Molecular Immunology 105 (2019) 190–197

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Selection and characterization of protective anti-chikungunya virus single domain antibodies

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ARTICLE INFO

Keywords:
CHIKV
Chikungunya
Virus like particles
Single domain antibodies
Immune phage display library
Neutralizing antibodies

ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes an arthralgia febrile illness that has affected millions of people on three continents. Previously, neutralizing monoclonal antibodies that have prophylactic and therapeutic activity were found to remove virus in joint tissues, thereby reducing the severity of symptoms in mice and non-human primates. In this study, we sought to develop thermostable small recombinant antibodies against CHIKV for future diagnostic, prophylactic and therapeutic applications. To develop these single domain antibodies (sdAb) a CHIKV immune library was constructed by displaying the consortium of variable heavy domains (VHH) amplified from peripheral white blood cells isolated from llamas immunized with CHIKV virus-like particles (VLPs). Five anti-CHIKV sdAb isolated using bio-panning were evaluated for their affinity and thermal stability. Their ability to detect CHIKV VLPs was demonstrated in both MagPlex- and ELISA-based assays. Finally, the ability of two sdAb, CC3 and CA6, to inhibit CHIKV infection were tested using a plaque reduction and neutralization test (PRNT), yielding $PRNT_{50}$ values of 0.6 and 45.6 nM, respectively.

1. Introduction

Chikungunya virus (CHIKV) is a mosquito-transmitted pathogen in the genus Alphavirus, family Togaviridae. CHIKV was first identified in 1952-53 in Tanzania as the causative agent of the severe joint pain associated with fever, headache, chills, photophobia, muscle pain, and rash. Most patients have an acute self-limiting infection lasting 7–14 days, 30% have ongoing joint pain and lethargy lasting for months, and an unfortunate 10% have symptoms that continue for years (Powers and Logue, 2007; Tsetsarkin et al., 2011).

By October 2014 the U.S. Center for Disease Control and Prevention (CDC) reported 760,000 suspected and 14,000 confirmed cases of CHIKV in 36 countries or territories in the Americas, and due to the presence in the United States of its mosquito vector, *Aedes albopictus*, indigenous spread of the disease could occur further north in the Americas as well. At present, there is no licensed human vaccine or therapeutic available to protect against CHIKV infection.

CHIKV, like other alphaviruses, has a single-stranded positive sense RNA genome of about 11.8 kb encoding two polyproteins: one nonstructural polyprotein that yields four nonstructural proteins involved in genome replication, capping and polyprotein processing, while the structural polyprotein produces the capsid as well as the E2 and E1 envelope glycoproteins (Kuhn, 2007). Virions are produced in the cytoplasm of the infected cells and later are enveloped and budded out of the membrane. Each virion is composed of the genomic RNA with 240 copies of the capsid protein and 240 copies of E1/E2 heterodimers embedded in the membrane. There are 80 trimeric E1/E2 spikes projecting outward from the membrane and the tip of each complex is formed by E2 protein that interacts with the cell receptor. E2 is the target of the most CHIKV–neutralizing antibodies (Goh et al., 2013).

CHIKV virus like particles (VLPs) are typically produced by encoding E1/E2 structural proteins along with capsid protein. They have been shown to effectively generate immunity in animals and non-human primates (Akahata et al., 2010). As part of a clinical trial, healthy human volunteers were immunized with CHIKV VLPs; the neutralizing ability of serum samples was examined and shown to neutralize the 9 different CHIKV strains that were examined (Goo et al., 2016). Thus, VLPs are a promising vaccine strategy that could provide for the long term solution to CHIKV infection (DeZure et al., 2016; Erasmus et al., 2016; Goo et al., 2016).

In addition to developing vaccines, the development of effective therapeutics are also critical to limiting the severity of CHIKV infection and hopefully reducing the long-term health effects. Several small molecule antiviral agents have been examined (Abdelnabi et al., 2018; Ching et al., 2017; Tardugno et al., 2018). In addition, antibody-based therapeutics are being investigated as a way to bridge the gap until an

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https://doi.org/10.1016/j.molimm.2018.11.016
Received 20 August 2018; Received in revised form 21 November 2018; Accepted 30 November 2018
Available online 11 December 2018
0161-5890/ Published by Elsevier Ltd.

effective immune response occurs (Chua et al., 2017; Pal et al., 2013; Smith et al., 2015). In one study, administration of neutralizing human monoclonal antibodies were shown to protect mice when given prophylactically, and were also effective therapeutics when given post exposure (Smith et al., 2015). Additionally there is a need for rapid, reliable, and affordable methods for diagnosis of CHIKV. Antibodies can also play a role in the diagnosis of CHIKV infection, as lower cost rapid alternatives to RT-PCR methods (Damle et al., 2016; Galo et al., 2017; Hueston, 2016; Huits et al., 2018; Okabayashi et al., 2015; Tuekprakhon et al., 2018). For example, a lateral flow assay was developed utilizing a pair of monoclonal antibodies that are specific for the CHIKV E1 protein, and was shown to enable diagnose of CHIKV infection in the acute phase (Okabayashi et al., 2015). To date most antibodies investigated for therapeutic or detection applications have been conventional IgGs. As therapeutics, there is the concern with their use, as antibodies are known to exacerbate the illness through an Fc mediated process (Lum et al., 2018; Petitdemange et al., 2015). As diagnostics, it would be desirable that antibodies be robust enough to be utilized in point-of-care assays where maintaining a cold chain becomes problematic.

