Published in final edited form as:

*Virology*. 2022 January 02; 565: 13–21. doi:10.1016/j.virol.2021.09.007.

# Exposing cryptic epitopes on the Venezuelan equine encephalitis virus E1 glycoprotein prior to treatment with alphavirus cross-reactive monoclonal antibody allows blockage of replication early in infection

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## Abstract

Eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV) can cause fatal encephalitis in humans and equids. Some MAbs to the E1 glycoprotein are known to be cross-reactive, weakly neutralizing *in vitro* but can protect from disease in animal models. We investigated the mechanism of neutralization of VEEV infection by the broadly cross-reactive E1-specific MAb 1A4B-6. 1A4B-6 protected 3-week-old Swiss Webster mice prophylactically from lethal VEEV challenge. Likewise, 1A4B-6 inhibited virus growth *in vitro* at a pre-attachment step after virions were incubated at 37 °C and inhibited virus-mediated cell fusion. Amino acid residue N100 in the fusion loop of E1 protein was identified as critical for binding. The potential to elicit broadly cross-reactive MAbs with limited virus neutralizing activity *in vitro* but that can inhibit virus entry and protect animals from infection merits further exploration for vaccine and therapeutic developmental research.

### Keywords

Alphavirus; Monoclonal antibody; Immunotherapy; Venezuelan equine encephalitis



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CRediT authorship contribution statement

**Amanda E. Calvert:** Conceptualization, Methodology, Formal analysis, Investigation, writing. **Susan L. Bennett:** Investigation, Validation, writing. **Ann R. Hunt:** Investigation, Validation, writing. **Rachel H. Fong:** Methodology, Validation, Investigation, Resources, Visualization. **Benjamin J. Doranz:** Methodology, Validation, Resources, writing, Supervision, Funding acquisition. **John T. Roehrig:** Conceptualization, Methodology, Formal analysis, writing, Supervision. **Carol D. Blair:** Conceptualization, Methodology, Formal analysis, writing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: B.J.D. and R.H.F. are shareholders of Integral Molecular.

## 1. Introduction

Encephalitic viruses in the *Alphavirus* genus of the family *Togaviridae* include eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV). These viruses are maintained in enzootic cycles between mosquitoes and rodents (VEEV) or birds (EEEV and WEEV), with equids and humans as incidental hosts (Calisher, 1994). VEEV and EEEV epizootic outbreaks occur across the Americas with resulting mortality rates of 30% or higher in in human spillover neurological infections and permanent neurologic sequelae in more than 50% of survivors presenting with neurologic disease (Lindsey et al., 2018, 2020). While the incidence of EEEV in the US has increased recently, with a multistate outbreak reported in 2019 (Lindsey et al., 2020), the incidence of WEEV has declined (Bergren et al., 2020). Although outbreaks due to epizootic subtypes of VEEV occurring mostly in South and Central America are rare, enzootic VEEV transmission occurs periodically. Recent seroprevalence studies in equids suggest infection rates may be higher than previously recognized (Carrera et al., 2020). Currently, no FDA approved vaccines or treatments are available for humans; however, prophylaxis and treatment with virus-neutralizing monoclonal antibodies (MAbs) are effective in reducing viral infection and disease in small animal models (Hunt et al., 2006, 2011; Hunt and Roehrig, 1995).

Alphaviruses have a positive-sense, single-stranded 11.5 kb RNA genome enclosed within an icosahedral nucleocapsid surrounded by a lipid bilayer obtained from the infected cell's plasma membrane during virus budding (Holmes et al., 2020; von Bonsdorff and Harrison, 1978). Traversing the lipid bilayer are two glycoproteins, E1 and E2, that are assembled as heterodimers and form 80 trimeric spikes on the mature virion surface (Jose et al., 2009). The E1 protein lies underneath E2 on the virion, where it serves as a scaffold for E2, remaining largely hidden on the mature virion and thus inaccessible to antiviral antibodies (Holmes et al., 2020; Jose et al., 2009). While E2 is involved in receptor binding and cell entry, E1 mediates low pH-triggered fusion of the viral envelope and endosomal membrane during virus entry. After viral replication and processing through the secretory pathway of the infected cell, E1-E2 heterodimers form lattices in a hexagonal array within cytopathic vacuoles (CPV-II) and are transported to the plasma membrane, where association with the nucleocapsid core occurs (Soonsawad et al., 2010). Alphaviruses may exit the cell through budding from the plasma membrane or by the formation of intercellular extensions that make closed-ended membrane bridges facilitating cell-to-cell transmission (Martinez and Kielian, 2016).

Antibodies can inhibit alphaviral replication at several steps in the infection cycle, including causing aggregation of extracellular virus particles, blocking virus attachment to cells, preventing structural rearrangements in the virion that are necessary for membrane fusion, or by inhibiting viral egress from the cell. Anti-E2 neutralizing antibodies have been shown to be virus-specific and highly neutralizing *in vitro*, with protective activity *in vivo* (Hunt et al., 2006; Kim et al., 2019; Mathews and Roehrig, 1982; Pereboev et al., 1996; Powell et al., 2020; Roehrig et al., 1988). Fox et al. (2015) also showed that broadly cross-reactive MAbs recognizing an epitope on the B domain of the alphavirus E2 protein elicited broad alphavirus protection *in vivo*. Even though the E1 protein elicits mostly non-neutralizing

cross-reactive MAbs, these MAbs may still confer protection *in vivo* (Boere et al., 1983; Burke et al., 2018; Kim et al., 2021; Rico et al., 2016; Schmaljohn et al., 1982; Williamson et al., 2021). This protection has been associated with MAb interactions with "cryptic" E1 epitopes not accessible on the mature virion surface. We sought to determine if the weakly-neutralizing, cross-reactive E1-specific MAb 1A4B-6 could protect *in vivo* and investigated the mechanism of protection with *in vitro* studies examining its ability to inhibit infection when applied pre- and post-attachment, pre-fusion, and before viral egress from infected cells. In this report we define the major mechanism of *in vitro* neutralization by MAb 1A4B-6 to include MAb blockage of the virus at a pre-attachment step under cryptic epitope-exposing conditions and cell membrane fusion.

## 2. Methods

### Cells, monoclonal antibodies (MAbs) and virus.

Vero (African green monkey kidney), C6/36 (*Aedes albopictus* larval) and SW13 (human adenocarcinoma) cell cultures were grown and maintained in DMEM supplemented with penicillin-streptomycin, L-glutamine, and fetal bovine serum (FBS). MAbs used in this study have been described elsewhere (Table 1). Stocks of VEEV strains TC83 (vaccine strain) and Trinidad Donkey were obtained from the Reference Collection at Division of Vector-Borne Diseases/CDC and grown and titrated in Vero cells.

### Animal Study.

The animal study was performed as previously described and pre-dates IACUC establishment (Hunt and Roehrig, 1985; Mathews and Roehrig, 1982). Essentially, 3-week-old Swiss Webster mice were inoculated intravenously (IV) via tail vein with known concentrations of purified MAbs or PBS. Twenty-four hours later, mice were challenged intraperitoneally (IP) with 100 $LD_{50}$ of VEEV strain Trinidad Donkey diluted in MEM (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Reheis Chemical Co. Phoenix, AZ). Mice were observed for 2 weeks and euthanized immediately upon signs of illness including hunched posture, weight loss and neurological signs.

