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Pragati Kapila April 10, 2023

Evaluating epitope-specific antibody immunity elicited by chikungunya infection and vaccination

by

Pragati “Pari” Kapila

Dr. Matthew Collins

Adviser

Biology

Dr. Matthew Collins

Advisor

Dr. Christina Gavegnano

Committee Member

Dr. Kate O’Toole

Committee Member

2023

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Pragati Kapila

Dr. Matthew Collins

Advisor

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology

2023

Abstract

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By Pragati Kapila

Chikungunya virus (CHIKV) is an alphavirus transmitted to human by *Aedes* mosquitoes and causes symptoms like fever, rash, and joint pain, which may persist from months to years after the initial infection. CHIKV poses as a public health risk to people across tropical and subtropic regions as CHIKV epidemics can overwhelm health systems and burden economies. There are currently no licensed vaccines or approved antivirals against CHIKV. Previous research has established that neutralizing antibodies (NAbs) are an important component of the human immune response to CHIKV and frequently target the E2 glycoprotein on the CHIKV surface. However, current serological measures of immunogenicity of CHIKV vaccines are insufficient. We hypothesize that vaccine candidates eliciting antibodies (Abs) at the same antigenic regions as those of natural CHIKV infection will provide durable immunity. In this paper, a blockage of binding (BOB) assay employing CHIKV-specific neutralizing monoclonal antibodies (NmAbs) was utilized to assess the specificity of Abs elicited by a virus-like particle (VLP) vaccine candidate and a live-attenuated vaccine candidate compared to natural CHIKV infection. BOB activity was measured using one mouse and two human NmAbs. We identified 115 CHIKV IgG positive samples of 296 cross-sectional serum samples obtained from participants residing in a CHIKV-endemic region of Colombia ~4-6 years after CHIKV emerged in the local population. CHIKV-immune sera consistently and strongly competed with the NmAbs tested by BOB. However, a minor portion of participants exhibited strong BOB responses to 1-2 NmAbs and negligible activity to other NmAb(s), suggesting person-to-person variation in immunodominance hierarchies in the Ab response generated by CHIKV infection. The VLP vaccine samples and the live attenuated vaccine samples both demonstrated high consistent BOB activity as well, supporting the hypothesis that similar antigenic regions on the E2 protein were targeted. Finally, BOB activity was positively correlated with NAb titers in all sample sets. Thus, the BOB assays implemented here demonstrated great potential as a robust measure of immunogenicity to guide CHIKV vaccine development.

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Acknowledgements

I would like to express my immense gratitude and appreciation towards my principal investigator (PI) and my mentor, Dr Collins, for guiding me towards becoming a successful investigator and researcher throughout my time in the lab and working on the thesis. His mentorship has been instrumental in my professional growth, and I am truly grateful for the time and effort he has invested into myself and the project.

I am also deeply grateful to all the members of the Collins lab, particularly Dr. Daniel Espinoza and Dr. Yerun Zhu for their unwavering support, invaluable knowledge, and generosity with their time. Their contributions have been instrumental in the progress and success of the project, and I am very grateful of their efforts and input.

In addition, I extend my thanks to our collaborators, partners, and colleagues who have contributed to this project. Their dedication and resources have elevated the quality of this work, and I am grateful for their support.

Table of Contents

Introduction

Chikungunya virus (CHIKV) is an alphavirus that is transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes.[1] Disease caused by CHIKV is called chikungunya fever (CHIKF) and was first recorded in Zanzibar in 1823 and in St. Thomas Island in 1827. CHIKV was then isolated and identified in Tanzania in 1952.[2] CHIKV is a public health problem in many tropical and subtropical regions of the world, exhibiting potential for explosive epidemics as seen most recently with its re-emergence in the Western hemisphere in 2013. CHIKV infection typically causes symptoms like fever, rash, headache, myalgia, and joint pains. Although CHIKF has a low case fatality rate (CFR) of 1 in 1000 in the Americas,[3] CHIKV infection can cause debilitating arthralgia that persists for weeks to years. Infection by CHIKV can also lead to neurological manifestations that include seizures, inflammation of the brain and nerves, optic neuritis, deafness, and Guillain-Barré syndrome.[4] In addition, epidemics of CHIKV disease often occur in developing regions, which can be overwhelming for local health systems and economies. Unfortunately, there are currently no licensed antivirals or vaccines against CHIKV.

CHIKV is an enveloped virus, approximately 70 nm in diameter, with a positive-sense, single-stranded RNA genome, and four CHIKV lineages have been identified: Asian; Indian Ocean; West African; and East/Central/South African (ECSA).[5] Upon host cell infection, the CHIKV genome is translated from two open reading frames encoding four non-structural proteins (nsP1-4) and five structural proteins: 6K, capsid (C), envelope 1 (E1), envelope 2 (E2), and envelope 3 (E3).[2] The surface of alphaviruses like CHIKV are composed of spike-like trimers of E1/E2 heterodimers. The E2 protein has recently been found to mediate infection by several arthritogenic alphaviruses including CHIKV by engaging the Mxra8 receptor on host cells.[6] Previous research indicates that E2 is a common target of CHIKV neutralizing antibodies (NAb)[2] and neutralizing monoclonal Ab (NmAb) against E2 prevent *in vitro* infection and protect against

CHIKV infection *in vivo*.[7] No precise correlate of protection has been defined for CHIKV, but mounting evidence suggests NAbs are prominent mediators of protective immunity against CHIKV infection in humans.[8] Researchers have isolated several monoclonal antibodies (mAb) from patients following CHIKV infection that bind to E2 and strongly neutralize CHIKV in serological tests. A chief goal CHIKV vaccine development is to elicit a durable memory response, and research indicates NAb activity mediated via E2 binding corresponds with an adaptive immune response that may provide long term protection during future CHIKV exposure.

An overarching purpose in our group is to understand the adaptive immune determinants of protection against arboviruses like CHIKV. In this project, a classical vaccinology perspective is taken in assuming that immunity arising from natural infection can provide a roadmap for developing efficacious vaccines. We hypothesized that two leading CHIKV vaccine candidates, a virus-like particle (VLP) vaccine and a live attenuated chimeric measles virus-CHIKV (MV-CHIKV) vaccine would elicit Abs at similar antigenic regions on the surface of the CHIKV virion as those targeted in natural infection. A panel of monoclonal antibody (mAb) probes were utilized in blockade of binding (BOB) assays to assess CHIKV-specific Ab responses at distinct E2 epitopes.

Methods

Human Subjects and Biospecimen. The appropriate institutional review boards and/or bioethics committees reviewed and approved all human subjects research. Participants provided their informed consent in native languages, and blood samples were then obtained via standard blood draw technique by trained phlebotomists. The samples made available and used in the study were archived serological specimens previously collected under previous IRB-approved studies (Table

1). Sera were separated, then aliquoted and stored at -20°C to -80°C until used. Samples were heat inactivated at 56 °C for 30 minutes prior to use in serologic assays.

Viruses and cells. CHIKV 181/clone25 and MAYV (Strain) were propagated and titrated on Vero-81 cells (ATCC).