To develop alternatives to conventional antibodies for both diagnostic and therapeutic applications, single domain antibodies (sdAb) that bind to both the CHIKV VLPs as well as recombinant E1 protein (rE1) were derived from llamas that had been immunized with CHIKV VLPs. Also referred to as nanobodies, sdAb are the recombinant variable heavy domain isolated from the llama's unique heavy-chain-only antibodies (VHH). Being only ~16 kDa, sdAb represent one of the smallest naturally derived antigen binding fragments known. In part by virtue of their small size, they possess many attractive features, such as: an ability to access hidden viral epitopes, the capacity to refold following thermal or chemical denaturation, efficient production in *E. coli* or Yeast, rapid tissue penetration, as well as rapid clearance by the kidneys, and the ability to make fusion constructs to create bivalent antibodies or add an effector domain (de Marco, 2011; Harmsen and De Haard, 2007; Muyldermans, 2013; Wesolowski et al., 2009). Importantly, sdAb have been described that show promise in both diagnostic applications and as therapeutics for human viral disease (Sherwood et al., 2007; Y. Wu et al., 2017). In this work we isolated several anti-CHIKV VLP sdAb, evaluated their affinity and thermal stability, and additionally demonstrated their ability to detect CHIKV VLPs in both MagPlex- and ELISA- based assays. Finally, two sdAb (CA6, CC3) were tested for their ability to inhibit CHIKV infection in a plaque reduction neutralization test (PRNT).

2. Materials and methods

2.1. Llama immunization and construction of phage display library

CHIKV VLPs purified from HEK293 cells (The Native Antigen Company, UK) were used for immunizing two llamas. The VLPs contained the capsid, E1 and E2 proteins; sequences were derived from the strain Senegal 37997. Each llama was immunized 4 times, 21 days apart, with 100 μg CHIKV VLPs. Two weeks after the final boost, ~200 mL samples of whole blood were collected. Peripheral blood lymphocytes were isolated by centrifugation using a Ficoll separation method and the total RNA was isolated using QIAamp RNA Blood Mini kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol. Next, cDNA was obtained by reverse transcribing the total RNA using superscript RTIII and oligo-dT (both from Life Technologies, Grand Island, NY) according to manufacturer's protocol. The heavy chain variable domain fragments from the llama heavy chain only antibodies (VHH) were amplified from the PCR reaction using degenerated primers (Vu et al., 1997) and our previously published modified protocol (Goldman et al., 2006).

The immune library derived from the two llamas immunized with CHIKV VLPs was obtained by cloning the amplified variable domain fragments into the phage display vector, pECAN21 (Goldman et al., 2006; Liu et al., 2007). Gel purified variable domain PCR fragments and pECAN21 DNA cut with SfiI (New England Biolab Inc, Ipswich, MA) were ligated overnight at 15 °C with a 3:1 ratio of insert to vector. The ligation mixture was then transformed into XL1 Blue cells (Agilent Technologies Inc, Clara, CA) using electroporation. Phage displaying sdAb were prepared from the library according to the previous protocol (Liu et al., 2013b). Representative clones were sequenced to assess the library quality and diversity.

2.2. Biopanning, selection and sequence analysis of CHIKV VLP potential binders

Recombinant CHIKV E1 (rE1) was obtained from the Native Antigen Company. Panning was carried out using a procedure similar to previous work (Goldman et al., 2006; Liu et al., 2013b). The VLP and rE1 at the concentration of 30 and 5 μg/mL respectively, were separately coated onto 4 wells of a 96-well plate and incubated at 4 °C overnight. The coated wells were then washed with PBS supplemented with 0.05% Tween 20 (PBST), blocked with PBS containing 4% (w/v) nonfat powdered milk, and incubated with phage displaying sdAb amplified from the immunized phage display library. Following extensive washing wells with PBST and PBS, the binding phage were eluted by 100 mM triethylamine, which was subsequently neutralized by adding 1 M Tri-HCl, pH8.0. The eluted phage titer was determined by infecting the log phase XL1 blue and referred to as round 1 (R1) eluted phage. The eluted phage were also grown and replicated in XL1 blue to obtain higher titer of amplified phage, which were referred as R1 amplified phage. R1 amplified phage were then used to repeat the above panning procedure once to obtain round 2 (R2) eluted phage and R2 amplified phage. Bacterial colonies were obtained by infecting XL1 blue with R2 eluted phage and individual colonies were inoculated into wells in a 96-well plate. Approximately 96 colonies (half a plate from the VLP selection and half from the rE1 selection) were grown to conduct monoclonal phage ELISA as previously described. Binding was assessed using the target VLPs and rE1. Monoclonal phage MagPlex assays (Anderson et al., 2008) were used to assess binding of the phage to immobilized VLPs and rE1. The phage MagPlex assays also included irrelevant targets (NS1 from dengue virus serotypes 1, 2, 3, 4; The Native Antigen Company) and confirmed what had been observed by ELISA. Fourteen of the clones judged positive and specific by ELISA and MagPlex assays were grown for plasmid purification. Plasmid DNA was sent out for sequencing (Eurofin Genomics, Louisville, KY). Amino acid sequences were obtained and aligned using multalin (http://multalin.toulouse.inra.fr/multalin/) to compare the variations in the complementarity determine regions (CDRs) (Corpet, 1988).