### Plaque reduction neutralization test (PRNT) for pre-/post-attachment assay.

Traditional PRNT to assess MAb pre-/post-attachment inhibition of VEEV infectivity was performed using VEEV TC-83 in 6-well Vero cell plates, as previously described (Hunt et al., 2006). Briefly, virus was diluted in BA-1 (Hanks M – 199 salts, 0.05 M Tris pH 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/ml penicillin, 100 mg/L streptomycin, 1 mg/L fungizone) and 60 μl of 2X input virus was added to an equal volume of 2-fold serial dilutions of MAb. After incubation at 4 °C overnight, 100 μl/well of virus-MAb mixture was added to confluent Vero cells. Back titration of the input virus was conducted by 2-fold serial dilutions (6 replicates each) of the 2X virus preparation in each assay. The virus or virus-MAb mixtures were adsorbed for 1 h at 37 °C and overlaid with medium containing 1% SeaKem LE agarose (Lonza, Rockland, ME, USA) in nutrient medium (0.165% lactalbumin hydrolysate, 0.033% yeast extract, Earle's balanced salt solution, and 2% FBS) before incubation at 37 °C with 5% $CO_2$. After 24 h, cells were stained with a secondary agarose overlay medium containing neutral red. Virus plaques

were counted on day 2 post-infection (PI) and percent neutralization was calculated based on input virus titer. Virus neutralization curves were generated by a 4-parameter non-linear regression dose response used to calculate the half-maximal inhibitory concentration ($IC_{50}$) values of each MAb using GraphPad Prism V6.

### Pre- and post-attachment neutralization assays.

Pre-attachment neutralization assays were performed by incubating virus-MAb mixtures at 4° or 37 °C for 1 h before adding to pre-chilled confluent Vero cell cultures and incubating on ice for 1 h. Cells were washed twice with chilled DMEM and equilibrated to room temperature before being overlaid with agarose medium and proceeding with the PRNT protocol as described above. Percent neutralization was calculated based on the untreated virus control. A two-way ANOVA with Tukey's multiple comparisons test was used to compare virus titers with and without MAb treatment at different incubation temperatures. Post-attachment neutralization assays were performed by adsorbing virus to Vero cells at 4 °C for 1 h before washing cells twice with chilled DMEM. After unbound virus was removed from the cell layer, dilutions of MAb were added and incubated with the cells for an additional hour at 4 °C. Vero cell plates were equilibrated to room temperature before being overlaid with agarose medium and proceeding as described above. A one-way ANOVA with Tukey's multiple comparisons test was used to compare percent virus neutralization with and without MAb treatment.

### Enzyme-linked immunosorbent assay (ELISA).

The ELISA was performed in 96-well plates (Nunc Maxisorp plates, ThermoFisher Scientific, Waltham, MA). Plates were coated with anti-VEEV rabbit polyclonal antibody diluted 1:500 in carbonate/bicarbonate buffer (50 mM sodium carbonate, 50 mM sodium bicarbonate, pH 9.6) and incubated overnight at 4 °C. Plates were washed five times with PBS/0.1% Tween wash buffer with an automatic plate washer. Non-specific binding sites were blocked with 3% rabbit serum in PBS (100 μl/well) for 30 min at 37 °C. Purified VEEV TC83 was added to the wells at a concentration of 1.2 μg/ml (50 μl/well) and incubated on the plates for 2 h at either 37 °C or 4 °C, after which the plates were washed as previously described. Purified antibody starting at 10 μg/ml was added in 4-fold dilutions (50 μl/well) and incubated for 1 h at either 37 °C or 4 °C. Plates were washed five times before the addition of rabbit anti-mouse antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) (50 μl/well), diluted 1:5000 in 3% rabbit serum in PBS. After an incubation period of 1 h at 37 °C, plates were washed again 10 times. Enhanced K-blue TMB substrate (Neogen, Lansing, MI, USA) was added to each well of the plate (100 μl/well) and incubated in the dark at room temperature for 10 min. The reaction was stopped with the addition of 2 N $H_2SO_4$ (50 μl/well), and the plates were read at 450 nm. Binding curves were generated by a 4-parameter non-linear regression dose response used to calculate the half-maximal effective concentration ($EC_{50}$) values of each MAb using GraphPad Prism V6.

### Quantitative flow cytometry.

To determine the number of specific antibody (Ab) binding sites on the surface of infected cells, SW13 cells were infected with VEEV TC-83 at a MOI of 0.1 and incubated for 24

h. Cells were harvested from cell culture flasks with trypsin and washed in IF wash buffer (5 mM EDTA, 0.1% sodium azide, 1.0% BSA). Cells were resuspended in ice-cold 1% paraformaldehyde and incubated on ice for 10 min before washing 3 times in IF wash buffer. A range of concentrations of purified MAb were incubated with cells for 1 h at 4 °C with gentle agitation. Cells were again washed 3 times in IF wash buffer and incubated with FITC- conjugated goat anti-mouse Ab (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in IF wash buffer and incubated as above. In parallel, Qifikit calibration beads coated with defined quantities of an IgG murine MAb (Agilent Technologies, Santa Clara, CA) were also treated with FITC- conjugated goat anti-mouse Ab. After the final incubation, cells were washed 3 times and resuspended in 200 μl of IF wash buffer and analyzed by flow cytometry using a BD FACSCalibur. A standard curve was generated in GraphPad Prism V6 from which to determine the number of Ab binding sites, or antibody binding capacity units (ABCs), on the surface of the infected cell by plotting the mean fluorescence intensity (MFI) for each bead population against the ABCs on the calibration beads. Specific ABCs (sABCs) were calculated by subtracting the interpolated ABCs of the MAb sample of interest from the interpolated ABCs of the corresponding isotype control of the same concentration as the test MAb on infected cells. A two-way ANOVA with Tukey's multiple comparisons test was used to compare differences in sABCs of MAbs tested.

### Virus egress inhibition assays.

Virus egress neutralization assays were performed by adsorbing VEEV TC-83 (MOI of 0.1 or 1.0) to Vero cells in 6-well plates in the presence of 25 mM $NH_4Cl$ and 100 μg of MAb 1A4B-6. One hour after virus infection, cells were washed 5 times in DMEM, and MAb was added to the cells. Samples from the cell culture medium were taken immediately after washing followed by 6- and 12-h post-infection (PI). Virus titers were determined by plaque assay and normalized virus titers were determined based on virus titers at 1 h PI. A two-way ANOVA with Tukey's multiple comparisons test was used to compare virus titers with and without MAb treatment, in the presence or absence of $NH_4Cl$.

### Fusion from within assay (FFWI).

Virus-mediated cell membrane FFWI was performed as previously described (Guirakhoo et al., 1993). Essentially, C6/36 cells were seeded onto glass chamber slides (ThermoFisher, Waltham, MA) before infection with VEEV TC83 at an MOI of 1.0 and incubation at 28 °C/5%$CO_2$ while maintaining the pH at 7.5. Twenty-four hours after infection, cells were incubated with various concentrations of MAb diluted in BA-1 for 2 h at 28 °C/5%$CO_2$. After MAb treatment, cells were exposed to fusion medium (DMEM buffered at pH < 5.0 with 2-N-morpholinoethanesulfonic acid) for 1 h, after which fusion medium was replaced with DMEM at neutral pH. Cells were incubated another 24 h at 28 °C/5%$CO_2$ before being fixed in absolute methanol and stained using the Wright-Giemsa stain kit (Abcam, Cambridge, MA). An isotype MAb (100 μg/ml), virus-infected non-treated cells at pH 7.5, and uninfected MAb-treated cells at pH 5.0 were included as controls. The number of nuclei and the number of cells in five microscopic fields (magnification 100-fold) were counted and the fusion index [1 - (number of cells/number of nuclei)] was calculated. A one-way ANOVA with Tukey's multiple comparisons test was used to compare differences in percent fusion.