Production and labeling of monoclonal probes: CHK-48, 4N12, and 3N23. CHK-48 was produced from a mouse hybridoma provided by Michael Diamond.[9,10] Hybridoma cells were grown in protein free media. Supernatant was harvested after days, and mAb purified over a protein A column. is a mouse mAb. Aliquots of purified human mAbs 4N12 and 3N23 were kindly provided by James Crowe, Jr.[10] Purified mAb were covalently bound to alkaline phosphatase via the Lynx Rapid Alkaline Phosphatase Antibody Conjugation kit per manufacturer's instruction.

Antigen Capture IgG ELISA. CHIKV or MAYV-binding IgG was measured by antigen capture ELISA as previously described.[11] Plates were coated with murine mAb CHK-48 in 0.1 M Carbonate buffer (pH = 9.6) overnight at 4°C. Mab-coated plates were blocked with 100ul of 5% Non-Fat dry milk in PBS with 0.05% Tween-20. Culture-derived virus was then added in 50ul block buffer for 1 hour at 37°C. Serum was added at 1:100 dilution, incubated for 1 hour at 37°C. Serum IgG were detected by an alkaline phosphatase-conjugated goat anti-human IgG for 1 hour at 37°C, volowed by addition of p-nitrophenyl phosphate (p-NPP) substrate for up to 30 minutes at room temperature, measuring optical density (OD) at 405nm by a plate reader every 5-10 minutes. To determine the titer of virus-binding IgG, a dilution series of serum was run in the same ELISA assay and IC50 was determined.

Blockage of Binding (BOB) assay. BOB assays were performed as we have previously done.[11] CHIKV or MAYV antigen is captured as in the antigen capture ELISA. Serum is incubated at a 1:10 dilution with antigen for 1 hour at 37°C. Plates are washed and a labeled competing mAb

(probe) is added at an optimized concentration, typically between 5-20ng/well (Figure 1). The percent blockade of binding is calculated using the equation: (1- [OD of sample/OD of negative control]). Samples are considered to have a high magnitude of BOB activity if the percentage is higher than an arbitrary value equal to or greater than 50%.

Neutralization assay. Serial dilutions of the serum were mixed with 50-75 focus-forming units of virus, incubated, and then transferred to a monolayer of Vero cells and was infected. OptiMEM overlay media supplemented with 2% FBS and 1% Carboxymethylcellulose which was added and left for 24 hours. Cells were fixed and virus foci detected by staining with CHK-48. Foci was counter used a user-supervised counting program on a CTL ELISPOT reader. Alphavirus-naïve controls were used to demonstrate 100% infection.

Statistical Analysis. For determining IgG seropositivity by ELISA, cutoff was calculated as mean OD of negative controls + 3 SD + 0.1. For determining quantitative levels of CHIKV-binding IgG, background subtracted OD values measured over a dilution series were graphed in Prism. A four-parameter dose response curve was fit to the data and IC50 was calculated in Prism. The following equation was used to calculate %BOB: (1 – [OD sample/ODctr]) x 100%.). NAb titers (FRNT50) were determined by interpolation from a fit curve (four-parameter, sigmoidal dose response, variable slope, Prism 7). FRNT50 values were required to have an R^2 >0.75, a Hill slope >0.5, and an FRNT50 falling within the range of the dilution series. For further analyses, FRNT50 values <20 was arbitrarily assigned a value of 5. The correlation between BOB activity versus NAb titer (FRNT50) and BOB versus CHIKV-binding IgG (IC50) was interrogated by non-parametric correlation analysis (Spearman correlation test) in Prism, with correlation coefficient and p values reported.

For the figures that are attached in the appendix and included the % BOB, any values that were negative values were converted to zero. Median and quartile 1 and quartile 3 for % BOB record were recorded per monoclonal probe. The BOB records were non-normally distributed, and a Wilcoxon paired signed rank test was performed to identify the significant differences between groups and groups. All tests and data graphs were performed in R (version 4.2.0) and R studio (1.4).

Results

To compare humoral immunity elicited by CHIKV infection versus vaccination, we first identified samples from research participants with prior CHIKV infection. We leveraged archived specimens collected from cohort studies involving people living in Risaralda, Colombia, a region endemic for *Aedes*-borne viruses (Table 1).[12] Mayaro virus (MAYV), an arbovirus closely related to Chikungunya virus, has also become endemic in Latin America[13] in recent years, were also measured to ensure the assay was specifically detecting CHIKV infections**.** Among 297 samples tested, 115 of 297 (38.8%) were positive by CHIKV IgG ELISA, consistent with prior CHIKV infection (Figure 3). Titers of NAb (FRNT50) and CHIKV-binding Ab (IC50) were measured in CHIKV IgG ELISA+ samples **(**Figure 4).

We next asked whether CHIKV infection consistently elicited Ab in polyclonal serum that targeted specific epitopes of NmAbs by performing BOB assays. Three mAbs reacting at distinct epitopes involving CHIKV surface glycoprotein E2 were selected (Table 2). 4N12 and 3N23 were specifically chosen because they are human CHIKV-specific mAbs that strongly neutralize several CHIKV strains, including those circulating in Latin America and our study site. The human mAbs do not bind MAYV (Figure 6) or recombinant CHIKV E2 monomers**,**[14] suggesting they are

CHIKV-specific mAbs that recognize complex structural epitopes requiring surface protein assembly on the intact virion. Most CHIKV-immune sera competed to a high degree with all three NmAb probes (Figure 7A). There was statistical significance found between the CHK-48 probe to the 3N12 probe and the CHK-48 probe and 4N12 probe in magnitude of BOB activity. However, a minority of samples with Ab that bound and neutralized CHIKV exhibited no BOB activity. Interestingly, we observed that some individuals, ~22% to 25%, mounted a strong Ab response at one or two epitopes but exhibited little or no BOB activity at other epitopes (Figure 7B), indicating that immunodominance hierarchies can differ between people. We also asked whether percent of BOB activity of the CZS sample set correlated with other quantitative serologic measures such as FRNT and binding titers of IgG, and a significant positive correlation was observed for BOB determined by all three mAb probes versus both parameters analyzed (Figure 8).

Next, we asked whether two leading CHIKV vaccine candidates (Table 1) generated Ab of similar specificities as those detected in serum after natural infection. We hypothesized that serum from the virus-like particle (VLP) vaccine platform[15] would generate Ab that compete at the complex epitopes identified by 4N12 and 3N23 since VLP vaccines are expected to closely mimic the three-dimensional structure of the native virion.[16] We tested serum from six longitudinal time points collected in the VLP vaccine study among three study subgroups receiving a vaccine dose at different amounts(10 mcg, 20 mcg, 40 mcg) (Figure 9). The magnitude of BOB values showed a variation at different time points in the three NmAb probes and an overall increase in magnitude of BOB activity increasing after each dose (Figure 10A) and subgroup of 20 mcg having the lowest magnitude of BOB activity versus the three probes. The peak immunogenicity timepoint was at timepoint 6, two weeks after the second dose of the vaccine for all the candidates in the three

subgroups. The differences in BOB activity across the three probes at the peak immunogenicity time point was statistically significant between mAbs 3N23 and CHK-48 ($p < 0.001$) (Figure 10B).