2.3. Expression and purification of potential binders

Representative sdAb genes from each sequence family were subcloned into a periplasmic expression vector, pET22b, for protein preparation and transformed into Turner (DE3) *E. coli* strain (EMD Millipore, Billerica, MA). The expression and purification procedure was performed according to the previously published protocol (Shriver-Lake et al., 2017). Following IMAC extraction, sdAb were further purified from other protein contaminates or aggregates through gel filtration chromatography using a Superdex 75 10/300 G L column (GE Healthcare) on a BioLogic DuoFLow chromatography system (Bio-Rad). Monomeric sdAb concentration was determined by UV absorption using a NanoDrop 1000 instrument.

2.4. Measurement of melting temperature (Tm) by fluorescent dye melt assay and circular dichroism (CD)

The fluorescent dye-based melting assay was performed as described previously (Liu et al., 2013b). Each of the sdAb was diluted to a

concentration of 500 µg/mL in a final volume of 20 µL PBS and Sypro Orange dye was added at a dilution of 1:1000. Samples were measured in triplicate using a StepOne Real-Time PCR machine (Applied Biosystems, Foster City, CA). The heating program was run in continuous mode from 25 °C – 99 °C at a heating rate of 1% (~2 °C per minute), and data was recorded using the ROX filter. The melting point was determined to be the peak of the first derivative of the fluorescence intensity. Each measurement was performed in triplicate, all three replicates giving essentially identical values for the melting temperature.

Circular dichroism (CD) measurements for the determination of melting temperature and refolding ability were as described previously (Liu et al., 2017, 2013b). The sdAb samples were diluted to 22 µg/mL in deionized water. CD was measured at an ultraviolet wavelength between 200 and 210 nm using a quartz cuvette with 1 cm path length in a Jasco J-815 Spectropolarimeter. Samples were heated from 25 °C to 95 °C at a rate of 2.5 °C/min, and a cooling stage at the same rate was used to assess the amount of refolding after heat denaturation.

2.5. Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) affinity and kinetics measurements were performed using the ProteOn XPR36 (Bio-Rad). Lanes of a general layer compact (GLC) chip were individually coated with rE1, CHIKV VLP, or left uncoated initially and then coated with rE1 or recombinant E2 protein (rE2; expressed as a fusion with a human Fc, The Native Antigen Company) for additional tests. Immobilization of the proteins was performed using dilution of 20 µg/mL in 10 mM acetate buffer pH 5.0 and attached to the chip following the standard 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) / N-hydroxysulfosuccinimide (sulfo-NHS) coupling chemistry available from the manufacturer. Binding kinetics of each sdAb was tested at 25 °C by flowing six concentrations typically varying from 300 to 0 nM at 100 µL/min for 90 s over the antigen coated chip and then monitoring dissociation for 600 s. Following each run, the chip was regenerated by flowing 0.085% phosphoric acid (~pH 2.2) across the surface for 18 s. Data analysis was performed with ProteOn Manager 2.1 software, corrected by subtraction of the zero antibody concentration column as well as interspot correction. The standard error on the fits was less than 10%. Binding constants were determined using the Langmuir model built into the analysis software.

2.6. MagPlex direct binding and sandwich immunoassays

MagPlex assays were performed essentially as described previously (Liu et al., 2017). Briefly, MagPlex beads were coated with the desired protein or sdAb using the recommended two step EDC/sulfo-NHS chemistry. The biotin-labeled sdAb (Bt-sdAb) were prepared by using a 10-fold molar excess of NHS-LC-LC-biotin, which after 30 min the excess biotin was removed using a Zeba spin 7 K desalting column (ThermoFisher). For direct binding assays the protein-coated MagPlex beads (~100/set) were mixed with various concentrations of the Bt-sdAb and incubated for 30 min. For sandwich immunoassays the sdAb-coated MagPlex beads (~100/set) were mixed with various concentrations of CHIKV VLPs diluted into PBSTB (PBS + 0.05% Tween (PBST) and 1 mg/mL bovine serum albumin (BSA)) in the wells of a 96-well polystyrene round bottom microtiter plate. After 30 min the beads were washed by placing the plate on a 96f magnet (BioTek, Winooski, VT) and washing three times with PBST. The beads were then incubated with the 1 µg/mL Bt-sdAb as indicated. For both the direct and sandwich assays, after 30 min the beads were washed 3 times and then, to generate the fluorescent signal, the beads were incubated for 30 min with 2.5 µg/mL streptavidin conjugated phycoerythrin (SAPE, Columbia Biosciences, Frederick, MD). After a final wash, the binding was measured on the MAGPIX instrument (Luminex Corp., Austin, TX). The median value obtained by the evaluation of ≥ 50 microspheres for each set plotted, and error bars plotted as the standard error of the mean (SEM), which is typically less than ± 10% the mean.