### Epitope Mapping.

Construction of a shotgun mutagenesis library with alanine mutations in chikungunya virus (CHIKV, *Alphavirus*) (s27 strain; UnitProt accession #Q8JUX5) envelope (Env) glycoprotein constructs was carried out at Integral Molecular. Library construction and flow cytometry analysis for MAb epitope identification has been described previously (Fong et al., 2014). For this study, recombinant E1/E2 expressed in HEK-293 cells from library clones containing individual mutations in the E1 fusion loop residues G83 to N100 were arrayed in quadruplicate in 384-well plates, and then assayed by flow cytometry for binding to MAbs 1A4B-6 and 3A5B-1 at 0.5 μg/ml (Table 3). Wild type (WT) CHIKV E1/E2 and cells transfected with empty vector alone were used as positive and negative controls, respectively. Control anti-CHIKV human MAbs IM-CKV067 and IM-CKV098 were screened to confirm protein expression and conformation.

## 3. Results

### MAb 1A4B-6 prophylactically protects mice from VEEV challenge.

Encephalitic alphaviruses, VEEV, EEEV and WEEV are considered potential biological weapons because of the severe disease they cause and the ability to infect via aerosol (Hart and Ketai, 2015; Sidwell and Smee, 2003). While relatively rare, natural human EEEV and VEEV infections still occur with high case fatality rates (Aguilar et al., 2011; Lindsey et al., 2018). There are no approved vaccines or virus-specific treatment options available (Stromberg et al., 2020). Most passive antibody approaches developed for prophylactic and therapeutic treatment to alphaviruses target highly neutralizing virus type-specific epitopes (Burke et al., 2019; Hunt et al., 1991, 2011; Hunt and Roehrig, 1995; Phillpotts, 2006). Cross-reactive antibodies with broad-protective capacity present a more appealing technology for use as an early therapy before specific viral diagnosis is made. Since MAb 1A4B-6 is broadly cross-reactive, targeting a conserved epitope on the E1 protein of alphaviruses, we wanted to determine its prophylactic potential for protection from VEEV-challenge in a mouse model. Weanling mice were inoculated IV with three concentrations of MAb 1A4B-6 (100 μg (n = 15), 40 μg (n = 10) and 20 μg (n = 5))or PBS (n = 25). Additionally, 100 μg of EEEV E1-specific MAbs 1B5C-3 and 1B1C-4, of the same isotype as 1A4B-6, were included. Results summarize 3 separate experiments. In two independent experiments, all mice prophylactically treated with 100 μg of MAb 1A4B-6 24 h before lethal virus challenge with VEEV Trinidad Donkey survived. In contrast, only 20% and 40% of mice were protected when prophylactically treated with 40 and 20 μg, respectively (Table 2). No animals survived challenge when prophylactically treated with either 100 μg of the EEEV E1-specific MAbs 1B5C-3 or 1B1C-4. These results demonstrate the potential prophylactic protection afforded by MAb 1A4B-6 in a dose-dependent manner.

### MAb 1A4B-6 Inhibits VEEV entry at a pre-attachment step under cryptic epitope-exposing conditions.

To assess the i*n vitro* virus neutralization capacity of MAb 1A4B-6 for VEEV PRNTs were performed with 1A4B-6 and compared to VEEV E2 glycoprotein-specific MAb 3B4C-4 (Table 1). An IgG2b isotype control antibody was included as a control and tested at high concentrations in the PRNT. The neutralization potency of 1A4B-6 was lower, with an $IC_{50}$

of 16.42 μg/ml (95% CI: 10.25 to 26.32) compared to 3B4C-4 with an $IC_{50}$ of 3.73 ng/ml (95% CI: 3.4 to 4.1) (Fig. 1A). No virus neutralization in Vero cells was observed in cells treated with the IgG2b isotype control antibody at concentrations ranging from 100 to 20 μg/ml. These results are not surprising, given the fact that the VEEV E2-specific MAb 3B4C-4 has been shown to be highly neutralizing *in vitro* with a $PRNT_{70}$ endpoint titer of 25 ng/ml, as well as being highly protective against VEEV *in vivo* (Hunt et al., 2006; Mathews and Roehrig, 1982; Roehrig et al., 1982).

To understand the mechanism of VEEV infection inhibition by 1A4B-6, we first examined the effect of temperature on the ability of MAb to block VEEV cell entry at a pre-attachment step. VEEV was incubated with 1A4B-6 at high concentrations (100 or 10 μg/ml) for 1 h at neutral pH at either 4 °C or 37 °C before virus-MAb complexes were adsorbed to cells at 4 °C for 1 h. When 100 μg/ml of MAb 1A4B-6 was incubated with virus prior to viral adsorption, percent neutralization was significantly higher at 37 °C (78.1%) than at 4 °C (48.0%) (Fig. 1B). Neutralization potency was dose-dependent since the higher concentration of MAb resulted in 78.1% neutralization compared to the lower concentration of MAb with 39% neutralization. In contrast, inhibition of virus entry pre-attachment by MAb 3B4C-4 was not affected by temperature (Fig. 1B). Similarly, when binding capacity was determined in an ELISA in which MAbs 1A4B-6 and 3B4C-4 detected captured purified VEEV TC83, the $EC_{50}$ for 1A4B-6 was significantly lower when incubated at 37 °C, 0.2161 μg/ml (95%CI: 0.188 to 0.25) (Fig D), compared to the $EC_{50}$ when incubated at 4 °C, 5.825 μg/ml (95%CI: 0.016 to ≥10) (Fig. 1E). While the level of overall reactivity in the ELISA incubated at 4 °C was lower (i.e.: lower $OD_{450nm}$ readings), the difference in temperature did not produce a significant difference in endpoint titer for MAb 3B4C-4 with $EC_{50}$ values calculated at 0.02 μg/ml (95%CI: 0.017 to 0.035) and 0.02 μg/ml (95%CI: 0.019 to 0.029) at 4 °C and 37 °C, respectively (Fig. 1D and E). These results suggest that the epitope recognized by 1A4B-6 is not as readily accessible on the virion at 4 °C and the MAb is better able to bind the virion when the structure of the particle is more dynamically active at 37 °C, thus resulting in higher neutralization potency.

To determine whether MAb 1A4B-6 was able to inhibit virus entry into the cell at a post-attachment step, virus was adsorbed to cells at 4 °C for 1 h, after which 1A4B-6 was added to cell medium and cultures were incubated for an additional hour at 4 °C. Compared to the untreated cells, 56.5% virus neutralization occurred when 100 μg/ml of 1A4B-6 was added to cell medium after virus was adsorbed, and only 18.4% virus neutralization was observed when cells were treated with 10 μg/ml of 1A4B-6 (Fig. 1C). As seen with the pre-attachment experiment, treatment of cells after viral adsorption with MAb 3B4C-4 ablated virus infection. Since the lower concentration of 1A4B-6 poorly inhibited virus infection, the major mechanism of inhibition of virus infection by this MAb probably does not occur post-attachment.