We also studied a live-attenuated recombinant measles-vectored vaccine (MV-CHK),[17] which is engineered to express chikungunya structural proteins C, E3, E2, 6K, E1.[18] Though the CHIKV virion (the nonstructural components) is not being replicated in the MV-CHK vaccine, the vaccine candidate is able to produce polyclonal NAbs that provide robust immune responses in individuals. Thus, we hypothesize that there will be Abs generated at the epitopes targeted by the CHIKV-specific NmAbs because there will likely elicit a strong Ab response that will compete with the chosen NmAbs at the quaternary epitopes. MV-CHK is separated into three subgroups, or cohorts, based on the vaccine schedule (Cohort 4,5,6) and samples chosen were taken from the theoretical peak immunogenicity timepoint based on the vaccine schedule (Figure 11). We conducted BOB assays with the MV-CHK vaccine candidate samples and found a high magnitude and general variation in magnitude of BOB activity in the three cohorts across the three probes. There was no statistical significance between the magnitude of the BOB between the three probes (Figure 12A); however, there was a small group of individuals that demonstrated variation in the epitope of Abs elicited based on differences in BOB activity (Figure 12B).

We further performed correlation analyses with the VLP vaccine candidate samples to determine whether the observed relationships between different serologic measures were similar in the vaccine sample sets. There was no correlation between the magnitude of BOB activity for any of the three probes and NAb titer (Figure 13A) There was a moderate positive correlation between the BOB values for all three probes and the binding titers of IgG (Figure 13B**).** We also performed to see whether the magnitude of BOB activity in the three probes correlated with other serological measures, such as the FRNT and binding titers of IgG for the MV-CHK vaccine

candidate samples. There was a moderate positive correlation between the BOB values for all three probes and the FRNT (Figure 14A) and to the binding titers of IgG (Figure 14B**)**.

Discussion

This study aimed to determine whether epitopes of potent NAbs are targeted by Abs generated after CHIKV infection and vaccination. Availability of a safe and effective vaccine is critical to diminish the global impact of CHIKV, as there have been over 2 million cases in the past 10 years and millions more residing in *Aedes*-endemic regions remain at risk.[19] Populations with no prior immunity are particularly vulnerable to large epidemics, but episodic transmission continues in endemic areas as well. While promising candidate CHIKV vaccines have been tested in early phase clinical trials, few vaccine candidates have advanced to phase 3 efficacy trials in humans,[19,20] largely because the unpredictable transmission of CHIKV complicates planning of these large-scale studies.

We found that two leading CHIKV vaccine candidates consistently generated Abs against all three epitopes probed, like the responses observed in the cross-sectional cohort of CHIKV-immune participants living in an *Aedes*-borne virus-endemic region of Colombia. These results support our hypothesis that vaccines delivering antigen that recapitulates native virion structure can elicit Ab responses to the same epitopes involving the E2 protein as in natural infection.

Vaccine development for CHIKV is at a pivotal juncture, with two vaccines currently seeking licensure and many others still in the pre-clinical and clinical pipeline. Due to the sporadic transmission patterns that characterize arboviruses like CHIKV, it may not be possible to rigorously demonstrate vaccine efficacy in prospective randomized controlled trials. Alternatively, licensure will likely depend on surrogate endpoints, including those defined by serologic outcome

measures. This is further complicated by the lack of a proven serologic correlate of protective immunity to CHIKV. It is widely agreed that NAb are crucial for CHIKV immunity and evidence from epidemiological studies, passive Ab transfer studies, and other animal models all support a role for NAb in protecting against CHIKV infection. The idea is based on a sound mechanistic hypothesis given that human Abs that interrupt the interaction of E1/E2 with the host cell receptor Mxra8 protect mice from death and disease.[7] Thus, the approach to serologic assessment of CHIKV immunity in evaluating vaccines is critical.

Quantifying NAb is clearly important. However, there are challenges with performing neutralization assays at large scale: 1) These assays often require culture of live virus, which presents a biohazard risk. Work with wild type CHIKV requires biosafety level-3 containment, which is not widely available. 2) Neutralization assays notoriously suffer from inter-laboratory variability. 3) Neutralizations are resource intensive in terms of turnaround time and the need for ample hands-on time from highly trained staff. Thus, NmAb BOB assays offer an alternative or complimentary serologic measure. ELISA-based assays measuring Ab binding are technically simpler, can be designed devoid of biohazardous reagents (there would only be need for standard precautions for human serum), and are more amenable to standardization and even automation. Our data indicate BOB assays detect seroconversion and Ab response kinetics similar to neutralization assays. In fact, % BOB was positively and significantly correlated with NAb titer (FRNT50) in samples from infection and the two leading vaccine candidate sample sets. In this regard, it may be possible to further develop BOB assays as a surrogate of protective immunity to viruses like CHIKV.

One key observation from our experiments after natural infection was that a minority of CHIKV-immune individuals mounting a markedly distinct response at the different epitopes

probed, with some serum samples exhibiting >75% BOB for 1 or 2 NmAb probes and nearly 0% to the third. This suggests that person-to-person variation in the specificity of the CHIKV Ab response occurs. It is immunologically interesting to understand this phenomenon, which could be related to one or multiple factors: Germline genetics, the naïve BCR repertoire, host infection history, infecting virus strain, random chance. Regardless of the mechanism, this finding justifies using a panel of BOB assays that probe multiple epitopes across a few antigenic regions of the virus surface to provide a more balanced and robust measure of CHIKV Ab immunity. Similarly, our data support the hypothesis that the VLP vaccine, the MV-CHIKV vaccine, and natural infection all induce Ab against quaternary epitopes given the observed competition with 3N23 and 4N12. However, the evidence from this study is only indirect and suggestive, and it would be valuable to further investigate this with future experiments specifically designed to test this idea.

There were limitations to this study. We only assessed Ab immunity with 3 mAb in the BOB assay. The polyclonal Ab response to CHIKV likely includes many specificities, and it would be important to test samples from this study with a larger panel of mAb to gain a more comprehensive understanding of Ab immunity to CHIKV. Additionally, the two vaccines investigated are likely not too antigenically different from one another, given that the MV-CHIKV vaccine generates VLPs upon infection of host cells.[18] It would be interesting to study a subunit vaccine that could have more significant differences in antigenicity. On the other hand, we did observe differences when comparing the two vaccines at peak immunogenicity and in examining the differences between study groups within each vaccine sample set. It is clear from our data that factors such as the time interval between vaccine doses may have an important effect on the magnitude and quality of Ab responses independent of antigenic properties. An additional aspect to ponder for future studies is the exclusion of immunocompromised individuals from the two

vaccine trials as well as individuals at the two extremes of the human lifespan. It is imperative to include these groups as they may mount diminished or altered immune responses to CHIKV vaccines compared to health adults. It is crucial to ensure that CHIKV vaccines are effective and safe for all members of populations at risk.

Overall, this study provides insights into the specificity and quality of epitopes targeted by Abs elicited by CHIKV infection and vaccination, and the BOB assay demonstrates utility as a measure for immunogenicity in CHIKV vaccine development. As CHIKV transmission continues to increase in tropical and subtropical regions of the world, a safe and effective vaccine is critical for diminishing the global impact of CHIKV, and the findings of this study provide insight to guide future vaccine development for CHIKV and other emerging viruses.