To evaluate sdAb specificity irrelevant targets were tested by direct binding assay. These targets were: NS1 from dengue virus serotypes 1, 2, 3, 4 (The Native Antigen Company), recombinant E3E2 from western equine encephalitis virus (IBT bioservices, Rockville, MD), recombinant E3E2 from eastern equine encephalitis virus (IBT bioservices), envelope protein from west nile virus (Prospec, Israel), and Lassa VLPs from Zalgen (Germantown, MD).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Mouse monoclonal antibody (mAb), CHK48 (anti-CHIKV E2; BEI Resources) was coated onto wells in a 96-well plate at the concentration of 2 µg/mL at 4 °C overnight. The next day, wells were washed with 3x PBST and blocked with PBST supplemented with 2% milk powder (2% MPBST) at room temperature (RT) for 1 h, followed by the addition of CHIKV VLP at various concentrations of 0, 1, 2.5, 5, 10, and 15 µg/mL at RT for 1 h. Bt-sdAb were then subsequently added onto wells at the concentration of 2 µg/mL at RT for 1 h after washing wells with PBST and PBS. Horseradish peroxidase (HRP) conjugated secondary antibody and Streptavidin were then added into wells at the concentration of 1 µg/mL at RT for 1 h. Peroxidase substrate, SureBlue TMB-1 component (KPL, Gaithersburg, MD) was added to each well and the reaction was stopped by the addition of 1 M HCl according to the manufacturer's protocol before measuring the absorbance at 450 nm.

2.8. Plaque reduction neutralization test (PRNT)

PRNT was performed by IBT Bioservices (Rockville, MD). Two sdAb, CC3 and CA6, were evaluated for neutralizing activity against CHIKV strain 181/25 in Vero cells. Twelve serial dilutions of each sdAb were prepared. For CC3 the starting concentration was 5 µg/mL and 3-fold dilutions were prepared while for CA6 the starting concentration was 10 µg/mL and 2-fold dilutions were examined. Each dilution was incubated with ~300 plaque forming units (PFU) of CHIKV in duplicate for one-hour inoculation. The sdAb-virus mix was then added to Vero cells seeded in 24-well culture plates for one-hour incubation, followed by adding 0.8% methylcellulose to each well and incubating for three days at 37 °C in a humidified 5% CO_2 atmosphere. On day three post incubation, the Vero cells were fixed and infected foci were counted by using crystal violet staining. 50% and 90% plaque reduction neutralization titer ($PRNT_{50}$ and $PRNT_{90}$) of each sdAb was calculated using XLfit dose response model.

2.9. Construction of sdAb-AP fusions

The alkaline phosphatase (AP) gene was amplified from C2-AP, a previously described sdAb-AP fusion clone, in a pECAN 45 based vector (Liu et al., 2013a). Primers were designed to incorporate flanking Not I and Xho I sites. A purified Not I – Xho I fragment containing AP was cloned into pET22b, C terminal to the sdAb sequence. The clones were all verified by Sanger sequencing.

2.10. Western blotting

Approximately 3 µg of CHIKV VLP and 1.5 µg of each rE1 and rE2 in 25 µL of 1x LDS sample buffer supplemented with 1x reducing agent was loaded onto pre-casted 10% BIS-TRIS NuPAGE gel in 1xMES-SDS running buffer, followed by applying electric current through the gel to separate the proteins by size. SeeBlue plus 2 prestained standard is loaded in separate lane as a size marker (Thermofisher Inc). Following the manufacturer's protocol, separated proteins on the gel were then transferred to a PVDF membrane in a cold room. The blot was then washed with 1xTBST (10 mM Tris pH 7.5, 150 mM NaCl and 0.01% Tween 20), and was subsequently blocked with 5% milk in 1xTBST (TBSTM) at RT for 1 h. Approximately 4 µg/mL of sdAb-AP were added

to the blot at RT for 1 h before washing with TBSTM for 15 min three times. The luminescent substrate was then applied to the blot and incubated for 5 min and the images were captured by using a GEL doc XR + system.

3. Results

3.1. Llama immunization and construction of phage display library

Total RNA was successfully isolated from the peripheral lymphocytes of CHIKV VLP immunized llamas. After amplifying the collection of VHH fragments using RT-PCR, the consortium of sdAb fragments were inserted into M13 phage display vector, pECAN21 and subsequently transformed into *E. coli* XL1 Blue to produce sdAb displayed on the phage tails. The CHIKV VLP immunized phage library has approximately 3.9×10^6 unique clones and ~80% of them have full length, based on the sequence analysis of randomly selected clones.

3.2. Biopanning, selection and sequence analysis of potential binders

Two rounds of biopanning were employed using rE1 and VLPs coated onto microtiter plates and resulted in 14 potential clones from phage ELISA, which were confirmed in a phage MagPlex assay. These clones were sequenced and the variation of CDR3 sequence were compared using the Multalin website (Fig. 1). Based on CDR3 homology, the selected clones were classified into four groups of sdAb (I–IV, Fig. 1). Group I has over half of the selected clones with an identical CDR3, while group II has the longest CDR3 with 19 residues.