### Anti-E1 protein MAbs recognize epitopes expressed on the surface of VEEV-infected cells.

Before proceeding to experiments examining virus egress from cells, we quantified the number of E1 epitopes accessible for binding by 1A4B-6 and other MAbs recognizing similar epitopes on the surface of infected cells. Human SW13 cells were infected

with VEEV and tested 24 h post-infection by fixing with paraformaldehyde to maintain membrane integrity, staining with MAb 1A4B-6 followed by staining with FITC-conjugated goat anti-mouse Ab, and analyzing by flow cytometry. The quantity of surface-expressed viral epitopes, or the specific antibody-binding capacity units (sABCs), per infected cell recognized by each MAb was extrapolated from a standard curve using calibration beads coated with known concentrations of murine IgGs and stained with FITC-conjugated goat anti-mouse IgG Ab. Antibody isotype controls (IgG2a, IgG2b and IgG1) were also used to stain infected cells. ABCs of isotype controls at varying concentrations were determined and subtracted from MAbs' ABCs to calculate sABCs for each MAb at different concentrations. MAbs 1A4B-6, 2A2C-3, 3A5B-1 and 3A1C-12 were previously shown to recognize a similar epitope on E1 protein, while 3B2A-9 recognized a unique epitope on VEEV E1 protein (Hunt and Roehrig, 1985; Roehrig et al., 1982, 1990). At a 100 μg/ml MAb concentration, the infected cells were nearly saturated with antibodies bound to their surface, with all MAbs tested (sABCs: 1A4B-6 (222,183), 2A2C-3 (202,469), 3A5B-1 (121,252), 3A1C-12 (244,175), 3B2A-9 (365,529)). At lower concentrations, significant differences in sABCs between 3B2A-9 and the other MAbs were observed (Fig. 2A). While no significant differences in sABCs of the E1 MAbs recognizing a similar epitope was observed, all MAbs had significantly lower (4–15-fold) sABCs compared to MAb 3B2A-9 (Fig. 2A). From these results, we concluded that at 37 °C and at high concentrations (100 and 10 μg/ml), MAb 1A4B-6 recognized and occupied a similar number of epitopes on the surface of the infected cell as highly neutralizing anti-VEEV E1 MAb 3B2A-9. We hypothesized that the number of epitopes available for MAb binding may be sufficient for treatment to affect the virus's ability for fusion-from-within (FFWI) and/or egress from the cell.

### Effect of MAb 1A4B-6 on infected cell plasma membrane fusion and virus egress from the infected cell.

After establishing that the E1 epitope recognized by 1A4B-6 was available for binding on the surface of infected cells, we next determined if the MAb could inhibit virus protein-mediated FFWI using C6/36 cells. One day after infection with VEEV TC83 at an MOI 1.0, cells were treated with varying concentrations of 1A4B-6 for 1 h before fusion medium (DMEM pH < 5.0) was added to the cells. After a 1-h incubation period, the fusion medium was replaced with cell culture medium (pH 7.5) and cells were incubated for an additional 24 h before assessing syncytia formation. A baseline for syncytia formation occurring naturally in uninfected cells treated at pH < 5.0) and infected cells in normal cell culture medium (pH 7.5) was determined to be 30.04 and 30.25%, respectively. Compared to the isotype control which displayed 95% fusion, 1A4B-6 was able to significantly inhibit cell membrane fusion. Treatment with 100 and 10 μg/ml of MAb resulted in 45% and 46% fusion (Fig. 2B). Treatment with 1 μg/ml of MAb resulted in 59% fusion, which was not significantly different from the isotype control.

Last, we looked at the ability of 1A4B-6 to inhibit virus particle assembly or release from infected cells. Beginning at 1 h after VEEV infection at an MOI of 0.1, 100 μg/ml of 1A4B-6 was added to the medium of infected Vero cells in the presence of $NH_4Cl$, a basic reagent used to raise the pH in the endocytic pathway, inhibiting virus re-entry and re-infection during the experiment to ensure only one round of infection took place. Medium

was sampled at 1, 6 and 12 h after MAb incubation on cells, and infectious virus titer in the medium was determined by plaque assay. At 6 h PI, there was nearly a 6-fold decrease in virus titer in medium of cells treated with 1A4B-6 (0.96 $\log_{10}$ PFU/ml) compared to untreated infected cells (5.65 $\log_{10}$ PFU/ml); however, no significant differences in virus titer were noted in cells at 12 h PI (Fig. 2C).

## MAbs 1A4B-6 and 3A5B-1 recognize epitopes on the fusion loop of alphavirus E1 glycoproteins.

To test alphavirus cross-reactivity of MAbs 1A4B-6 and 3A5B-1, which both recognize a similar epitope on encephalitic alphavirus E1 glycoproteins, a panel of alphavirus envelope proteins including chikungunya virus (CHIKV), Ross River virus (RRV), Sindbis virus (SINV) and Semliki Forest virus (SFV) were expressed on the surface of HEK-293T cells, which had been fixed in paraformaldehyde or left unfixed. The cells were stained and then analyzed by flow cytometry. MAbs 1A4B-6 and 3A5B-1 cross-reacted with all 4 antigens when tested on fixed cells (Fig. 3A). However, MAbs did not bind to E1/E2 antigens left unfixed on the cells (Fig. 3B). These data suggest that 1A4B-6 and 3A5B-1 bind epitopes that are broadly conserved among alphavirus E1 proteins, but that the epitope these MAbs recognize is hidden in some circumstances. Binding only to fixed cells is consistent with the binding of previously characterized anti-CHIKV MAbs recognizing the CHIKV E1 protein fusion loop (Fong et al., 2014).

Because we had shown that MAbs 1A4B-6 and 3A5B-1 were cross-reactive with a wide range of alphavirus E1 glycoproteins, including that of CHIKV, and because the fusion loops of alphavirus E1 glycoproteins are highly conserved, we proceeded to determine the critical amino acid residues recognized by our MAbs. Integral Molecular used alanine scanning mutagenesis on an expression construct of the CHIKV envelope proteins. An individual expression clone was created for each point mutant. For this study, HEK-293 cells transformed with clones containing individual mutations across the E1 fusion loop (residues G83 to N100) were arrayed in quadruplicate in 384-well plates, and then assayed for binding to MAbs 1A4B-6 and 3A5B-1 to identify primary critical residues for binding. Control anti-CHIKV human MAbs IMCKV067 and IM-CKV098 were also screened to confirm protein expression and conformation. Mean binding reactivities expressed as the percent binding to wild type (WT) CHIKV E1 were calculated. Primary critical residues for binding were identified as those that were negative (<25% WT) for 1A4B-6 and 3A5B-1, but positive (>50% WT) for the control antibodies (Table 3). Mutations C94A, C96A and N100A had decreased binding for both MAbs 1A4B-6 and 3A5B-1, while mutation E99A had decreased binding for 3A5B-1 alone (Table 3, Fig. 4). Clone C94A also resulted in decreased binding (<50%) for both control MAbs, IM-CKV067 and IM-CKV098, suggesting that C94A likely disrupts the disulfide bond interaction with C62, resulting in a protein misfold or in reduced CHIKV E1 expression (Table 3, Fig. 4). Likewise, even though control MAb reactivity with C96A was minimally impacted, this mutation likely disrupts the disulfide bond interaction with C63. Residue N100 was identified as critical for 1A4B-6 binding, while residues E99 and N100 were identified as critical for 3A5B-1 binding. The reactivity of 3A5B-1 to clone G90A did not meet the <25% of WT threshold to be considered a critical residue. However, the mutation still caused a decrease in 3A5B-1 binding to just over 30% of WT. Therefore,

G90 may also be involved in 3A5B-1 binding. Visualization on a high-resolution crystal structure of the CHIKV E1 protein (PDB 3N41) revealed that the region for binding of these alphavirus cross-reactive MAbs is localized to the fusion loop, a highly conserved region of the alphavirus E1 protein essential for virus-membrane fusion (Fig. 4).