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Appendix

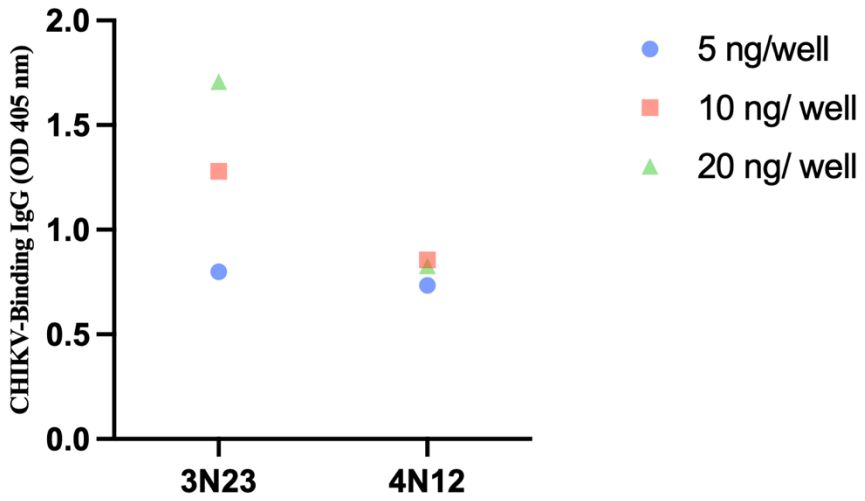


Figure 1. Validation of CHIKV binding by AP-conjugated monoclonal antibody probes for BOB assay. The indicated mAbs were conjugated to AP and tested for binding to CHIKV at the concentrations indicated in the legend by antigen capture ELISA. OD, optical density; AP, alkaline phosphatase; BOB, blockade of binding.

Study Source	# of Subjects	# of Specimens	Parent Cohort or Study Description	Key Features	Clinical Trials Registration)
CZS	115	115	CHIKV Infection	Cross-sectional samples post-CHIKV emergence collected in Arbovirus endemic region (Risaralda, Colombia)	N/A
VRC 311	25	145	Virus-like particle (VLP) Vaccine	CHIKV VLP Phase 1 Trial; 25 participants, 3 groups, 6 longitudinal time points per vaccine candidate	NCT01489358
DMID 15-0038	63	70	Measles-CHIKV Live Attenuated Vaccine (MV-CHK)	live attenuated chimeric vaccine well-tolerated in Phase I trials.	NCT02861586

Table 1: Characteristics of Sample sets used in assays.

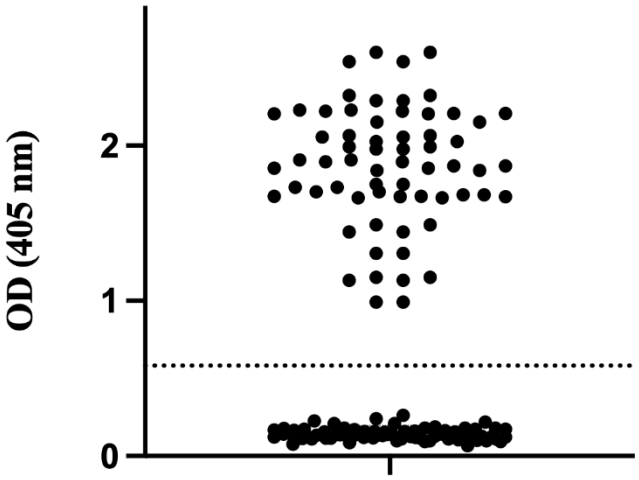


Figure 3. Identification of seropositive CZS Samples based on CHIKV IgG ELISA. This is a representative sample (n=116) of the 297 CZS samples that were tested to determine CHIKV IgG seroprevalence. The y-axis has the OD and the checkered line at y = 0.581 is the cutoff for positivity (mean of negative controls + 3 SD). OD, optical density.

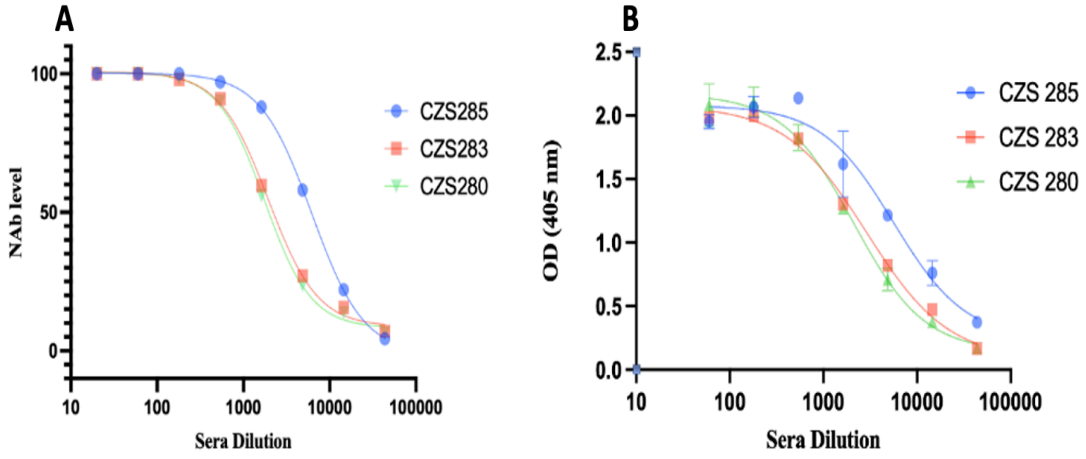


Figure 4. Representative titration curves for NAb and binding IgG indicate robust humoral immune response to CHIKV.
A) Neutralizing (left) and binding IgG (right) antibody levels against CHIKV were determined over the indicated dilutions for the three CHIKV IgG+ serum samples listed in the legend. OD, optical density; NAb, neutralizing antibody; CHIKV, chikungunya virus.

mAb*	Species (isotype)	Epitope (contact residues), antigenic region	Source of mAb generated	Other alphaviruses neutralized by mAb
CHK-48	Mouse (IgG2c)	E2 B Domain (AA184, 190, 197, 209, 210)	Neutralizes multiple alphaviruses, binds to recE2	ONNV, MAYV, UNAV, BEBV, GETV
4N12	Human (IgG2)	E2 arch (AA 250)	Potent neutralization against all CHIKV genotypes, does not bind to recE2, does not bind to MAYV	
3N23	Human (IgG1)	E2A domain arch (AA 60, 68, 98, 170, 171, 233, 234)	Potent neutralization against all CHIKV genotypes, does not bind to recE2, does not bind to MAYV	

Table 2: Characteristics of monoclonal antibodies used in the panel.
*mAb, monoclonal antibody