One or two representatives from each group were then selected for soluble sdAb production and further evaluation (Fig. 2). The yields for the sdAb ranged from a low of 1.5 mg/L for CA5 to a high of 17 mg/L for CH6 with 90%–95% purity following the final gel filtration purification step.

3.3. Measurement of Tm for purified sdAb

To measure thermal stability, Tms were determined by fluorescent dye melt assay for each recombinant sdAb (Table 1). All Tms were above 50 °C, while CD11 in group II exhibits the highest Tm at 71 °C, more than 10 °C higher than others. The melting temperatures of two clones, CC3 and CA6, were confirmed by CD. Both sdAb melted at 59 °C, within a few degrees of the Tm measured by the fluorescent dye melt assay. The CD measurements show refolding for CC3 and CA6 after heat denaturation of 64% and 53% respectively. Several mutagenesis strategies have been demonstrated that serve to increase the Tm of sdAb while preserving their binding ability (Goldman et al., 2017), and could be applied to these sdAb if desired.

3.4. Analysis of binding affinity and specificity for rE1, rE2, and CHIKV VLPs

The binding kinetics for each sdAb to rE1 and CHIKV VLPs were assessed using the SPR based biosensor, ProteOn XPR36 (Table 2). Following the coating of GLC chips with either rE1, rE2, or VLP, purified sdAb were flowed through to measure the binding kinetics. With the exception of CH5, the sdAb bind to immobilized rE1, while only CC3 and CH5 in group I bind to VLP (Table 2 and Fig. S1), which suggests group I CDR3 sequence, RAFGPADY, has the binding specificity to VLP. CC3 possesses binding to both VLP and rE1 in the sub nM range, while CA6 in group IV has the best binding affinity for rE1 (sub nM range). None of the sdAb were observed to bind rE2 in the SPR experiments.

These proteins (rE1, rE2, and VLPs) were also covalently attached to MagPlex microspheres along with a variety of other proteins to further examine the specificity of the selected sdAb (Fig. S2 and Fig S3). These results mirrored the SPR results, with binding observed to rE1 and VLPs, but no binding to non-target proteins or the rE2. A sandwich format was used to demonstrate the sdAb's potential to detect virus; the sdAb were immobilized to MagPlex magnetic microspheres and used to detect CHIKV VLPs at a range of concentrations. Following the addition of VLPs, the biotinylated (Bt)-sdAb (Bt-CC3 and Bt-CA6) were then added separately as a tracer. Our data indicated that group I sdAb, CC3 and CH5, capture VLPs well, and combined with tracers Bt-CC3 and Bt-CA6 have VLP detection limits below 0.37 and 1.1 μg/mL, respectively (Fig. 3A and B). Since CC3 and CA6 exhibited the highest binding affinity to VLP and rE1 respectively, they were selected for further characterization by traditional ELISA and plaque reduction neutralization tests (PRNT).

3.5. Plaque reduction neutralization test (PRNT) for CHIKV

To conduct PRNT for CHIKV, the biosafety level 2 vaccine strain CHIKV 181/25 was used. To determine the concentration ranges for sdAb used for PRNT, we conducted an ELISA to measure the limit of VLP detection for CC3 and CA6. Both sdAb were used as VLP capture reagents and tracers in ELISA, while the conventional monoclonal antibody, CHK48, serves as a positive control for both capture and tracer (Fig. 4A). Consistent with MagPlex data, CC3 was able to better capture VLPs and generated a much higher signal than CA6 (Fig. 4B vs C) and Bt-CC3 was the best tracer. The limit of VLP detection for CC3/Bt-CC3 is below 1 μg/mL, while the detection limit for CC3/Bt-CA6 is at least 10-fold higher. Based on the ELISA measurements, an initial starting concentration of 5 μg/mL for CC3 and 10 μg/mL for CA6 were used along with additional dilutions for PRNT to obtain an inhibition curve for each sdAb (Fig. 5A and B). $PRNT_{50}$ for CC3 and CA6 are 8.4 ng/mL (0.6 nM) and 676.3 ng/mL (45.6 nM), respectively (Table 3), suggesting that CC3 is much more effective than CA6.

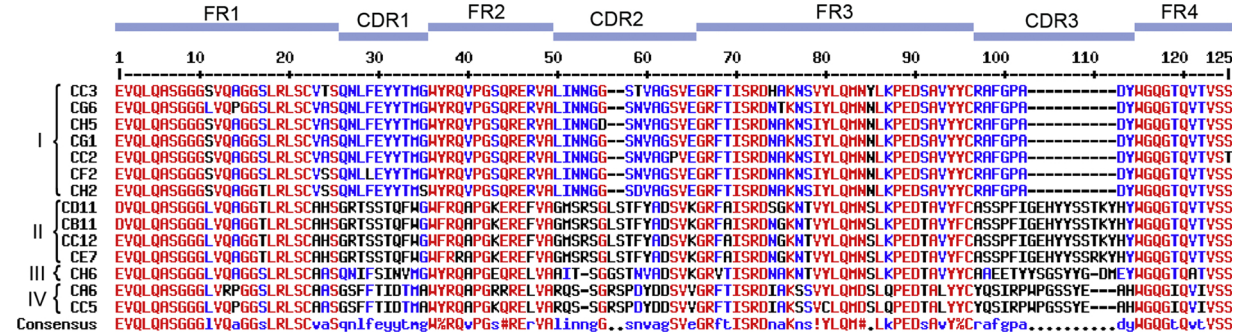

Fig. 1. Sequence analysis of 14 potential CHIKV VLP binding sdAb from two rounds of bio-panning. Based on CDR3 diversity, the clones were divided into four groups. Group I has 7 clones with identical CDR3, while group II has 4 clones most with an identical CDR3. Group III has only CH6 and group IV has 2 clones with an identical CDR3. Framework (FR) and CDR regions are indicated.