## 4. Discussion

The encephalitic alphaviruses are a group of highly pathogenic viruses circulating in the Western Hemisphere. Once developed as a bio-weapon, VEEV has been the causative agent of several laboratory acquired infections transmitted by aerosolization (Rusnak et al., 2004). An alternative Ab prophylaxis or therapeutic is needed in the absence of an FDA-approved human vaccine for VEEV and other alphaviruses. Potent neutralizing MAbs specific to the E2 glycoprotein of VEEV, EEEV and WEEV have been shown to protect in mouse models (Burke et al., 2018; Hunt et al., 2011; Kim et al., 2019). Non-neutralizing antibodies may also confer protection after lethal alphavirus challenge, and this protection may be inhibiting viral egress from the infected cell and facilitating monocyte-dependent antibody effector functions (Boere et al., 1983; Kim et al., 2021; Parker et al., 2010; Rulker et al., 2012; Schmaljohn et al., 1982; Williamson et al., 2021).

In this study we investigated the ability of a broadly cross-reactive MAb to inhibit VEEV infection *in vitro*. Antibodies can neutralize viruses by several mechanisms, including prevention of viral particle attachment to cells, which can occur through virus aggregation or at the cell receptor-virus protein interface, prevention of virus envelope-cell endosomal membrane fusion, or by impeding virus egress from the infected cell. The potency of a MAb to neutralize virus by any of these mechanisms is dependent upon the virus-specific epitope involved in MAb binding and the accessible antibody-binding sites on the virion or infected cell-surface, which may be impacted by temperature and/or other reaction conditions such as changes in pH or exposure to reducing agents (Fong et al., 2014; Lok et al., 2008; Meyer et al., 1992; Schmaljohn et al., 1983; Sevvana and Kuhn, 2020; Williamson et al., 2021; Zhou et al., 2020).

At high concentrations of MAb (>10 μg/ml), 1A4B-6 was able to inhibit virus infection at a pre-attachment step, with a significant increase in virus inhibition when virus and Ab were pre-incubated at a higher temperature (37 °C compared to 4 °C) before addition to cells. The reactivity of other broadly alphavirus cross-reactive MAbs specific to the fusion loop has also been shown to be affected by temperature and reactivity conditions such as fixation with paraformaldehyde, which indicated that epitopes on the fusion loop were hidden in the native virion conformation but exposed in conditions that altered the virion protein configuration (Fong et al., 2014). Alterations in the virion structural proteins have also been shown to occur after virion attachment to cells, but before internalization of the virus particle, as indicated by increased binding of anti-E1 and anti-E2 MAbs recognizing so-called ‘transitional’ epitopes (Flynn et al., 1990). Increased Ab binding to virus particles with increasing temperature also occurs with dengue virus (*Flavivirus*). CryoEM crystal structures revealed that incubation of the Ab-dengue virus complex at higher temperatures caused large changes in the organization of the envelope proteins (called virus ‘breathing’) that resulted in exposure of epitopes previously hidden on the virion surface (Lok et al.,

2008). By increasing the virus-MAb incubation temperature before attachment to cells, we were able to show that 1A4B-6 could inhibit virus infection in a dose-dependent manner more effectively than incubation at 4 °C. Likewise, we showed 1A4B-6 bound more efficiently to the virus particle at the higher temperature, and more efficient binding of the antibody at the higher temperature may be the basis for 1A4B-6 neutralization. In contrast, when virus was adsorbed to cells at a low temperature (4 °C), and then cells were treated with MAb at the same low temperature post-attachment, virus infection was less effectively inhibited, even at 100 μg/ml. The binding of 3B4C-4 was unaffected by temperature which is not surprising given that 3B4C-4 recognizes and binds to an epitope expressed on the surface of the viral particle (Porta et al., 2014). Taken together, these results concur with other studies that indicate the fusion loop is hidden in the pre-attachment state of the infectious virion, leaving MAbs unable to bind to the virus particle and prevent infection until the virion undergoes dynamic configuration changes at a higher temperature, which allows for cryptic epitopes to become exposed.

Determination of the E1 glycoprotein antigen density on the infected cell surface was performed with quantitative flow cytometry, an approach used in human Ab therapeutic evaluations (Engelberts et al., 2013; Zenger et al., 1998). Four MAbs (1A4B-6, 2A2C-3, 3A5B-1 and 3A1C-12) that recognize similar epitopes on the fusion loop of alphaviruses were found to have moderate quantities of antibody-binding sites on the surface of VEEV infected cells. Protection by cross-reactive, non-neutralizing antibodies in alphaviral infections has been attributed to Fc effector functions such as Ab-dependent cell-mediated cytotoxicity (ADCC) and Ab dependent cell phagocytosis (Burke et al., 2018; Kim et al., 2019, 2021; Pal et al., 2013; Wust et al., 1987). Based on the quantitative flow cytometry data showing that the epitope recognized by 1A4B-6 is readily available on the surface of paraformaldehyde-fixed infected cells and our data showing *in vivo* protection from VEEV infection with 1A4B-6, we hypothesize that protection may be afforded by ADCC and warrants further investigation.

Treatment of VEEV-infected cells with MAb 1A4B-6 resulted in 54% cell fusion inhibition at an Ab concentration of 10 μg/ml. Alphaviruses induce modifications in the cellular cytoskeleton that result in the formation of intercellular extensions between cells allowing for cell-to- cell virus transmission. Direct intercellular viral transmission was only able to occur with mature viral particles capable of fusion (Martinez and Kielian, 2016). The FFWI assay described here may mimic aspects of virus transmission via intercellular extensions, resulting in cellular syncytia formation. MAb 1A4B-6 was also found to inhibit virus egress at high concentrations 6 h PI. Since the assay to determine inhibition of viral egress relied on detection of infectious virus that may or may not have been inhibited by residual antibody in the culture medium, we are unable to conclusively say 1A4B-6 inhibited VEEV egress; however, others have shown that broadly alphavirus cross-reactive MAbs recognizing similar epitopes on the fusion loop do not inhibit viral egress *in vitro* (Williamson et al., 2021).

The epitopes recognized by MAbs 1A4B-6 and 3A5B-1 both map to specific amino acid residues of the fusion loop of CHIKV E1 glycoprotein. Residue N100 is very likely critical to the major epitope for MAbs 1A4B-6 and 3A5B-1, while residues E99 and to a lesser extent G90 also contribute to the epitope recognized by MAb 3A5B-1. Although CHIKV

E1 fusion loop mutants C94A and C96A had the lowest binding activities with both MAbs, suggesting that these side chains make the highest energetic contributions to the Ab-epitope interaction (Bogan and Thorn, 1998; Lo Conte et al., 1999), mutations at these residues most likely altered the structure of the epitope by disrupting disulfide bonds. Reactivity with control MAb IM-CKV067 was significantly reduced with mutant C94A, while reactivity with control MAb IM-CKV096 was lower than other mutants but not significantly. In cross-reactivity studies, we found that MAb binding only to paraformaldehyde-fixed cells was consistent with binding of previously characterized anti-CHIKV MAbs that map to the E1 protein fusion loop (Fong et al., 2014). These results agree with previous studies that suggest the fusion loop remains hidden in the native virion and is only exposed when conditions change the conformation of the virion structural proteins.