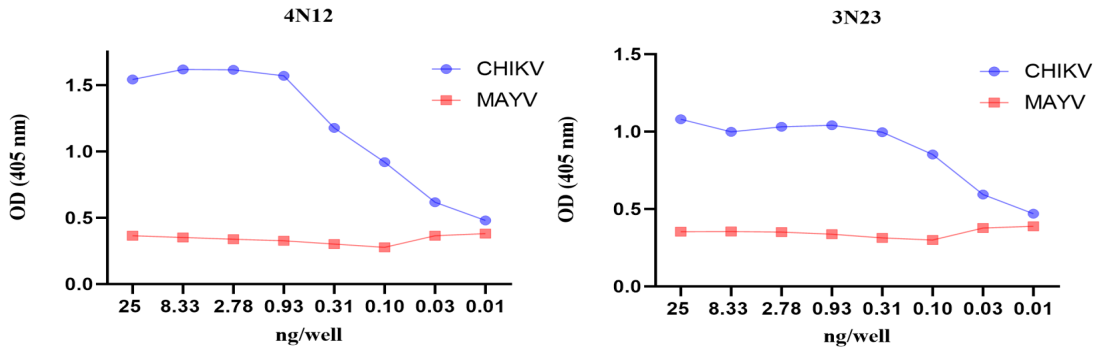
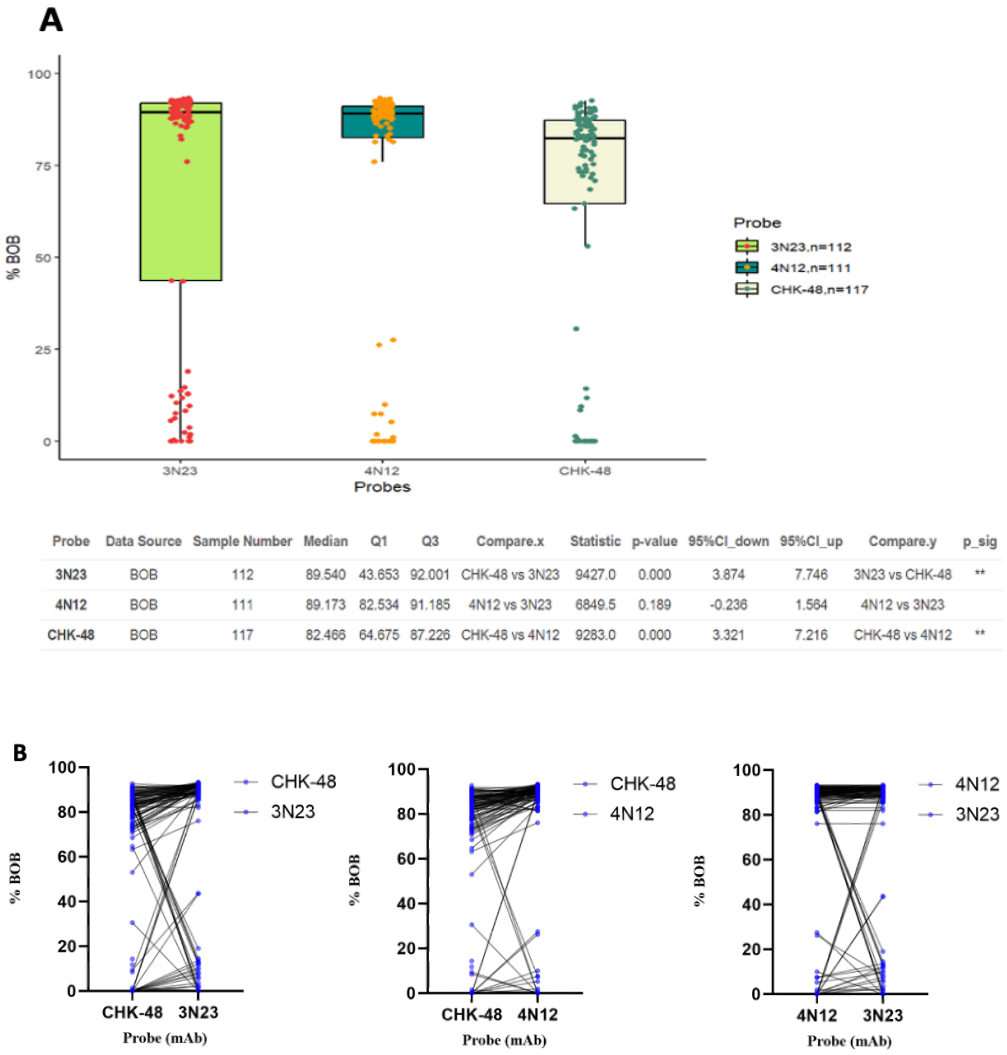


Figure 6. Human monoclonal antibodies that strongly neutralize CHIKV do not cross-react with MAYV. Antibody binding to CHIKV (blue) and MAYV (red) was determined by antigen capture ELISA for IgG over a dilution series for 4N12 and 3N23. CHIKV, chikungunya virus; MAYV, Mayaro virus; OD, optical density.

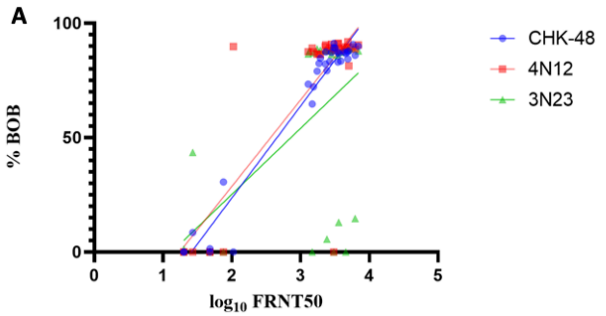


Probe	Data Source	Sample Number	Median	Q1	Q3	Compare.x	Statistic	p-value	95%CI_down	95%CI_up	Compare.y	p_sig
3N23	BOB	112	89.540	43.653	92.001	CHK-48 vs 3N23	9427.0	0.000	3.874	7.746	3N23 vs CHK-48	**
4N12	BOB	111	89.173	82.534	91.185	4N12 vs 3N23	6849.5	0.189	-0.236	1.564	4N12 vs 3N23	
CHK-48	BOB	117	82.466	64.675	87.226	CHK-48 vs 4N12	9283.0	0.000	3.321	7.216	CHK-48 vs 4N12	**

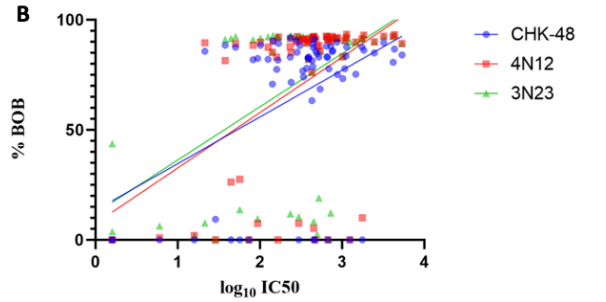
Figure 7. Ab immunity elicited by CHIKV infection is readily detected by BOB assays.

A) Detection of BOB activity against NmAb probes CHK-48, 3N23, AND 4N12 (n= 115). The data demonstrates the spread of the individual values of BOB activity in each probe and displays the spread with the median, interquartile range, and the individual points. Data analysis for p-value was conducted in comparing the three probes and found statistical significance between 3N23 versus CHK-48 at a p-value of 0.000 and found statistical significance between 4N12 and CHK-48 at a p-value of 0.000. The BOB values were formulated using a formula using the OD-values from the samples and that of the negative control samples.

B) Linked pairwise BOB testing reveals individual variation in immunodominance hierarchy of Ab response to CHIKV. (n=115). This figure maps the BOB activity for an individual versus two different probes by linking them. This data shows similar levels of BOB magnitude per person using different probes. However, there is a small group that has heterogenous response, demonstrating distinct levels in BOB magnitude.



Correlation	Log10FRNT vs. CHK-48	Log10FRNT vs. 4N12	Log10FRNT vs. 3N23
Spearman r			
r	0.8265	0.5826	0.4656
95% confidence interval	0.6688 to 0.9130	0.2894 to 0.7758	0.1291 to 0.7060
P value			
P (two-tailed)	<0.0001	0.0004	0.0072
P value summary	****	***	**
Exact or approximate P value	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	Yes	Yes	Yes



Correlation	Log10IC50 Binding Titer vs. CHK-48	Log10IC50 Binding Titer vs. 4N12	Log10IC50 Binding Titer vs. 3N23
Spearman r			
r	0.2046	0.5094	0.2708
95% confidence interval	-0.04080 to 0.4268	0.3037 to 0.6698	0.03118 to 0.4810
P value			
P (two-tailed)	0.0917	<0.0001	0.0234
P value summary	ns	****	*
Exact or approximate P value	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	No	Yes	Yes

Figure 8. Moderate, positive correlations found using Spearmen correlation test between BOB activity and NAb titers and BOB activity and IgG titers of Natural CHIKV infection samples.