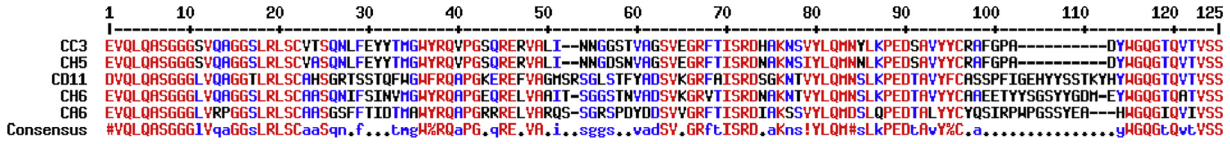

Fig. 2. Representative sdAb sequences evaluated for each group. CC3 and CH5 are group I. CD11, CH6 and CA6 belong to group II, III, IV, respectively.

Table 1
Measurement of Tms.

SdAb Clone	Tm (Dye melt, °C)
CC3	58
CH5	54
CD11	71
CH6	59
CA6	55

3.6. Characterization of sdAb-AP fusions

The traditional ELISA is a standard diagnostic assay used in the lab. To simplify the assay procedure, both CC3 and CA6- AP fusions were developed. The yields of both AP fusions were approximately ~3-6 mg/L. Purified AP fusions dimerized and could bind both rE1 and CHIKV VLP by SPR (Fig. S4). They were also used as tracers in ELISA and Western blotting to analyze the binding of CHIKV VLP and rE1. CC3-AP was able to detect the captured CHIKV VLP by CC3 and CA6 and the detection limit is comparable to Bt-CC3 (Fig. 6A). Both AP fusions showed binding to denatured E1 and E2 from VLPs, rE1 and rE2 in western blotting (Fig. 6B and C).

4. Discussion

Although there are currently several neutralizing CHIKV mAbs available, no sdAb for CHIKV neutralization have been reported. Our main objective in this study was to select sdAb that showed promise for future diagnostic, prophylactic, or therapeutic applications; ideally we hoped to isolate a good neutralizing sdAb. We have achieved this goal by selecting high affinity sdAb from a CHIKV VLP immunized sdAb phage display library. CHIKV VLP generate good immunity in both human and animal hosts (Ahola et al., 2015; Goo et al., 2016). In fact, a couple of promising VLP based vaccine candidates are currently in phase I/II trials. Human hosts immunized with CHIKV VLP of West African genotype were able to elicit a high titer neutralizing antibodies against multiple strains of CHIKV (Akahata et al., 2010). Likewise, llamas immunized with CHIKV VLP composed of structural protein E1, E2 and capsid protein also generated a good immune response with a good antibody titer, including production of heavy-chain-only antibodies against CHIKV VLPs. A robust immune response was vital in facilitating the construction a CHIKV VLP immunized sdAb phage display library for biopanning. We successfully selected a number of sdAb that bind to rE1 and/or CHIKV VLPs. Two sdAb, CC3 and CA6, that had differential binding behavior to CHIKV VLPs and rE1 glycoprotein were evaluated in a PRNT assay and found to have neutralizing ability against the attenuated CHIKV vaccine strain 181/25 comprising two critical point mutations in E2 (Gorchakov et al., 2012). The subtle differences in E1 and E2 between 181/25 and wild type may not significantly change the binding of CC3 and CA6 to wildtype CHIKV and other strains.

Most of the described neutralizing antibodies target the CHIKV E2 glycoprotein, thereby blocking viral entry and release, resulting in virus neutralization (Jin et al., 2015). Here, our stringent panning selected for sdAb binding either to CHIKV VLPs or to E1, which is critical for viral fusion to cell membrane. All of the selected binders show

Table 2
Analysis of binding affinity and specificity by SPR.

SdAb Clone	rE1 ka(1/Ms)	rE1 kd(1/s)	rE1 K_D (nM)	VLP ka(1/Ms)	VLP kd(1/s)	VLP K_D (M)
CC3	6.6×10^5	2.2×10^{-4}	3.4×10^{-10}	5.2×10^5	3.3×10^{-4}	6.5×10^{-10}
CH5	–	–	–	3.4×10^5	4.1×10^{-3}	1.2×10^{-8}
CD11	1.5×10^6	1.0×10^{-3}	6.6×10^{-10}	–	–	–
CH6	3.7×10^5	9.1×10^{-5}	2.5×10^{-10}	–	–	–
CA6	8.5×10^5	1.1×10^{-4}	1.3×10^{-10}	–	–	–

- Indicates either no binding or data insufficient to calculate a reliable value.