In conclusion, MAb 1A4B-6 demonstrates the potential ability to inhibit VEEV infection *in vitro* by two separate mechanisms. The MAb was able to bind virions in an altered conformation at mammalian physiologic temperatures (37 °C) and inhibit viral entry into the cell, albeit when the MAb was applied at high concentrations. MAb 1A4B-6 also inhibited infected-cell fusion mediated by viral glycoproteins displayed on the surface of the infected cell, possibly blocking direct intercellular transmission. While 1A4B-6 and similar MAbs may not be potent virus neutralizers *in vitro*, they may play an important role in the inhibition of viral infection *in vivo*.

## Acknowledgements

This work was supported by DTRA grant HDTRA1-14-1-046, as well as a contract from the NIH (HHSN272201400058C to BJD). We thank Edgar Davidson for assistance in editing and analysis.

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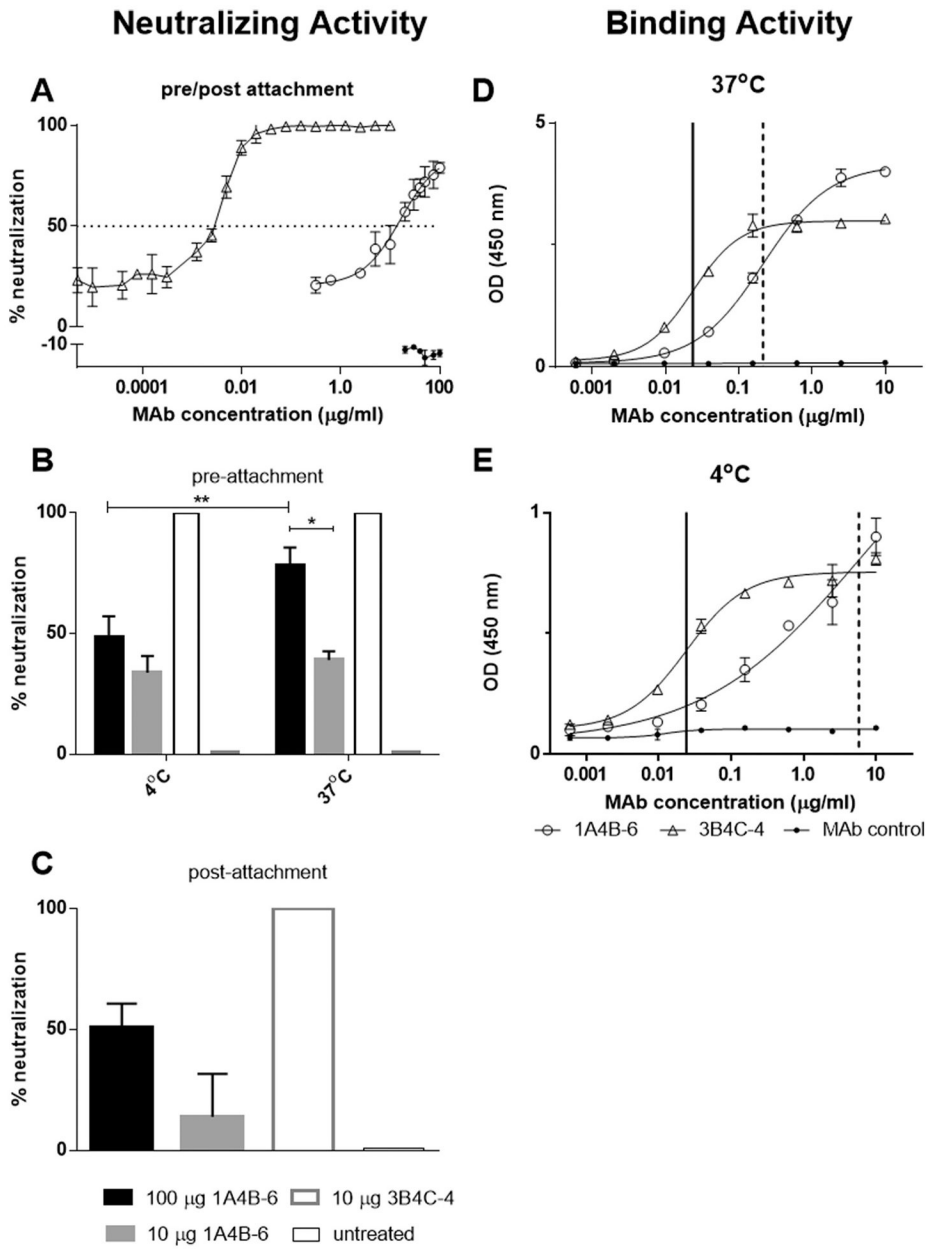


**Fig. 1. Neutralizing activity of MAb 1A4B-6 by Virus Entry Inhibition.**

MAb 1A4B-6 was evaluated for its ability to inhibit VEEV cell entry pre/post attachment at different temperatures. (**A**). Two-fold serial dilutions of MAb 1A4B-6, VEEV E2 glycoprotein-specific MAb 3B4C-4, and an IgG2b isotype MAb control were incubated with VEEV TC83 for 1 h at 37 °C and the mixture was added to a Vero cell monolayer. Cells were overlaid with agar medium and plaques were read 24 h after infection. Dotted line represents 50% neutralization ($IC_{50}$). Calculated $IC_{50}$ values: 1A4B-6, 16.42 μg/ml; 3B4C-4, 3.73 ng/ml (**B**). MAb 1A4B-6 (100 and 10 μg/ml) and MAb 3B4C-4 1(10 μg/ml) were incubated with VEEV TC83 at 4 or 37 °C for 1 h before allowing virus to adsorb to Vero cell monolayers at 4 °C (**C**). VEEV TC83 was adsorbed to Vero cells at 4 °C. Un-attached virus was removed by washing and 1A4B-6 (100 and 10 μg/ml) and 3B4C-4

(10 μg/ml) were added. After further incubation for 1 h at 4 °C, cells were overlaid with agar medium and plaques were read 24 h after infection. (**D** and **E**). Binding curves of 1A4B-6, 3B4C-4 and an isotype control binding to VEEV TC83 at 37 °C (D) or 4 °C (E) in an antibody-capture ELISA. The solid line denotes the $IC_{50}$ values for 3B4C-4 and the dotted line denotes the $IC_{50}$ values for 1A4B-6. Data for **B** and **C** are the mean and SEM of at least two independent experiments each performed in triplicate (**B**) two-way ANOVA with Tukey's post-test and (**C**) two-tailed paired *t*-test, $P = 0.1276$). Asterisks indicate significant differences (*: p = 0.0328, **: p = 0.0045).