A) Correlation of BOB activity in the NmAbs and FRNT50 test in the CZS samples (n=32). Regression analysis was used to assess correlation between FRNT50 and BOB activity. Pearson Spearmen correlation test was found to have a correlation with the CHK-48 mAb (p= 0.001), 4N12 mAb (0.0004), and 3N23 mAb (0.0072).

B) Correlation of BOB activity in the NmAbs and FRNT50 test in the CZS samples (n=70). Regression analysis was used to assess correlation between IgG binding titers and BOB activity. Pearson Spearmen correlation test was found to have a correlation with 4N12 mAb (p= 0.001 and 3N23 mAb (0.0234). The Pearson spearman coefficient test did not find a statistical correlation versus the CHK-48 probe (p= 0.0917)

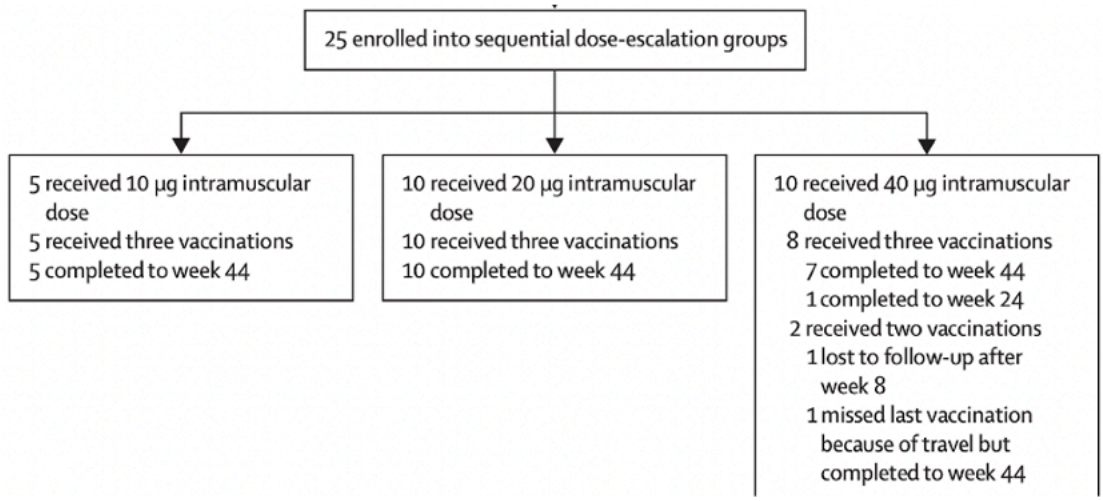
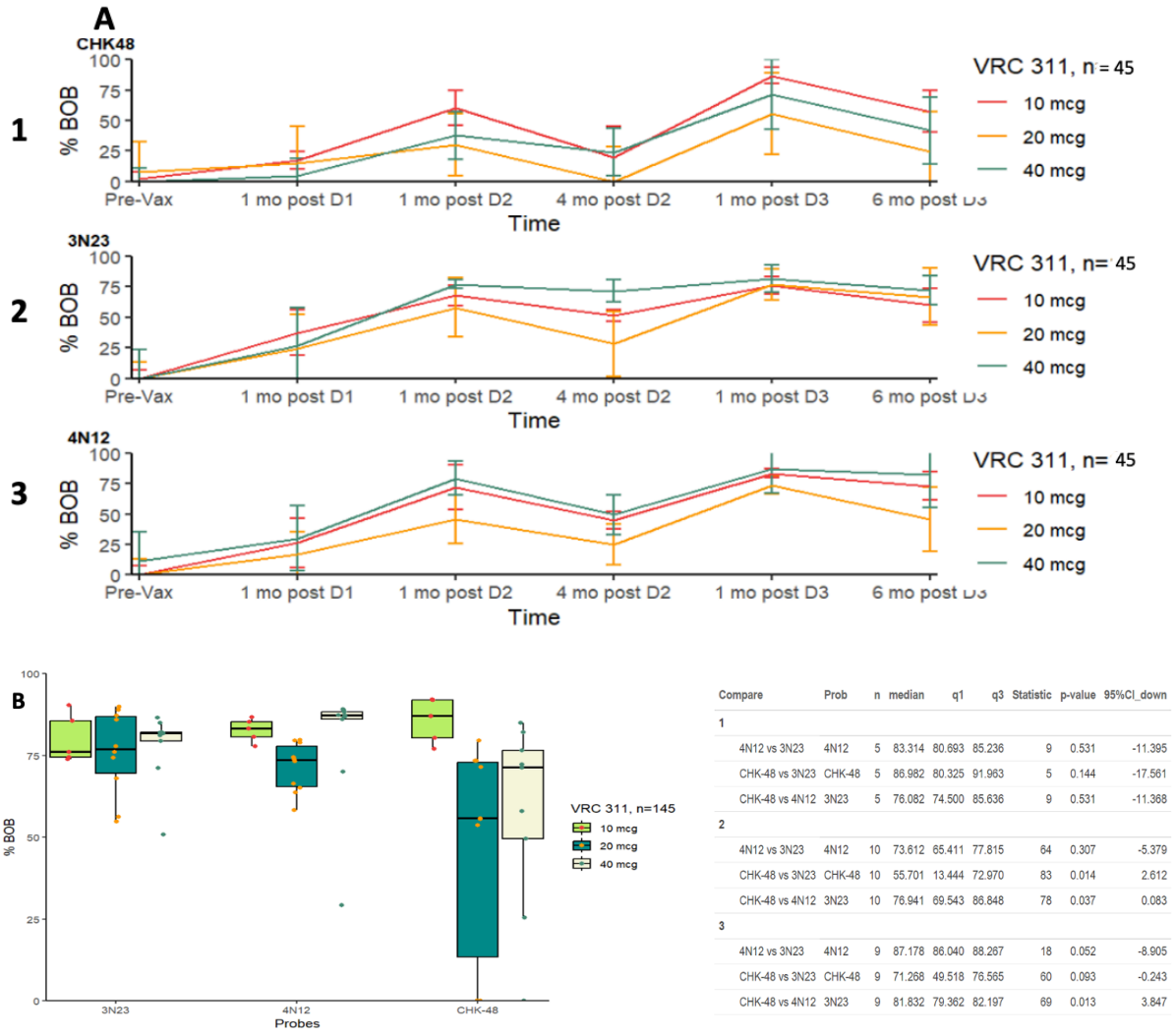


Figure 9: The vaccine dosage schedule of the VRC 311 vaccine candidate trial. This flow diagram shows the vaccine dosage schedule of the subgroups (10 µg, 20 µg, and 40 µg).