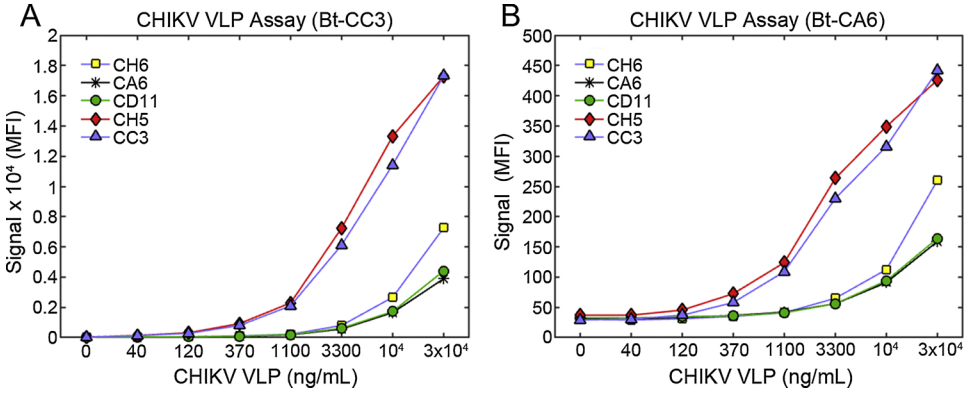

Fig. 3. MagPlex sandwich assay for CHIKV VLPs using sdAb as captures and tracers. Five sdAb were immobilized onto the surface of MagPlex microspheres and serve to capture various VLP concentrations. Bt-CC3 was used as a tracer sdAb in A while Bt-CA6 was used in B.

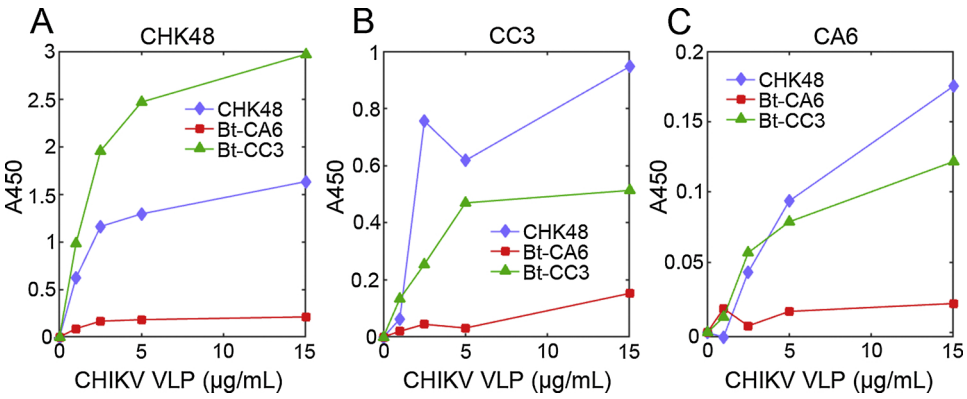

Fig. 4. ELISA assay for CHIKV VLPs using sdAb and anti-E2 mAb, CHK48, as capture reagents and tracers. CHK48, CC3 and CA6 were used as captures in A, B and C, respectively. CHK48, Bt-CA6 and Bt-CC3 were used as tracers in all the reactions. Capture identities are labeled above each plot and the individual traces within each plot represent the three different tracers.

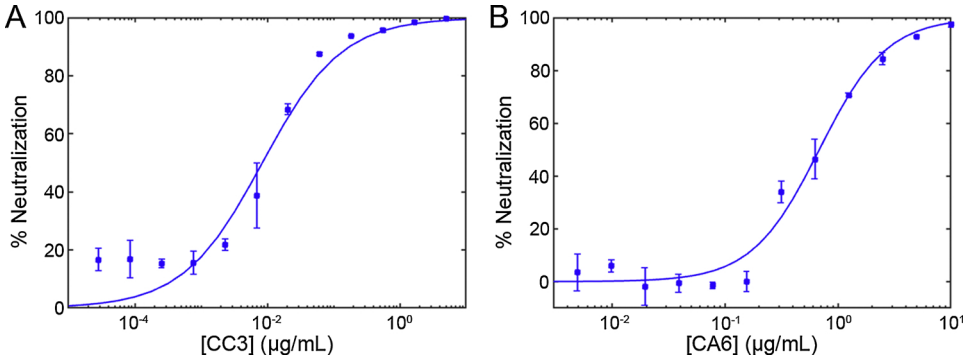

Fig. 5. Dose dependent neutralization of CHIKV using plaque reduction neutralization test (PRNT). Vero cells were used to measure the plaque numbers after the incubation of CHIKV and two sdAb, CC3 and CA6. The reduction of plaque numbers is due to the neutralization of CHIKV by CC3 (A) and CA6 (B). The concentration for 50% ($PRNT_{50}$) and 90% ($PRNT_{90}$) reduction of plaques were also determined.

Table 3
Measurement of sdAb concentration for $PRNT_{50}$ and $PRNT_{90}$.