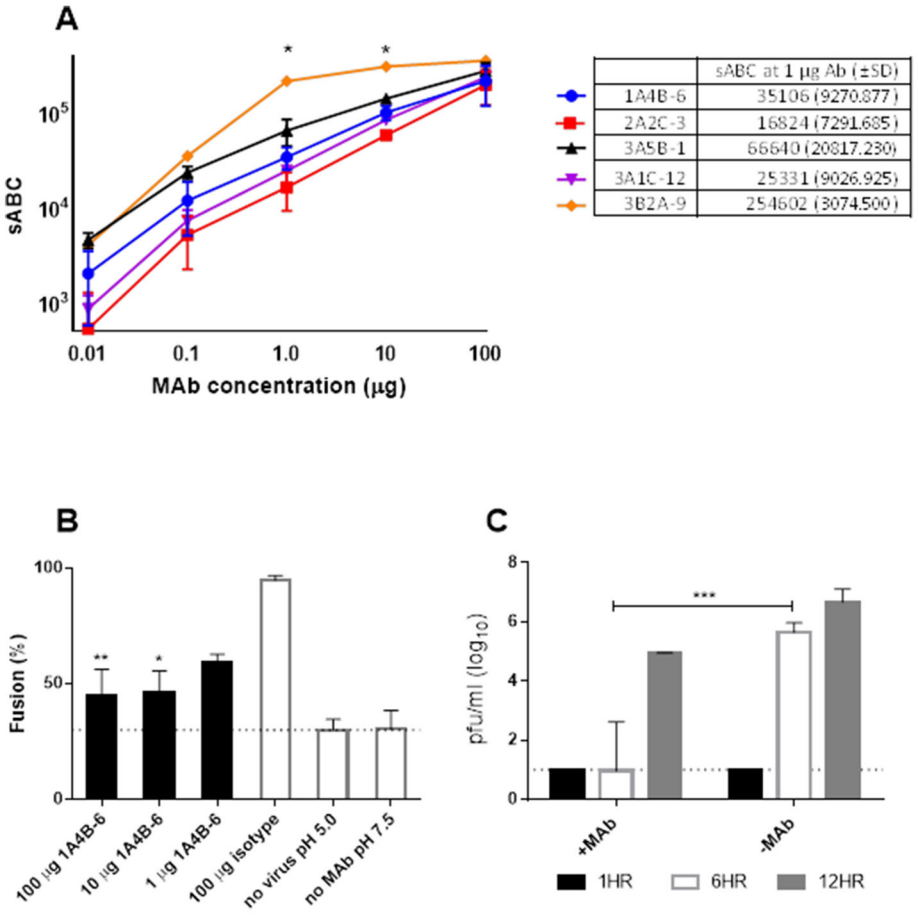


**Fig. 2. Activity of MAb 1A4B-6 in inhibition of cell membrane fusion and VEEV egress inhibition.**

(**A**) Specific Ab binding capacity units (**sABCs**) on the surface of VEEV TC83-infected SW13 cells were determined for E1 glycoprotein-specific MAbs 24 h after infection. Cells were stained with various concentrations of MAbs, and ABCs were extrapolated from a standard curve generated with Qifikit beads displaying a known quantity of murine IgGs. sABCs were calculated by subtracting the ABCs of the MAb sample from the ABCs of the corresponding isotype control of the same concentration. Asterisks indicate significant differences between sABCs of 3B2A-9 and other MAbs (*: $p \leq 0.0001$). (**B**) Inhibition of cell plasma membrane fusion was measured by a fusion from within assay. Cells were infected and treated with MAb for 1 h before unbound MAb was removed and low pH fusion medium was incubated on the cells for 1 h. Syncytia were counted 24 h later, and the fusion index was calculated as described. Percent fusion was calculated by dividing the fusion index of the test sample from the fusion index of the No MAb control sample. The dotted line represents the baseline of percent fusion occurring naturally in cells based on uninfected cells treated at pH 5.0 and infected cells at pH 7.5. Asterisks indicate significant differences in percent fusion of 1A4B-6 and isotype control treated cells (*: $p = 0.01$, **: $p = 0.0081$). (**C**) Inhibition of virus egress with 100 μg/ml of 1A4B-6 was measured by infecting Vero cells with VEEV TC83 at an MOI of 0.1 in the presence of 25 mM $NH_4Cl$ and determining virus titer by plaque assay 1-, 6- and 12-h PI. Unbound virus was removed,

cells were washed extensively and medium containing antibody and 25 mM $NH_4Cl$ was added back to cells. Extracellular infectious virus was measured by plaque assay at 1 h, 6 h and 12 h PI. The dotted line represents the limit of detection of the assay. Data from each study represent the mean of at least 2 independent experiments. Asterisks indicate significant differences (***: $p = 0.0003$).

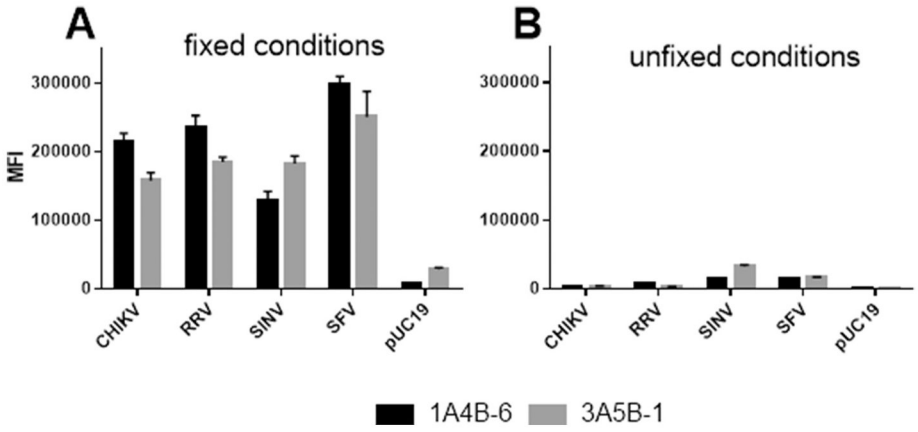


**Fig. 3. Cross-reactivity of MAbs 1A4B-6 and 3A5B-1 with alphavirus E1 proteins.**

1A4B-6 (black) and 3A5B-1 (gray) were tested for cross-reactivity to E1 proteins of CHIKV, RRV, SINV and SFV expressed on HEK293T cells by flow cytometry. Cells were fixed with 4% paraformaldehyde (**A**) or left unfixed (**B**) and reported as mean fluorescent intensity (MFI) with SEM. Cells transfected with empty expression vector pUC19 were used as a negative control. The data shown represent the mean and standard deviation of four data points.