Compare	Prob	n	median	q1	q3	Statistic	p-value	95%CI_down
1								
4N12 vs 3N23	4N12	5	83.314	80.693	85.236	9	0.531	-11.395
CHK-48 vs 3N23	CHK-48	5	86.982	80.325	91.963	5	0.144	-17.561
CHK-48 vs 4N12	3N23	5	76.082	74.500	85.636	9	0.531	-11.368
2								
4N12 vs 3N23	4N12	10	73.612	65.411	77.815	64	0.307	-5.379
CHK-48 vs 3N23	CHK-48	10	55.701	13.444	72.970	83	0.014	2.612
CHK-48 vs 4N12	3N23	10	76.941	69.543	86.848	78	0.037	0.083
3								
4N12 vs 3N23	4N12	9	87.178	86.040	88.267	18	0.052	-8.905
CHK-48 vs 3N23	CHK-48	9	71.268	49.518	76.565	60	0.093	-0.243
CHK-48 vs 4N12	3N23	9	81.832	79.362	82.197	69	0.013	3.847

Figure 10. Variation in BOB activity by vaccine dosage timepoint and between probes in VLP vaccine samples.

A) Longitudinal BOB testing in VRC 311 samples. (n=25). This figure demonstrates a three panels of longitudinal BOB activity, one per probe (1 is CHK-48 probe, 2 is 3N23 probe and 3 is 4N12 probe). The longitudinal BOB activity is graphed per subgroup (timepoint of vaccine dosage received) and standard error bars are added based on statistical analysis on R software.

B) Detected high magnitude of BOB activity against NmAb probes CHK-48, 3N23, and 4N12 (n=25). The data demonstrates the spread of the individual values of BOB activity in each probe and displays the spread with the median, interquartile range, and the individual points. Data analysis for p-value was conducted in comparing the three probes and found statistical significance between 3N23 versus CHK-48 at p-value < 0.001 and 3N23 versus CHK-48 when comparing in the cohort of vaccine candidates that received the second vaccine dosage 29 days after.

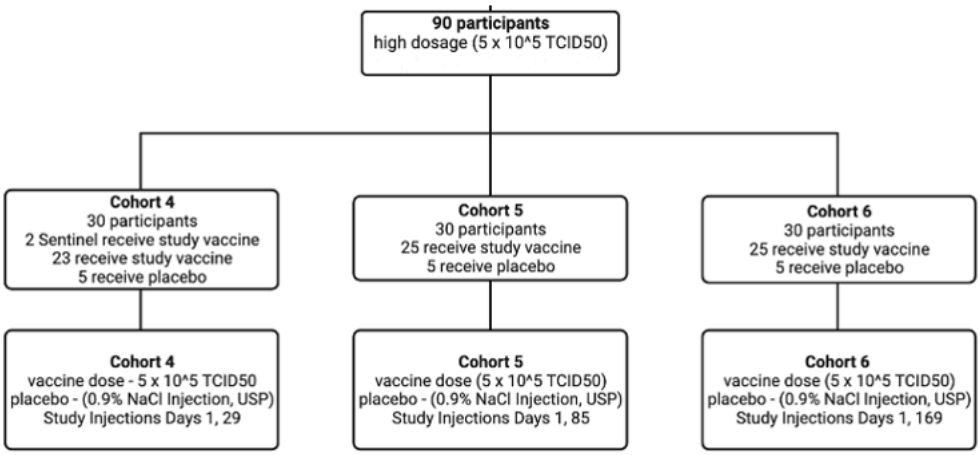
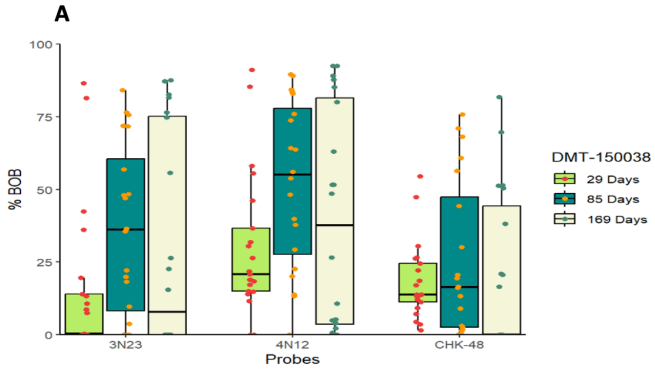


Figure 11. The vaccine dosage schedule of the DMID 15-0038 vaccine candidate trial. This flow diagram shows the vaccine dosage schedule of the subgroups (Cohort 4,5,6) which received the second vaccine dosage on different days. All the samples used from this source set included specimen given the study vaccine and no placebos.



Group Rows

Group	median	q1	q3	compare	Statistic	p-value	95%CI_down	95%CI_up
3N23								
4	0.247	0.000	13.960	4 vs 5	123.0	0.022	-42.886	0.000
6	7.741	0.000	75.221	4 vs 6	191.0	0.614	-26.360	0.088
5	36.060	8.199	60.568	5 vs 6	229.0	0.431	-10.735	32.894
4N12								
4	20.845	14.935	36.690	4 vs 5	121.0	0.021	-44.258	-3.103
6	37.594	3.538	81.408	4 vs 6	202.0	0.845	-34.499	14.884
5	54.968	27.569	77.818	5 vs 6	240.5	0.279	-8.957	37.541
CHK-48								
4	13.731	11.152	24.581	4 vs 5	211.0	0.990	-15.908	10.041
6	8.256	0.000	41.217	4 vs 6	241.0	0.423	-13.589	13.429
5	16.413	2.474	47.346	5 vs 6	240.0	0.277	-8.041	16.583

Group Rows

Compare	Probe	n	median	q1	q3	Statistic	p-value	95%CI_down	95%CI_up
4									
3N23 vs 4N12	3N23	21	0.247	0.000	13.960	107.0	0.004	-21.819	-6.269
3N23 vs CHK-48	CHK-48	21	13.731	11.152	24.581	127.0	0.019	-13.731	-1.507
4N12 vs CHK-48	4N12	21	20.845	14.935	36.690	300.0	0.047	0.105	17.277
5									
3N23 vs 4N12	3N23	20	36.060	8.199	60.568	134.0	0.076	-37.413	1.165
3N23 vs CHK-48	CHK-48	20	16.413	2.474	47.346	243.0	0.249	-4.144	31.801
4N12 vs CHK-48	4N12	20	54.968	27.569	77.818	303.5	0.005	9.664	50.493
6									
3N23 vs 4N12	3N23	20	7.741	0.000	75.221	145.0	0.134	-32.007	2.428
3N23 vs CHK-48	CHK-48	20	8.256	0.000	41.217	221.0	0.553	-0.272	24.954
4N12 vs CHK-48	4N12	20	37.594	3.538	81.408	276.0	0.038	0.000	48.620

