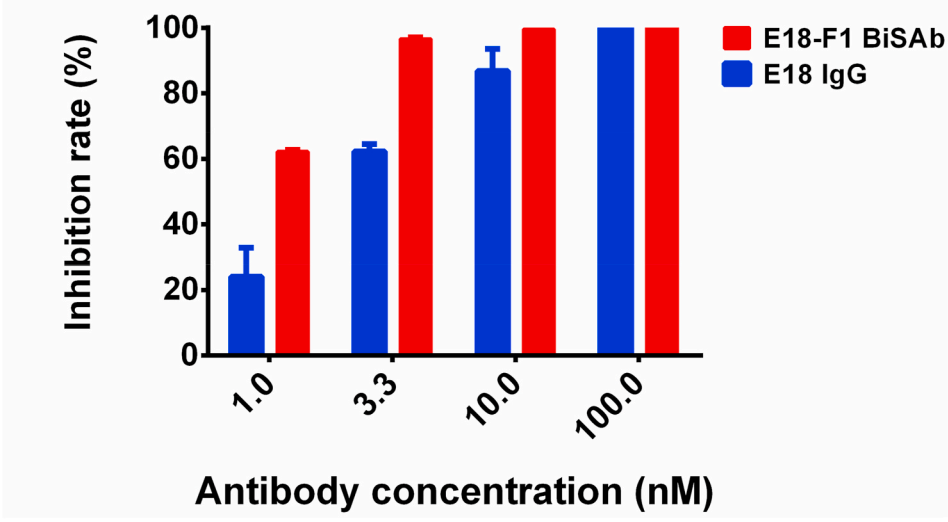
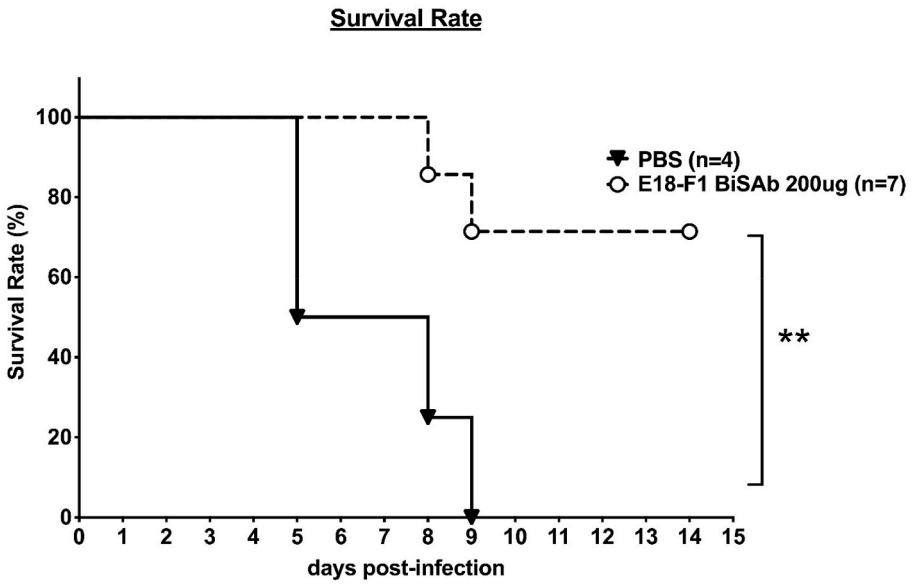


Fig. 4. Evaluation the antiviral activity of E18 IgG and E18-F1 BiSAb by plaque reduction assay. (A) Plaque assays were performed with agarose overlay and incubated for 2 days. Plaque formation assay shows the reduction of plaque generation with a dose dependent response. (B) Histogram shows that E18 IgG and E18-F1 BiSAb are able to inhibit infection by EVA71 after normalization to untreated control. Results are expressed as mean ± SD from a representative experiment performed in triplicate. (C) The inhibitory activity of E18 IgG and E18-F1 BiSAb at different concentrations was calculated.

(A)



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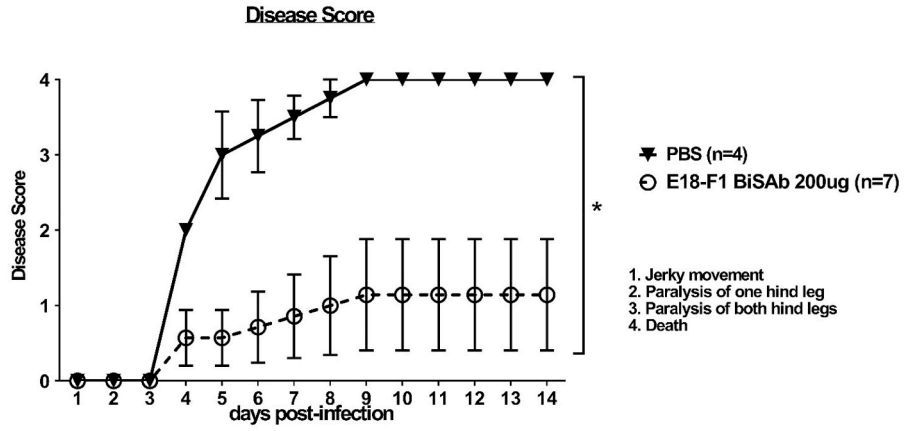

Fig. 5. Prophylactic efficacy of E18-F1 BiSAb in hSCARB2 transgenic mice. Kaplan–Meier survival curve (A) and clinical scores (B) of hSCARB2 transgenic mice administered with E18-F1 BiSAb one day before EVA71 infection at lethal dose. The total number (N) of mice per group is shown. Logrank test was used to analyze the statistic difference of survival rate. One-way ANOVA with the Kruskal-Wallis test was used to analyze the statistic difference of the individual groups with disease score. The symbols * and ** are used to indicate P values < 0.05 and < 0.01, respectively.

Validation, Data curation, Writing - original draft. **Peng-Nien Huang:** Methodology, Formal analysis, Resources, Writing - original draft. **Yu-An Kung:** Methodology, Formal analysis, Resources. **Sung-Nien Tseng:** Methodology, Formal analysis. **Yun-Ming Wang:** Conceptualization, Resources. **Shin-Ru Shih:** Conceptualization, Resources, Investigation, Supervision. **John Tsu-An Hsu:** Conceptualization, Investigation, Writing - review & editing.

Declaration of competing interest

We have no conflicts of interest to disclose.

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