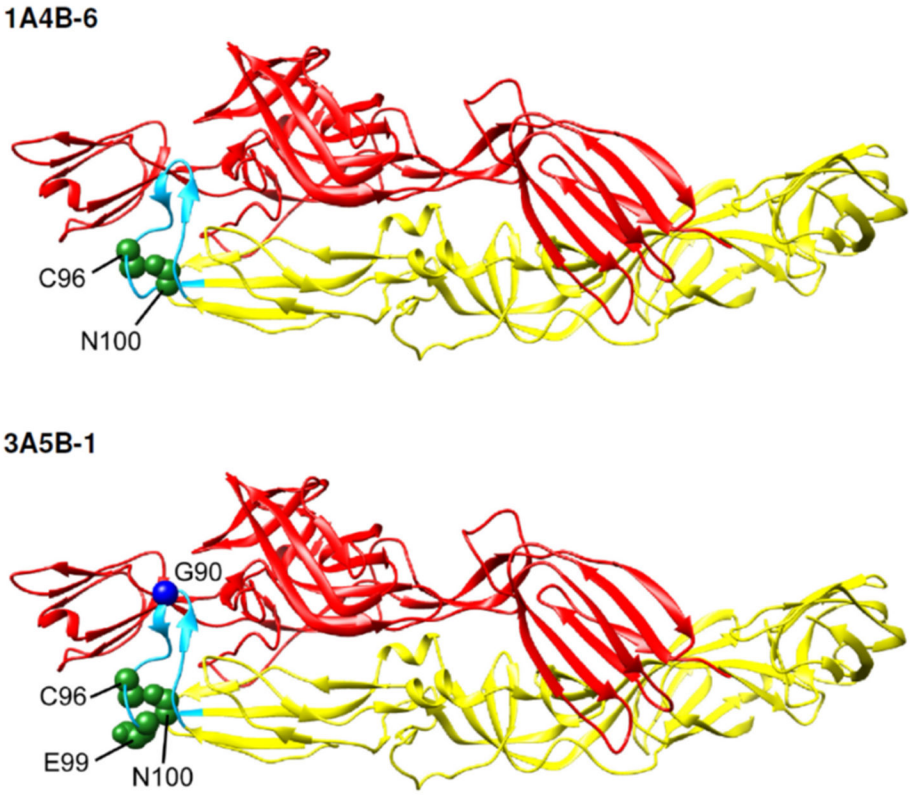


**Fig. 4. MAbs 1A4B-6 and 3A5B-1 bind the CHIKV E1 protein Fusion Loop.**

The critical residues comprising the epitopes for 1A4B-6 and 3A5B-1 are visualized on the CHIKV envelope protein heterodimer crystal structure, showing E2 (red), E1 (yellow), and the fusion loop (cyan). The epitopes (green and blue spheres) are depicted on the structure of CHIKV E1/E2 heterodimeric structure obtained at neutral pH (PBD 3N41).

*Virology*. Author manuscript; available in PMC 2022 January 18.

**Table 1**

Murine MAbs used in this study.

| MAb | Immunogen[a] | Virus-Specificity | Epitope Specificity[b] | Reference |
|---|---|---|---|---|
| 1A4B-6 | EEEV NJ-60 | Alphavirus group reactive | E1[d] | Roehrig et al., (1990). AJTMH 42, 394 |
| 1B5C-3 | EEEV NJ-60 | EEEV North American-specific | E1 | Roehrig et al., (1990). AJTMH 42, 394 |
| 1B1C-4 | EEEV BeAn 5122 | EEEV complex-specific | E1 | Roehrig et al., (1990). AJTMH 42, 394 |
| 2A2C-3 | WEEV McMillan | Alphavirus group reactive | E1[d] | Hunt and Roehrig. (1985). Virology 142, 334 |
| 3A5B-1 | VEEV TC83 | Alphavirus group reactive | E1[d] | Roehrig (1982). Virology 118, 269 |
| 3A1C-12 | VEEV TC83 | Alphavirus group reactive | E1[d] | Roehrig (1982). Virology 118, 269 |
| 3B2A-9 | VEEV TC83 | VEEV complex-specific | E1[b] | Roehrig (1982). Virology 118, 269 |
| 3B4C-4 | VEEV TC83 | VEEV complex-specific | E2 | Roehrig (1982). Virology 118, 269 |

[a] Virus used to elicit MAb.

[b] Epitope on protein recognized by MAb.

## Table 2

MAb 1A4B-6 protects mice prophylactically from VEEV challenge.

| MAb [μg][a] | Survivors/Total[b] | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 1A4B-6 [100] | 5/5 | nd[c] | 10/10 |
| 1A4B-6 [40] | nd | 2/10 | nd |
| 1A4B-6 [20] | 2/5 | nd | nd |
| 1B1C-4 [100] | 0/5 | nd | nd |
| 1B5C-3 [100] | 0/5 | nd | nd |
| PBS | 0/5 | 0/10 | 0/10 |

nd = not determined.

[a] Various concentrations of MAbs 1A4B-6, 1B1C-4, 1B5C-3 or PBS were administered to 3-week-old Swiss Webster mice prophylactically IV 24 h before IP challenge with 100 $LD_{50}$ VEEV strain Trinidad Donkey.

[b] Three independent experiments were performed with varying concentrations of MAb.

## Table 3

Binding reactivities of 1A4B-6 and 3A5B-1 with CHIKV E1 protein with fusion loop mutations.

| AA substitution[a] | Mean Binding Reactivity (±SD)[b] | | | |
|---|---|---|---|---|
| | 1A4B-6 | 3A5B-1 | IM-CKV067 | IM-CKV098 |
| WT | 100(12.4) | 100 (39.7) | 100 (11.6) | 100.0 (47.3) |
| Vector | **0(2.0)** | **0 (2.8)** | **0.0 (0.4)** | **0.0 (1.4)** |
| G83A | 126.3 (13.0) | 131 (18.9) | 74.4 (6.6) | 194.6 (39.2) |
| V84A | 128.2 (21.2) | 154.5 (15.4) | 97.0 (11.2) | 207.5 (35.2) |
| Y85A | 118.8 (19.5) | 113.5 (6.8) | 91.9 (6.7) | 194.2 (19.5) |
| P86A | 109.1 (24.8) | 185.0 (15.6) | 92.3 (3.3) | 156.2 (45.2) |
| F87A | 112.8 (13.1) | 160.5 (16.7) | 93.0 (9.8) | 140.1 (12.8) |
| M88A | 110.3 (5.4) | 149.7 (23.1) | 93.2 (13.0) | 127.0 (17.8) |
| W89A | 133.8 (20.6) | 134.6 (23.4) | **5.9 (2.2)** | 224.8 (13.6) |
| G90A | 82 (10.2) | **32.6 (10.3)** | 74.8 (12.4) | 159.8 (29.2) |
| G91A | 98.1 (8.8) | 121.3 (25.8) | 72.1 (9.1) | 108.2 (41.2) |
| A92S | 111.7 (18.5) | 105.0 (14.6) | 73.6 (10.9) | NT |
| Y93A | 117.0 (18.5 | 112.3 (13.7) | 77.9 (15.9) | 116.8 (18.7) |
| C94A | **0.6 (1.1)** | **0.4 (1.4)** | **3.1 (0.9)** | 45.2 (12.4) |
| F95A | 59.0 (4.8) | 161.3 (7.3) | 104.5 (14.9) | 72.7 (26.5) |
| C96A | **3.9 (1.7)** | **1.5 (1.1)** | 54.0 (7.5) | 85.2 (6.7) |
| D97A | 101.3 (2.9) | 124.3 (11.7) | 91.6 (14.1) | NT |
| A98S | 115.1 (18.2) | 198.4 (14.2) | 91.9 (16.6) | 160.8 (13.4) |
| E99A | 79.5 (11.0) | **15.0 (4.7)** | 91.2 (20.4) | 156.9 (27.2) |
| N100A | **20.9 (1.0)** | **6.7 (3.5)** | **13.3 (3.8)** | 180.6 (32.4) |

[a] amino acid (AA) mutation on CHIKV E1 and expressed in HEK293 cells.

[b] Immunoreactivities expressed as a percentage of the immunoreactivity of the WT in quadruplicate, with SD given in parentheses. Values in bold show critical residues for Ab binding. Control MAbs IMCKV067 and IM-CKV098 were screened to confirm protein expression and conformation.