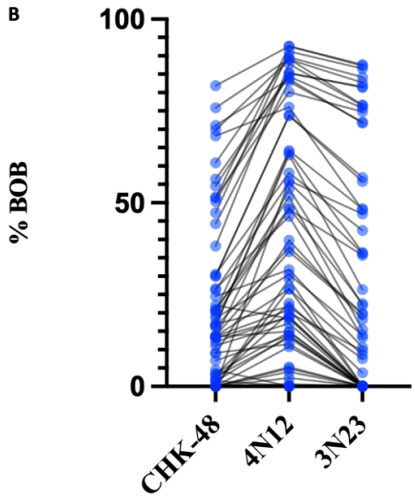
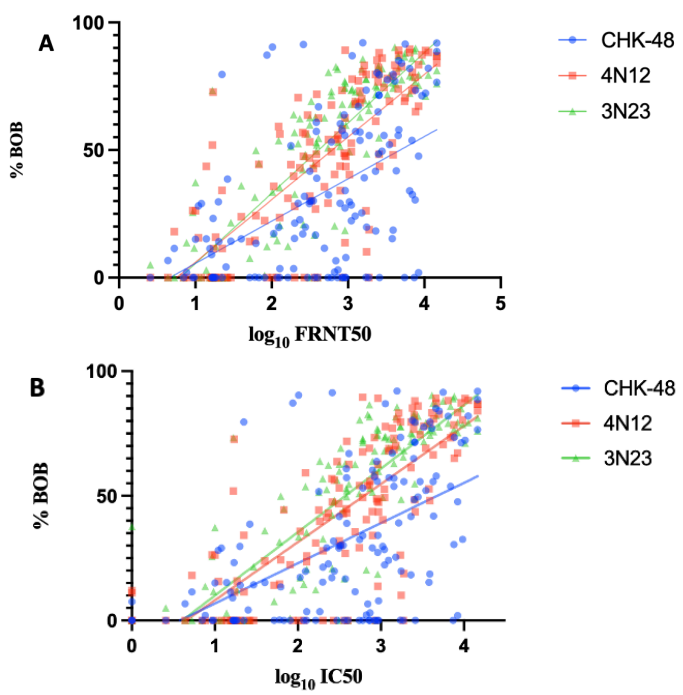


Figure 12. BOB activity varies by MV-CHIK vaccine dose schedule and between probes.

A) The % BOB is shown against each NmAb probe indicated on x-axis among serum from recipients of MV-CHIKV at x days post vaccination (n=63). Box and whiskers denoting median, interquartile range, and full range of the individual data points. Groups with statistically different median values are indicated.

B) Linked pairwise BOB activity against each NmAb probe per individual reveals individual variation in immunodominance hierarchy of Ab response in MV-CHK vaccine candidates (n=63). This figure maps the BOB activity for an individual versus the three different probes by linking them. This data shows similar levels of BOB magnitude per person using different probes. There is a small group that has heterogenous response, demonstrating distinct levels in BOB magnitude.



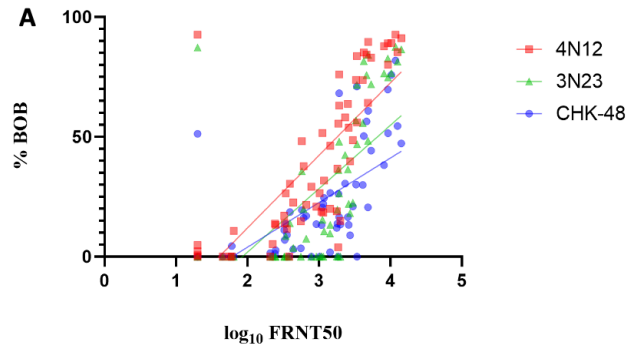
Correlation	Log10FRNT vs. CHK-48	Log10FRNT vs. 4N12	Log10FRNT vs. 3N23
	Y	Y	Y
Spearman r			
r	0.5271	0.7772	0.8485
95% confidence interval	0.3922 to 0.6398	0.6996 to 0.8367	0.7928 to 0.8901
P value			
P (two-tailed)	<0.0001	<0.0001	<0.0001
P value summary	****	****	****
Exact or approximate P value?	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	Yes	Yes	Yes

Correlation	LOG IgG Binding Titer vs. CHK-48	LOG IgG Binding Titer vs. 4N12	LOG IgG Binding Titer vs. 3N23
	Y	Y	Y
Spearman r			
r	0.5627	0.7869	0.8456
95% confidence interval	0.4362 to 0.6675	0.7134 to 0.8433	0.7899 to 0.8875
P value			
P (two-tailed)	<0.0001	<0.0001	<0.0001
P value summary	****	****	****
Exact or approximate P value?	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	Yes	Yes	Yes

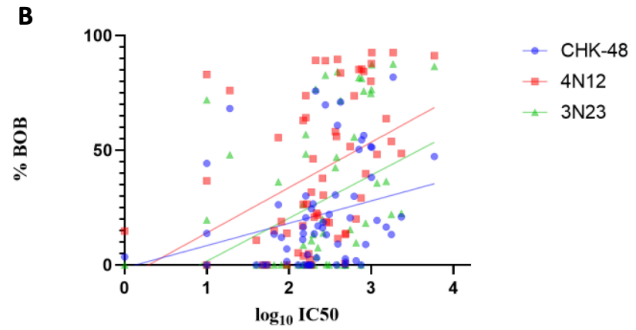
Figure 13. Moderate, positive correlation found using Spearmen correlation test between BOB activity and IgG Binding Titers of VRC vaccine samples.

A) Correlation of BOB activity in the NmAbs and FRNT50 test in the VRC 311 samples (n=25). Regression analysis was used to assess correlation between FRNT50 and BOB activity. Pearson Spearmen correlation test was run and found no statistical correlation of the FRNT50 values to CHK-48 mAb (p= 0.01837), 4N12 mAb (0.8736), and 3N23 mAb (0.6974).

B) Correlation of BOB activity in the NmAbs and IgG binding tests in the VRC 311 samples (n=25). Regression analysis was used to assess correlation between IgG binding titers and BOB activity. Pearson Spearmen correlation test was run and found to have a correlation with CHK-48 probe ($p < 0.0001$), 4N12 mAb ($p < 0.001$) and 3N23 mAb ($p < 0.001$).



Correlation	LOGFRNT vs. CHK-48	LOGFRNT vs. 4N12	LOGFRNT vs. 3N23
Spearman r			
r	0.7355	0.8262	0.7986
95% confidence interval	0.5887 to 0.8354	0.7216 to 0.8939	0.6804 to 0.8763
P value			
P (two-tailed)	<0.0001	<0.0001	<0.0001
P value summary	****	****	****
Exact or approximate P value	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	Yes	Yes	Yes



Correlation	LogIC50 vs. CHK-48	LogIC50 vs. 4N12	LogIC50 vs. 3N23
Spearman r			
r	0.3670	0.4978	0.4826
95% confidence interval	0.1194 to 0.5716	0.2742 to 0.6703	0.2556 to 0.6592
P value			
P (two-tailed)	0.0036	<0.0001	<0.0001
P value summary	**	****	****
Exact or approximate P value	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	Yes	Yes	Yes

Figure 14. Moderate, positive correlations found using Spearmen correlation test between BOB activity and NAb titers and BOB activity and IgG titers of MV-CHK vaccines.

A) Correlation of BOB activity in the NmAbs and FRNT50 test in the MV-CHK samples (n=63). Regression analysis was used to assess correlation between FRNT50 and BOB activity. Pearson Spearmen correlation test was run and found negative statistical correlation of the FRNT50 values to the BOB activity in the CHK-48 mAb ($p < 0.0001$), 4N12 mAb ($p < 0.001$) and 3N23 mAb ($p < 0.001$).

B) Correlation of BOB activity in the NmAbs and IgG binding tests in the MV-CHK samples (n=63). Regression analysis was used to assess correlation between IgG binding titers and BOB activity. Pearson Spearmen correlation test was run and found to have a positive correlation with CHK-48 mAb ($p < 0.0001$), 4N12 mAb ($p < 0.001$) and 3N23 mAb ($p < 0.001$).