SdAb	$PRNT_{50}$ (nM)	$PRNT_{90}$ (nM)
CC3	**0.6**	**12.5**
CA6	**45.6**	**214.9**

MW for CC3 is 13794 Da and CA6, 14825 Da.

specificity to rE1, albeit CH5 bound weakly. However, it was CH5 along with CC3 which bound CHIKV VLP most robustly. No cross reactivity was observed towards recombinant envelope proteins from several other alphaviruses, or to recombinant proteins or VLPs from unrelated virus (Fig. S3 and S4). Group IV binder, CA6, bound to VLPs poorly, but the AP fusions improved its binding likely due to the avidity advantage provided by dimerization. CA6 bound to rE1 strongly in contrast to CC3, providing the main reason to select it along with CC3 in the subsequent PRNT assays. Not surprising, CC3 exhibited much better $PRNT_{50}$ than CA6, as the assay is often linearly related to the binding affinity (Wu et al., 2005).

While our MagPlex and SPR assays failed to show any binding of our sdAb to rE2, Western blotting contradicted those results. Although it is difficult to differentiate VLP derived E1 and E2 on the gel and blot, we were able to observe by western blotting that both CC3 and CA6 appeared to bind to both denatured rE1 and rE2. The commercial rE1 runs at ~60 kDa position (private communication with the Native Antigen Company), higher than the E1 assembled into VLPs, possibly due to extra glycosylation during VLP production in cells (Fig. 6B and C). Due to the difference in both the extent of glycosylation and the 3D structure between rE1 and E1 in VLP, it is not surprising that binders selected against rE1 may not bind or bind more poorly to E1 displayed as part of a VLP. On a gel, the rE2 fusion with human Fc runs consistent with the manufacturer's information, higher than 62 kDa position. While inconclusive at this point, if our neutralizing sdAb also bind to rE2, then they may also inhibit the entry and release of viruses (Warter et al., 2011).

AP fusion does not alter sdAb binding affinity and specificity and indeed increases sdAb avidity resulting from its spontaneous dimerization in solution (Swain et al., 2011). Consistent with the avidity increase, the AP fusions greatly improve the SPR signal observed on binding to rE1

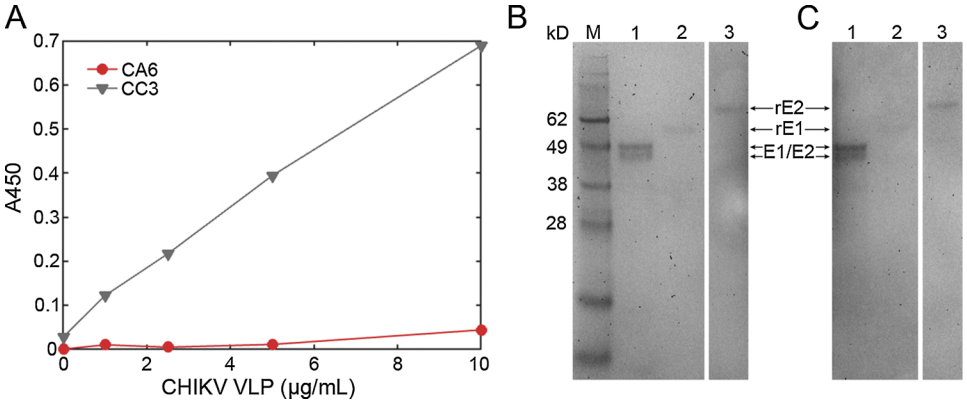

Fig. 6. Characterization of CC3/CA6-AP fusions. A. CC3-AP was used as tracer agent for captured VLPs by CA6 and CC3 in ELISA. CC3-AP (B) and CA6-AP (C) were used as primary antibodies to blot against CHIKV VLP (lane 1), rE1 in lane 2, and rE2 in lane 3 respectively. M represents the size markers in kDa. The arrows indicate the proteins rE1, rE2 as well as E1 and E2 in the VLP.

and VLP versus sdAb alone. Our CC3-AP fusion also exhibits a better detection limit than CA6-AP while pairing with CC3 as a VLP capture. Each VLP contains multiple copies of E1 and E2, so it is expected the same sdAb can be paired together as capture and tracer.

5. Conclusions

Llamas were successfully immunized with CHIKV VLPs, which facilitated the development of a good quality phage display immune library of sdAb for biopanning. Several selected sdAb were characterized and exhibited specificity and affinity to rE1 and CHIKV VLPs. Among them, CC3 possessed the best affinity to VLPs and effectively neutralized live CHIKV. In contrast, CA6 possessed a relatively low affinity to VLPs, but was still able to neutralize live CHIKV at higher concentrations, suggesting that CA6 also binds to E1/E2 in live viruses. The neutralization mechanism of CHIKV for these two sdAb still needs confirmation, however the isolated sdAb, especially CC3, show potential for future diagnostic and therapeutic applications.

Funding sources

This work was supported by the Joint Science and Technology Office and DTRA RD Chemical and Biological Technologies Department, project number HDTRA1518114 (CB3636).

Acknowledgements

The following reagent was obtained through BEI Resources, NAID, NIH: Monoclonal Anti-Chikungunya Virus E2 Envelope Glycoprotein, Clone CHK-48 (produced *in vitro*), NR-44002.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.11.016.

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